CHAPTER 4

ASSESSMENT OF SELENIUM, SELENOPROTEINS AND REDOX STATUS IN ALZHEIMER’S DISEASE PATIENTS

4.1 INTRODUCTION

Oxidative stress play an important role in the pathogenesis of AD (Zhao & Zhao 2013). Numerous studies have reported a change in antioxidant status of AD patients with increase in lipid peroxidation (Mecocci et al 2002; Rinaldi et al 2003; Zito et al 2013). Clinical studies in AD patients showed significant alteration in mineral, trace elements and antioxidant status. Only a few studies have reported the protective role of Se and selenoproteins in brain function and in brain diseases (Steinbrenner & Sies 2013; Zhang et al 2010) including Alzheimer’s disease (Loef et al 2011).

Se though widely distributed throughout the body, it is particularly well maintained in the brain, even upon prolonged dietary Se deficiency. Studies have shown a significant association between low Se levels and rapid cognitive decline in elderly (Chen & Berry 2003; Steinbrenner & Sies 2013; Zhang et al 2010). SeP is a highly glycosylated, Se rich plasma protein. Aside from its role as Se carrier protein, an antioxidative function of SeP has also been suggested (Richardson 2005). Genetic inactivation of SeP in animal models lead to altered synaptic dysfunction in the hippocampus (Peters et al 2006) and general neurological dysfunction (Hill et al 2004). SeP knockout
mice showed increased learning impairment and deficient long term potentiation, indicating the role of SeP in maintaining the cognitive function. Analysis of spatial distribution in postmortem brain tissues showed a significant association between amyloid-\(\beta\) plaques and selenoprotein P (Bellinger et al 2008), suggesting the possible role of SeP in the pathology of Alzheimer’s disease. Two polymorphisms namely exon 6 - A234T and 3’ UTR – G/A in the gene encoding for SeP has been found with low baseline values of plasma SeP (Cooper et al 2008; Méplan et al 2007).

The present study mainly focuses on analysing the levels of Se, selenoproteins, antioxidants and lipid peroxidation in blood and CSF of AD patients and correlating their levels with the pathological markers of AD viz. total tau and A\(\beta\)42. Since oxidative stress is also implicated in normal ageing and in vascular dementia (VD), comparative analysis of these parameters in AD, VD and healthy controls would highlight specific oxidative stress markers of AD.

4.2 METHODOLOGY

The blood and CSF samples were collected from AD, VD and controls as described in Chapter 2, section 2.2.1. Se, SeP and antioxidant levels in AD, VD patients and controls were estimated using protocol given below:

4.2.1 Selenium Estimation

Se in blood and CSF was determined according to a modified procedure given by Safaralizadeh et al (2005). One mL of blood and CSF was digested with 15 mL of HNO\(_3\)/HClO\(_4\) (7:3 v/v) mixture in a Kjeldahl digester at 50–60 °C. The CSF samples were spiked by the addition of a known concentration of sodium selenite. The temperature was raised to 175 °C till
fumes of HClO₄ appeared and continued for 1 hour. The mixture was then subjected to digestion at 200 °C for 1 hour and at 225–250 °C for 1 hour. The mixture was then brought to room temperature, and 10 mL of 6 N HCl was added and heated at 170 °C to reduce Se (VI) to Se (IV). After cooling, the mixture was made up to a known volume and Se concentration was determined by HG-AAS using a Shimadzu 6300 atomic absorption spectrometer.

4.2.2 Estimation of Selenoprotein P in Plasma and CSF

The concentration of SeP in plasma and CSF was determined using a commercially available ELISA kit (CUSABIO, CSB-EL021018HU). The kit consists of a 96 well plate coated with capture antibodies specific to SeP, Standards, biotinylated detector antibody, horse radish peroxidase (HPR)-avidin, tetramethylbenzidine (TMB) substrate, stop solution and suitable diluents.

The standards and samples were suitably diluted using a diluent provided within the kit. 100 μL of standards, plasma and CSF samples were added to the wells and incubated at 37 °C for two hours. The samples were then aspirated and then added 100 μL of biotin-antibody and incubated for one hour at 37 °C. After incubation the unbound biotin-antibody in the well was aspirated and the well were washed thrice using an automated ELISA plate washer. Then, 100 μL of HRP-avidin was added and incubated for one hour at 37 °C. The excess liquid was aspirated after incubation and the wells were washed five times. Then 90 μL of TMB was added on to each well and incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 50 μL stop solution. The yellow colour developed because of the HRP reaction was read at 450 nm with a wavelength correction at 540 nm in a plate reader (Thermo Labsystems, model No. 352).
4.2.3 Selenoprotein P Isoform Profiling

SeP in blood and CSF was purified using nickel-affinity chromatography followed by immunoprecipitation using a commercially available SeP H-300 antibody (Santa Cruz Biotechnology, sc-30162) and isoforms were identified by using western blot (Tujebaieva et al 2000).

4.2.3.1 Nickel affinity chromatography

SeP is a histidine rich protein comprising of a penta-histidine residue, thus making it easy to purify using a nickel affinity column. Before purification thorough the column, the plasma and CSF samples were cleared of IgGs by incubation with protein A agarose for 1 hour. The IgGs in the samples bind to the protein A-agarose and is pelleted out after centrifugation. The supernatant is used for affinity purification. The nickel affinity column (1 mL) was procured from Merck, India. (Cat no. PC137). The reagents used for the purification is as below:

1. Equilibration buffer: 30mM Tris-HCl, 0.3 M NaCl, 100mM β-mercaptoethanol and 10% glycerol.
2. Wash buffer: 30mM Tris-HCl, 0.5 M NaCl, 100mM β-mercaptoethanol and 10% glycerol.
3. Elution buffer: 250mM Imidazole in wash buffer

The column was first equilibrated using the equilibration buffer and suitably diluted plasma sample (0.5 mL diluted to 3 mL) was loaded and was allowed to percolate through the column. The column was washed thoroughly, till the protein content of the eluent was negligible. The bound SeP was then eluted out by the addition of elution buffer containing 250 mM
imidazole, till the protein content in the eluent is negligible. The total eluent was collected and used for immunoprecipitation. After elution, the column was washed thoroughly using wash buffer and recharged with 100 mM nickel sulphate for reuse.

