CHAPTER – III

Amyloid precursor protein in Platelets
3.1 Introduction

Pathological changes in cells even outside the central nervous system characterize AD (Joachim et al., 1989; Scott 1993; Casoli et al., 2007). The definite diagnosis of AD is neuropathologically defined by the presence of amyloid plaques, so called senile plaques in the neocortex and NFT’s within specific regions of the cerebral cortex (Jellinger et al., 1998, Newell et al., 1999). NFT’s consist of aggregates of hyperphosphorylated tau protein, a microtubule-associated protein which results in its dissociation from microtubules and its aggregation in intraneuronal tangles composed of paired helical filaments (Hyman et al., 1997; Iqbal et al., 2006). The amyloid plaques are made of a core of insoluble Aβ and several other proteins including α1-antichymotrypsin, ubiquitin, synaptophysin, sulphated glycosaminoglycans and complement (Armstrong 2006). There are two major forms of Aβ, the Aβ1-40 and