4.2.3.2 Immunoprecipitation

Immunoprecipitation is a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a cell extract so that the antibody will bind the protein in solution. The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled agarose beads. This physically isolates the protein of interest from the rest of the sample. The sample can then be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for Western blot analysis. The purified plasma and CSF samples were immunoprecipitated using SeP H 300 antibody. According to the manufacturer’s instructions, the antibody was added (2μg/500μg of total protein-1 mL) to the samples and incubated at 4 °C overnight with constant shaking. The antibody bound SeP complex was precipitated by the addition of Protein A-agarose. The pellet obtained after centrifugation (12000 rpm for 3 minutes) was washed thrice with 25mM phosphate buffer (pH 7.4) and was dissolved in sample buffer for analysis using SDS-PAGE.

4.2.3.3 Western analysis

Western blotting is a process by which the protein bands in the gel are transferred or blotted on to a stable and immobilizing supports like nitrocellulose and nylon membrane. A variety of analysis like Immunoblotting can be performed with this blot, in which specific molecules
can be detected, if a specific or heterologous antibody is available, using a “double antibody” technique (Nagarajan & Kumar 2002).

The reagents used are as below:

**Materials**

1. Semidryblot electrophoretic transfer unit
2. Whatman 3 mm filter paper
3. Nitrocellulose membrane

**Reagents**

**Transfer Buffer:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (pH 8.3)</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.92 M</td>
</tr>
<tr>
<td>Methanol</td>
<td>20 %</td>
</tr>
</tbody>
</table>

SDS-PAGE was carried out in a discontinuous buffer system using 8 % acrylamide gel. The gel was equilibrated after electrophoresis with transfer buffer for 5 min in a trough. Whatmann filter papers of the size of the gel were also equilibrated in the transfer buffer. A piece of nitrocellulose paper was cut to the size of the gel and immersed in the buffer. On one lid of the transfer cassette, placed 3 bits of filter paper and the gel was carefully placed on the filter paper. Then placed the nitrocellulose paper carefully on the gel without entrapping air bubbles between the gel and nitrocellulose paper. Another set of filter papers were placed on the nitrocellulose paper and the cassette was closed with another lid and placed in the transfer tank filled with transfer buffer. Blotting was carried out overnight at 20 mA at 4 ºC.
The regents used for western blotting are as below:

1. Phosphate Buffered Saline (PBS): 10 mM sodium phosphate buffer (pH 7.0), with 0.9% NaCl.
2. Phosphate Buffered Saline with Tween 20 (PBST) : 0.10 % Tween 20 in PBS
3. Blocking Solution: 2 % BSA in PBST
4. Rabbit anti-SeP antibody (SeP H 300, Santacruz biotechnology, sc-30162, epitope 82-381 amino acids) with specific dilution (1:1000)
5. Anti-rabbit IgG – HRP conjugate
6. Chemiluminescence substrate (Luminol: Amersham Biosciences, Catalogue No. RPN2108)

After electro blotting, the nitrocellulose membrane was transferred to a clean staining tray and washed with 25 mL of PBS (pH 7.0) for 5 min at room temperature. The membrane was then incubated with 25 mL of blocking solution for 1 hour at room temperature, with gentle agitation. After blocking, the membrane was washed thrice with 15 mL of PBST for 10 min each. The membrane was then incubated with anti-SeP antibody (1:1000) with gentle agitation at 4°C for 1 – 2 hours. The unbound antibody was washed off by washing the membrane with 15 mL of wash buffer for 10 min with three changes. The membrane was then incubated with anti-rabbit IgG antibody (HRP-conjugated) for 1 hour at room temperature with gentle agitation. The excess antibody was removed by washing with 15 mL of PBST for 15 minutes with three changes and the membrane was transferred to a clean tray for detection.
Chemiluminescence detection was performed using a luminol substrate. The substrate solutions consisted of reagent A (luminol) and reagent B (H$_2$O$_2$). Equal volumes of both solutions were mixed together and were spread evenly on the membrane. The luminol reacted with the HRP present in the membrane to emit light, which was captured by the CCD camera. SeP isoforms were visualized as light bands and the intensity of the band is expected to be proportional to the concentration of the protein in the membrane.

### 4.2.4 Selenoprotein P Genotyping

#### 4.2.4.1 Amplification of selenoprotein P gene

Selenoprotein P gene (SEPP) polymorphisms, namely exon 6 - A234T and 3’ UTR – G/A mutation were identified by PCR-RFLP. DNA was isolated as specified in section 2.2.2.1. The gene was amplified by PCR using the following primers as described by Meplan et al (2007):

Forward: 5’-CACGCATTATCTCTATCTATAAGCTTG - 3’

Reverse: 5’-GAAATTGTGTCTGGACTAAATTGGAGG- 3’

A mutation (G changed to A) was created in the reverse primer to introduce a restriction site for Ppi I for detection of 3’ UTR – G/A mutation and to decrease the primer-primer complementarity, A to G mutation was made. PCR was performed in a 30 µl reaction volume containing the following

1. 25 ng of each primers
2. 100-300 ng DNA
3. 10 mM Tris-HCl buffer (pH 9.0), 1.5 mM MgCl₂,
4. 0.3 U of Taq polymerase.

The thermal cycler program was set as follows:

Step 1: Initial denaturation at 95 °C for 5 min

Step 2: 35 cycles of amplification by

2.1 Denaturation at 95 °C for 1 minute
2.2 Primer annealing at 55 °C for 1 minute
2.3 Extension at 72 °C for 2 minutes

Step 3: Final extension at 72 °C for 10 min.

4.2.4.2 RFLP analysis

The exon 6 - A234T and 3’ UTR – G/A mutation were identified by restriction digestion (RFLP) using the enzymes Bst AP I and Ppi I (New England Biotechnology), respectively. The 20 μL reaction mixture consisted of 5 μL PCR product, 1X BSA, 1X Restriction buffer and enzyme 1 U. The mixture was incubated at 60 °C for Bst AP I and 30 °C for Ppi I for 3 hours. The reaction was stopped by incubating the mixture at 80 °C and the restricted fragments were visualized in 2% agarose gels.

4.2.5 Antioxidant Analysis

The activities of antioxidant enzymes, GSH and MDA levels were estimated using suitable spectrophotometric procedures.
4.2.5.1 Glutathione peroxidase

GPx activity was determined by using a modified procedure of Paglia and Valentine (1967). The reaction mixture consisted of 5.0 mM EDTA, 50 mM sodium phosphate buffer, 10 mM sodium azide, 1 mM reduced GSH, 0.2 mM NADPH, 0.01 % (v/v) Triton X-100 and 1 U GR. A suitably diluted enzyme sample was added and the reaction was initiated by the addition of 2.6 mM H$_2$O$_2$, and the absorbance was read at 340 nm for about 3–5 min. GPx activity was calculated using a molar extinction coefficient of $6.22 \times 10^3$ M$^{-1}$ cm$^{-1}$.

4.2.5.2 Thioredoxin reductase

TR activity was assayed by the method of Arner et al (1999). The reaction mixture consisted of 100 mM potassium phosphate buffer, 2 mM EDTA buffer (pH 7.4) and 3 mM DTNB (100 mM stock solution in ethanol), and 200 μM NADPH was used for initiating the reaction. The increase in absorbance at 412 nm was monitored for about 3 min. The activity was calculated using a molar extinction coefficient of 13.6 mM$^{-1}$ cm$^{-1}$.

4.2.5.3 Superoxide dismutase

The assay of SOD was done according to the procedure of Das et al (2000). SOD was assayed based on the principle of inhibition reaction, where superoxide radical reduced to form an insoluble blue formazan, measured at 550nm. The reaction of xanthine oxidase with xanthine was the source for generating O$_2^-$. The reaction was performed in a total volume of 1 mL containing 0.05M sodium carbonate (pH 10.2), 0.1 mM EDTA, 2.5 mM NBT, 0.1 mM xanthine, 220 mM xanthine oxidase, and enzyme. The reaction was started with the addition of xanthine oxidase which gave an increase in absorbance at 550nm with a rate of 0.02/min at 25°C due to the
formation of blue formazan. One unit of SOD was defined as that amount which was required to inhibit the reduction of NBT by 50% under the assay conditions as described above.

4.2.5.4 Peroxidase

Plasma POD activity was measured by the method of Lobarzewski & Ginalska (1995). The activity was measured spectrophotometrically by following the change in absorbance at 460nm due to O-dianisidine oxidation in the presence of H₂O₂ and enzyme. The reaction mixture consisted of 15 mM ortho-dianisidine, 0.1 M phosphate buffer (pH 5.0), 0.2 M H₂O₂ and a suitably diluted sample. The reaction was started by the addition of H₂O₂ and the increase in absorbance at 460nm was followed against a blank, spectrophotometrically for about 3-5 mins at 37 ºC. Unit of enzyme activity was defined as μmol of O-dianisidine oxidized/min at 37 ºC using the molar extinction coefficient of 11.3 x 10³ M⁻¹ cm⁻¹.

4.2.5.5 Catalase

CAT activity was measured by the procedure of Aebi (1984). The reaction mixture consisted of 25 mM phosphate buffer (pH 7.0), 10mM H₂O₂ and a suitably diluted plasma and erythrocyte lysate. The reaction was started with the addition of H₂O₂. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve was used for calculation of the enzymatic activity. One unit of catalase activity was defined as the amount of enzyme, causing the decomposition of μmol H₂O₂/mg protein/min at pH 7.0 at 25 ºC using the molar extinction coefficient of 43.6 M⁻¹ cm⁻¹.
4.2.5.6 **Glutathione reductase**

GR activity was measured by the method of Calberg & Mannervik (1985). The enzymatic activity was determined spectrophotometrically by the decrease in absorbance of NADPH at 340nm. The reaction mixture consisted of 25 mM potassium phosphate buffer (pH 7.6), 1 mM NADPH, 165 mM oxidised GSH and 1% BSA. The reaction was initiated by the addition of sample and the decrease in absorbance at 340nm was measured for 3 minutes against a blank. GR activity was calculated using extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

4.2.5.7 **Glucose-6-phosphate dehydrogenase**

G6PD activity was assayed by the method of Balinsky et al (1963) by measuring the increase in absorbance which occurs at 340nm due to NADP being reduced to NADPH. The reaction takes place when two electrons were transferred from glucose 6 phosphate to NADP catalysed by the enzyme glucose-6-phosphate dehydrogenase in the reaction. The reaction mixture consisted of 0.1 M Tris-HCl buffer (pH 8.2), 0.2 mM NADP, 0.1 M MgCl₂. The reaction was started by the addition of enzyme sample and the increase in absorbance was measured at 340nm for about 3 minutes.

4.2.5.8 **Reduced glutathione**

GSH concentration was determined by the procedure of Ellman (1959). The method is based on the reaction of reduced GSH with DTNB to give a compound that absorbs at 412 nm. About 1.0 mL portions of suitably diluted samples were treated with 4.0 mL of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30g NaCl in 100 mL water). After centrifugation, GSH in protein free supernatant was mixed with 2.0 mL of 0.4M Na₂HPO₄ and 1.0mL of DTNB.
Reagent (40 mg DTNB in 100 mL aqueous 1% disodium citrate) and the absorbance was read at 412 nm.

4.2.5.9 Lipid peroxidation product – Malondialdehyde

Erythrocyte and CSF lipid peroxidation levels was measured by estimating the levels of malondialdehyde (MDA) using a modified method of Buege & Aust (1978). 1.0 mL of sample was added with 3 mL of phosphoric acid solution containing 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl in 100 mL of water and 1 mL of 0.6% thiobarbituric acid. The mixture was incubated at 80 °C in water bath for 45 minutes. The mixture was extracted with 4 mL of 2-butanol. The organic layer was removed and absorbance was measured at 535 nm using spectrophotometer.

All the spectrophotometric analyses were performed in a Shimadzu double-beam UV-visible spectrophotometer (UV-1601).

4.2.6 Statistical Analysis

The Se, SeP and antioxidant levels were expressed as mean ± standard deviation and analysed using MANOVA and Tukey’s post hoc comparison using SPSS version 15.0 software. Receiver operating characteristic (ROC) curve analysis was performed to study the reliability of total tau, Aβ42 and SeP in plasma and CSF as diagnostic markers. Pearson’s correlation was done wherever applicable.
4.3 RESULTS AND DISCUSSION

4.3.1 Status of Plasma and CSF Selenium in Alzheimer’s Disease Patients

Se levels in plasma and CSF was determined by HG-AAS using Shimadzu 6300 atomic absorption spectrometer. The results are represented in Figure 4.1. In all the three study groups, Se level was observed to be within the normal range of 80–250 ppb (blood) and 10-50 ppb (CSF). No significant difference was seen in Se levels between the patient and the control groups in both plasma and CSF.

![Graph showing Se levels in plasma and CSF](image)

**Figure 4.1** Plasma and CSF levels of Selenium in Alzheimer’s disease patients

Se is reported to have an antioxidant role and changes in Se level has been reported in many neurodegenerative diseases like AD (Steinbrenner & Sies 2013). In the present study, Se levels in blood and CSF did not vary significantly among the patients and controls and were within the normal reported range (Klapcinska et al 2005). Our results are in accordance with Gerhardsson et al (2008) and Meseguer et al (1999) who indicated that Se levels did not vary between AD and controls. No correlation was observed
between plasma and CSF Se levels (Figure 4.2) indicating that Se level in brain are managed differently and efficiently from the rest of the body. It has also been established that Se metabolism in the brain is independent from the rest of the system, and even in severe Se-deficient conditions, the brain maintains optimal concentrations of Se required for its function (Steinbrenner & Sies 2013). Meseguer et al (1999) also indicated that CSF and brain Se levels may not be correlated with blood Se level despite the fact that Se supplementation increases brain Se levels and starvation decreases brain Se levels.

![Figure 4.2](image)

**Figure 4.2 Correlation between plasma and CSF selenium levels in Alzheimer’s disease patients and controls**

Reports indicate that sodium selenite attenuates Aβ production in neuronal cell lines (Gwon et al 2010) and selenite mitigates tau pathology and neurodegeneration in AD models (van Eersel et al 2010). However, Se level was not correlated with Aβ42 and tau (Figure 4.3) indicating that Se may not be directly involved in regulating Aβ/tau mediated mechanism in AD. Strozyk et al (2009) observed that Se does not occur in its ionic form in the tissues and elicits its antioxidant function as selenoproteins like SeP, GPx and TR.
Figure 4.3 Correlation between selenium status and pathological markers of Alzheimer’s disease in Plasma (A) and CSF (B)
Our results suggest that Se may not be directly involved in mitigating oxidative stress in AD. Hence, the levels of selenoproteins, viz. SeP, GPx, and TR were investigated in blood and CSF of AD patients.

4.3.2 Status of Selenoproteins in Alzheimer’s Disease Patients

4.3.2.1 Selenoprotein P

SeP is reported to be the principle supplier of Se to the brain and also has an antioxidant function. Studies on the levels of this protein with respect to AD pathology are limited. The present study was aimed at quantifying the levels of SeP in plasma and CSF and to establish a relation between plasma and CSF SeP levels with the pathological markers of AD, to validate the use of plasma SeP as a possible biomarker for AD diagnosis. The results are shown in Figure 4.4.

** - Significantly different from control (P<0.001)
* - Significantly different from control (P<0.05)

Figure 4.4 Plasma and CSF Selenoprotein P status in Alzheimer’s disease

The levels of plasma SeP was significantly higher in both AD (8.89±2.47μg/mL, P<0.001) and VD (8.33±1.64 μg/mL, P<0.05) patients
compared to the controls (7.14±2.22μg/mL) (Figure 4.4), indicating increased expression of the protein in neurodegenerative condition. However, the levels of this protein in CSF were observed to be significantly lower in AD patients (1.96±1.05 ng/mL, P<0.05) compared to controls (2.76±1.52 ng/mL). The isoforms of SeP were detected after purification by Ni-Affinity column, followed by immunoprecipitation and detection using western blot. In both plasma and CSF samples, two isoforms of SeP corresponding to molecular weight around 60 and 66 KDa were observed and both isoforms were expressed equally (Figure 4.5).

<table>
<thead>
<tr>
<th>Plasma</th>
<th>CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  2</td>
<td>1  2  3  4  5  6  7</td>
</tr>
</tbody>
</table>

![Sel P](image)

In figure A, lane 1 corresponds to sample from AD patient and lane 2 from control. In figure B, lanes 1-3 correspond to samples from control and lanes 5-7 from AD patients.

**Figure 4.5** Selenoprotein P isoforms in plasma (A) and CSF (B) of Alzheimer’s disease patients
In plasma, SeP expression in AD patients was marginally higher compared to controls. SeP quantified by sandwich ELISA in these samples also showed a similar trend. However, in CSF samples, SeP expression did not vary between AD and controls though ELISA based quantification showed marginal increase in control samples compared to AD.

Plasma SeP is the principal supplier of Se to the brain and also performs an antioxidant function (Hill et al 2007). In present study, plasma SeP was increased significantly in AD patients. The observed increase in the levels in dementia might help to counter the excess free radicals as a result of increased oxidative damage. It has also been suggested that SeP is a peroxynitrite scavenger and protects the endothelial cells from oxidation (Hill et al 2007). The increase in plasma SeP, observed in AD patients, might contribute to supply of enough Se to the brain to counter the excessive oxidative stress prevalent in the disease condition.

In order to study the effectiveness of plasma SeP as an useful biomarker, ROC analysis was performed. The results indicated that that plasma SeP levels can fairly differentiate AD and VD from controls (AUC – 0.700, P<0.05) with a cutoff value of > 7.85 µg/mL (Sensitivity 66%, Specificity 60%) (Table 4.1, Figure 4.6).
Table 4.1 Receiver operating characteristic curve analysis of Selenoprotein P as diagnostic markers of Alzheimer’s disease

<table>
<thead>
<tr>
<th></th>
<th>AD vs Control</th>
<th>AD vs VD</th>
<th>VD vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>AUC: 0.7 (P&lt;0.01)</td>
<td>AUC: 0.58</td>
<td>AUC: 0.65(P&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td>Cutoff: 7.85 μg/mL</td>
<td></td>
<td>Cutoff: 7.85 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Sensitivity: 66%</td>
<td></td>
<td>Sensitivity: 60%</td>
</tr>
<tr>
<td></td>
<td>Specificity: 62%</td>
<td></td>
<td>Specificity: 60%</td>
</tr>
<tr>
<td>CSF</td>
<td>AUC: 0.649 P&gt;0.05</td>
<td>Not analysed</td>
<td>Not analysed</td>
</tr>
</tbody>
</table>

The area under the curve (AUC) determines the quality of diagnostic test.

0.9 – 1 excellent, 0.8 - 0.9 good, 0.7 – 0.8 fair, 0.6 – 0.7 poor, 0.5 – 0.6 fail

Figure 4.6 Receiver operator characteristic curve for plasma Selenoprotein P as diagnostic markers for Alzheimer’s disease
The concentration of SeP in the CSF has been reported for the first time and the level of this protein in AD patients were observed to be significantly decreased (P<0.05) compared to controls. However, western blot analysis indicated no significant variation in SeP expression between AD and control. A recent study by Rueli et al (2015) demonstrated the presence of SeP isoforms in choroid plexus/frozen CSF and their enhanced expression in AD patients after autopsy using western blot analysis. The present study employed a sandwich ELISA method to quantify SeP from live AD patients. The difference in results between the two studies could be attributed to various factors like time of sample collection, storage and the antibody employed. While, Rueli et al (2015) analysed SeP from postmortem brain tissues and CSF, the present study analysed SeP from live patients which could reflect the actual status of brain and may be a more reliable prediction.

It has been reported that SeP is synthesised de novo in the brain and is influxed from the blood via the apolipoprotein E receptor (ApoER2). SeP is also reported to be co-localised within the amyloid plaques in AD brain (Bellinger et al 2008). Deletion of either SeP or ApoER2 leads to severe neurological dysfunction in mice with reduction in activity of GPx (Loef et al 2011), indicating a strong role for SeP in maintaining normal neurological function. The observed decrease in CSF selenoprotein P levels in AD patients could be due to decreased release of SeP in to the CSF by the ependymal cell due to excessive neurodegeneration and/or increased transport of CSF selenoprotein P into the brain through APO ER2 receptor to counter the increased oxidative stress due to overproduction of amyloid peptides.

Since SeP has been reported to be a survival factor for neurons and its levels in CSF were observed to be decreased when compared to controls, the ability of CSF SeP as a marker for AD diagnosis was analysed by ROC analysis. However, ROC analysis indicated that CSF SeP may not be a
reliable marker in differentiating between AD and controls (AUC – 0.649, P>0.05, Table 4.1 & Figure 4.7).

Figure 4.7 Receiver operator characteristic curve for CSF Selenoprotein P as diagnostic marker for Alzheimer’s disease

No correlation was observed between plasma and CSF SeP indicating the independent regulation of the protein in plasma and CSF (Figure 4.8). Schweizer et al (2005) observed that brain selenoprotein synthesis is not altered in hepatic selenoprotein knock out mice, indicating that brain maintains a separate pool of SeP.
Figure 4.8  Correlation between plasma and CSF Selenoprotein P levels in Alzheimer’s disease and controls

Though the levels of SeP depend on Se status, both plasma (Figure 4.9) and CSF (Figure 4.10) SeP levels were not correlated with the Se levels in the present study.
Figure 4.9  Correlation between plasma Selenoprotein P and selenium status in Alzheimer’s disease (A), vascular dementia (B) and controls (C)
Figure 4.10  Correlation between CSF selenoprotein P and selenium status in Alzheimer’s disease and controls

The levels were also not correlated with Aβ, tau and tau-to-amyloid ratio in both blood and CSF (Figure 4.11).

The study concludes that plasma SeP is elevated in AD patients, however, it has to be always correlated with its corresponding levels in CSF for its use as possible marker for AD diagnosis. A study in a larger cohort with age, sex and APOE correlation, would help in validating the use of SeP as biomarker for clinical diagnosis of AD.
Figure 4.11  Correlation between selenoprotein P and pathological markers of Alzheimer’s disease in plasma

Figure 4.12  Correlation between selenoprotein P and pathological markers of Alzheimer’s disease in CSF
4.3.2.2 Selenoprotein P gene polymorphism

Since exon 5 - A234T and 3’ UTR – G/A polymorphisms in the SEPP gene is reported to regulate SeP expression, the frequency of the SNPs were analysed in AD and controls using PCR-RFLP. The desired sequence (722 bp) which includes both the polymorphic regions was amplified using PCR as shown in Figure 4.13.

Lane 1 corresponds to the 100 bp DNA marker. The DNA from AD patients (lanes 2-8) and controls (lanes 9-13) were amplified using gene specific primer.

**Figure 4.13 Amplification of polymorphic regions in selenoprotein P gene**

The exon 5 - A234T polymorphism was identified by restriction digestion of the PCR product using Bst API (Recognition sequence: 5’ GCANNNN^NTGC 3’). The enzyme recognizes the wild type (G/G) phenotype leading to the restriction pattern as shown Table 4.2 and Figure 4.14.
Table 4.2  Restriction pattern for the identification of exon 5 polymorphism in selenoprotein P gene

<table>
<thead>
<tr>
<th>Wild type (G/G)</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous (A/A)</td>
</tr>
<tr>
<td></td>
<td>Heterozygous (G/A)</td>
</tr>
<tr>
<td>221</td>
<td>722</td>
</tr>
<tr>
<td>501</td>
<td>221</td>
</tr>
</tbody>
</table>

Lane 1 : 100 bp ladder  
Lane 2,3,5,6,7,8 : Wild Type  
Lane 4 : Heterozygous mutant  
Lane 6 : Homozygous mutant  

Figure 4.14  Genotypic variations of selenoprotein P exon 5 polymorphism in dementia patients (A) and controls (B)

The 3’ UTR G/A polymorphism was identified by restriction digestion of the PCR product using *Ppi I* (Recognition sequence:
5’...^7(N)GAAC(N)5CTC(N)13^...3’). The enzyme recognizes the mutant (A/G or A/A) phenotype leading to the restriction pattern as shown Table 4.3 and Figure 4.15.

Table 4.3  Restriction pattern for the identification of 3’- UTR polymorphism in selenoprotein P gene

<table>
<thead>
<tr>
<th>Wild type (G/G)</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous (A/A)</td>
</tr>
<tr>
<td>722</td>
<td>687</td>
</tr>
<tr>
<td></td>
<td>Heterozygous (G/A)</td>
</tr>
<tr>
<td>722</td>
<td>722</td>
</tr>
<tr>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>687</td>
</tr>
<tr>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

Lane 1 : 100 bp ladder
Lane 3, 12 : Heterozygous mutant
Lane 2,4-11,13 : Wild type

Figure 4.15  Genotypic variants of selenoprotein P 3’ UTR polymorphism in dementia patients and controls

The frequency of both the SNPs did not vary significantly in dementia patients (AD & VD) and controls (Table 4.4), indicating that both
the polymorphisms may not be associated with the development of AD. Both the polymorphisms also did not affect the levels of SeP and the isoenzyme pattern in the present study.

**Table 4.4 Frequency of selenoprotein P polymorphism in dementia patients**

<table>
<thead>
<tr>
<th>Samples (nos)</th>
<th>3’- UTR polymorphism</th>
<th>Exon 5 polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type G allele</td>
<td>Mutant A allele</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>AA</td>
</tr>
<tr>
<td>Control (55)</td>
<td>0.93</td>
<td>0.07</td>
</tr>
<tr>
<td>Dementia (90)</td>
<td>0.94</td>
<td>0.06</td>
</tr>
</tbody>
</table>

A234T (G/A) mutation present in exon 5 could affect the activity of the protein and 3’ UTR (G/A) mutation is present near the SECIS which could affect the expression of SeP. The frequency of exon 5- A234T and 3’ UTR polymorphism was similar in both dementia and controls indicated that both the polymorphisms are not associated with the disease pathology. Méplan et al (2007) reported that A234T GA (exon5) individuals have higher SeP levels and has a correlation with Gpx activity. Cancer patients with GG genotype have significantly lower levels of SeP levels than controls. In the present study, the both the polymorphisms did not have any influence on the protein levels since the levels of SeP did not vary significantly between individuals with mutant and wild type allele. Since, the study employs only limited sample size, SeP levels and the influence of SEPP gene polymorphism has to be validated in a larger co-hort to obtain a statistically significant result to emphasize the importance of SeP in AD pathology.
4.3.2.3 Status of plasma and CSF selenoenzymes in Alzheimer’s disease and Vascular Dementia

Among the selenoenzymes studied, plasma and erythrocyte GPx activity was significantly elevated in both AD and VD groups (Table 4.5) compared to controls, whereas no significant change was observed in CSF GPx activity, though a slight increase in activity was observed in AD patients. TR activity was significantly reduced in both plasma and CSF of AD patients (Table 4.5).

Table 4.5 Status of selenoenzymes in Alzheimer’s disease and Vascular Dementia

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer’s Disease</th>
<th>Vascular Dementia</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (μmolNADPH oxidized/min/mg protein)</td>
<td>2.11±0.70***a</td>
<td>1.96±0.60***a</td>
<td>1.34±0.35</td>
</tr>
<tr>
<td>Thioredoxin reductase (NADPH dependent production of 1 μmol of 2-nitro-5-thiobenzoate/min/mg protein)</td>
<td>0.42±0.12***a</td>
<td>0.36±0.13***a</td>
<td>0.69±0.27</td>
</tr>
<tr>
<td><strong>Cerebrospinal Fluid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (nmolNADPH oxidized/min/mg protein)</td>
<td>18.01±3.01</td>
<td>Not analysed</td>
<td>16.61±2.17</td>
</tr>
<tr>
<td>Thioredoxin reductase (NADPH dependent production of 1 nmol of 2-nitro-5-thiobenzoate/min/mg protein)</td>
<td>0.28±0.10*a</td>
<td>Not analysed</td>
<td>0.37±0.116</td>
</tr>
</tbody>
</table>

Values are expressed as mean±standard deviation

***a – Significantly different from controls (P<0.001)
*a – Significantly different from controls (P<0.05)
Contradictory reports exist regarding the activities of plasma and CSF selenoenzymes with some studies reporting an increase (Zafrilla et al 2006; Vural et al 2010; Torres et al 2011), decrease (Rinaldi et al 2003; Puertas et al 2012) and no change (Archasson et al 2001; Schrag et al 2013). No correlation was observed between Plasma and CSF GPx ($r=0.003, P>0.05$) and TR ($r=0.156, P>0.05$). Blood Se and the activity of both plasma GPx [$r=-0.214$ (AD), $r=-0.027$ (control), $P>0.05$] and TR [$r=-0.021$ (AD), $r=-0.018$ (control), $P>0.05$] were not correlated and no correlation was observed between CSF Se and the activity of CSF GPx [$r=0.034$ (AD), $r=0.140$ (control), $P>0.05$] and TR [$r=0.140$ (AD), $r=0.060$ (control), $P>0.05$]. Also, no correlation was observed between GPx and amyloid ($r=0.64, P>0.05$) and tau ($r=-0.48, P>0.05$) in AD patients. Similarly, TR activity was also not correlated between amyloid ($r=-0.22, P>0.05$) and tau ($r=0.259, P>0.05$). Since only total GPx was measured in the present study, the observed increase might be due to Se-independent GPx which helps to combat the enhanced oxidative stress. Despite no alteration in Se level among patients and controls, variation in the activities of GPx and TR indicates the probable role of selenoenzymes in combating oxidative stress.

**4.3.3 Evaluation of cellular redox status and antioxidant enzymes in Alzheimer’s disease and vascular dementia patients**

It is evident that oxidative stress plays a major role in the pathology of AD. The body is endowed with many antioxidants to tackle the excessive oxidative stress. Reports indicate that majority of the plasma antioxidants do not cross the BBB into the CSF and brain (Gilgun-Sherki et al 2001). Thus, the levels of antioxidants have to be measured in plasma, CSF and brain of AD patients to validate the extent of damage caused due to oxidative stress. In the present study, the levels of different enzymatic antioxidants along with GSH
and lipid peroxidation product – MDA are measured in erythrocytes, plasma and CSF, which represent both cellular and fluid compartments.

In plasma, SOD, POD and GPx are the important enzymes involved in ROS scavenging. The activity of SOD was significantly elevated (P<0.01) in AD and VD compared to control group, with the AD group showing the highest activity (Table 4.6). Among the peroxidative enzymes studies, POD activity was observed to be the lowest in AD, whereas CAT and GPx activity increased in both AD and VD groups (P<0.01) with the VD group showing the highest activity. This indicates that CAT and GPx are mainly involved in scavenging the hydroperoxides in AD. The increased GPx activity is associated with decreased GR activity in AD patients with a concomitant decrease in GSH concentration (Table 4.6), indicating a disturbance in the GSH homeostasis in dementia.

In erythrocytes, SOD activity was significantly increased in both AD and VD, indicating increased production of superoxide radical in dementia. While GPx activity was enhanced in both forms of dementia, no significant change in CAT activity was observed among all groups. GR activity decreased significantly in AD compared to both VD and healthy controls with a corresponding decrease in G6PD activity. The concentration of GSH was significantly lower in dementia compared to the control group with AD patients exhibiting the lowest level.

In CSF, SOD and GPx are the main enzymes involved in scavenging free radicals. While SOD activity was significantly elevated (P<0.05) in AD patients, no significant change was observed in GPx activity. GR activity decreased significantly in AD compared to controls with corresponding decrease (P<0.01) in GSH level, indicating an altered redox status in AD patients.
Table 4.6  Status of antioxidant enzymes in Alzheimer’s disease and Vascular Dementia patients

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Alzheimer’s disease</th>
<th>Vascular dementia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=45)</td>
<td>(n=45)</td>
<td>(n=55)</td>
</tr>
<tr>
<td><strong>PLASMA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (50% nitrate inhibition/mg protein)</td>
<td>6.97±1.63***a</td>
<td>6.74±1.32***a</td>
<td>4.27±1.82</td>
</tr>
<tr>
<td>Glutathione peroxidase (µmolNADPH oxidized/min/mg protein)</td>
<td>2.11±0.70***a</td>
<td>1.96±0.60***a</td>
<td>1.34±0.35</td>
</tr>
<tr>
<td>Catalase (µmoles of H₂O₂ decomposed/min/mg protein)</td>
<td>0.52±0.13***a</td>
<td>0.54±0.10***a</td>
<td>0.40±0.11</td>
</tr>
<tr>
<td>Peroxidase (µmoles of o-dianisidine oxidized/min/mg protein)</td>
<td>2.78±1.42***a,***b</td>
<td>3.82±0.78</td>
<td>4.19±1.21</td>
</tr>
<tr>
<td>Glutathione reductase (µmoles NADPH oxidized/min/mg protein)</td>
<td>1.29±1.21***a</td>
<td>1.31±0.97***a</td>
<td>2.62±0.74</td>
</tr>
<tr>
<td>GSH (µmol/mL)</td>
<td>2.67±0.37***a</td>
<td>2.80±1.46***a</td>
<td>4.38±0.44</td>
</tr>
<tr>
<td><strong>ERYTHROCYTES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (50% nitrate inhibition/g Hb)</td>
<td>2.48±0.46***a</td>
<td>2.80±0.58***a</td>
<td>1.19±0.85</td>
</tr>
<tr>
<td>Glutathione peroxidase (µmolNADPH oxidized/min/g Hb)</td>
<td>3.79±0.64***a</td>
<td>3.96±0.73***a</td>
<td>3.45±0.68</td>
</tr>
<tr>
<td>Glutathione reductase (µmoles NADPH oxidized/min/g Hb)</td>
<td>0.69±0.15***a</td>
<td>0.82±0.35***a</td>
<td>1.48±0.47</td>
</tr>
<tr>
<td>Glucose 6 Phosphate dehydrogenase (µmoles NADP reduced/min/g Hb)</td>
<td>1.26±0.64***a</td>
<td>1.17±0.88***a</td>
<td>1.90±0.62</td>
</tr>
<tr>
<td>GSH (µmol/g Hb)</td>
<td>7.73±0.94***a</td>
<td>7.83±1.19***a</td>
<td>8.79±1.46</td>
</tr>
</tbody>
</table>
Table 4.6 (Continued)

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=45)</th>
<th>Alzheimer’s disease (n=45)</th>
<th>Vascular dementia (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CSF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (50% nitrate inhibition/mg protein)</td>
<td>0.58±0.17^a</td>
<td>Not analysed</td>
<td>0.38±0.09</td>
</tr>
<tr>
<td>Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)</td>
<td>18.01±3.01</td>
<td>Not analysed</td>
<td>16.61±2.17</td>
</tr>
<tr>
<td>Glutathione reductase (nmoles NADPH oxidized/min/mg protein)</td>
<td>11.70±1.93^**a</td>
<td>Not analysed</td>
<td>14.61±1.55</td>
</tr>
<tr>
<td>GSH (μmol/mL)</td>
<td>0.15±0.04^a</td>
<td>Not analysed</td>
<td>0.17±0.02</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± standard deviation

*^a – Significantly different from controls – P<0.05

***^a – Significantly different from controls – P<0.001

**^a – Significantly different from controls – P<0.01

**^b – Significantly different from Vascular dementia – P<0.001

Blood and CSF MDA levels were significantly higher in both AD and VD compared to healthy controls, indicating increased lipid peroxidation in both forms of dementia (Table 4.7).
Table 4.7 Evaluation of lipid peroxidation product – MDA in Alzheimer’s disease and Vascular dementia patients

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Plasma μmol/mL</td>
<td>2.39±0.54**a</td>
</tr>
<tr>
<td>Erythrocyte μmol/g Hb</td>
<td>13.93±1.93**a</td>
</tr>
<tr>
<td>CSF nmol/mL</td>
<td>0.88±0.29**a</td>
</tr>
</tbody>
</table>

*a - Significantly different from healthy control (P<0.05)

**a - Significantly different from healthy control (P<0.001)

ROS-mediated damage has been implicated in many neurodegenerative diseases like AD (Padurariru et al 2010). ROS like superoxide radical and hydrogen peroxide can cause a wide range of modifications like DNA damage, protein phosphorylation, lipid peroxidation, alteration of mitochondrial function and decreased production of NADPH and ATP (Naziroğlu 2009). The existence of oxidative stress in both AD and VD patients is evidenced by increased activities of plasma, erythrocyte and CSF SOD in order to counter the excess superoxide radical. Under physiological conditions, a balance must exist between the rate of hydrogen peroxide production by SOD and its removal by CAT and GPx (Puertas et al 2012).

Numerous studies have reported contradictory results regarding the levels of antioxidants in AD patients (Schrag et al 2013). Overall, in the present study, SOD activity was increased in all the compartments with increase in lipid peroxidation which is indicative of the increased oxidative stress in AD. The increase in SOD activity was well correlated with the increase in GPx and CAT activities in plasma to scavenge the excess
hydrogen peroxide in both AD and VD. Since, the antioxidant content in the brain is generally low, CAT activity was not observed in CSF. No significant change in GPx activity was observed indicating excessive oxidative stress in brain. CSF GSH and TR, which regulate the redox state, were low in AD patients indicating an altered redox status. Gil et al (2006) also indicated an age-dependent increase in the level of MDA in AD and VD patients.

The GSH system effectively maintains the redox balance and is also important in the scavenging of hydroperoxides independently (Naziroğlu 2009). The reduction in GSH level in dementia could also be attributed to the decrease in the activity of GR which replenishes the GSH by reducing the oxidised glutathione (GSSG) formed by GPx activity. The activity of GR and TR is dependent on the level of NADPH which is supplied by G6PD. A significant decrease in G6PD activity observed in the present study could have depleted the NADPH, resulting in reduced activities of GR and TR. Mandal et al (2012) indicated that GSH level is also lowered in AD brains. Our results indicated that GSH level is decreased in plasma, erythrocytes and CSF. Hence, GSH level could be used as an effective marker to diagnose AD. The decrease in activity of redox enzymes like TR, GR and GSH observed in AD and dementia patients clearly point out the possibility of oxidative stress and imbalance in the redox state of the cell which could be a cause or consequence of the disease.

4.4 CONCLUSION

The study was aimed to analyse the role of Se and selenoproteins in the pathology of Alzheimer’s disease. In our study, AD patients showed significantly elevated plasma SeP compared to controls. ROC analysis indicated that plasma SeP level can fairly differentiate AD and VD from controls. The levels of this protein in CSF was observed to be significantly
lower in AD patients compared to controls. No correlation was observed between plasma and CSF selenoprotein levels. Blood and CSF Se levels were similar in both AD and controls. Brain maintains a separate SeP pool from plasma and SeP is also a neuronal survival factor. Hence, decrease in CSF SeP levels could cause neuronal damage by increasing the oxidative stress and decreasing neurogenesis, further alleviating the disease condition. In the present study, both plasma and CSF GSH levels were significantly lower in AD patients and Mandal et al (2012) reported reduction in brain GSH level. Hence plasma GSH may reflect the actual reduction in brain GSH level.

Detailed analysis of CSF and brain selenoprotein levels in a larger co-hort with taking the baseline correction of parameters like age, sex, APO E status and method of analysis into account would help in determining the usefulness of Se, selenoproteins, Aβ, tau and tau-to-amyloid ratio as possible markers for AD and would help us in better understanding the possible role of selenoproteins in Alzheimer’s pathology.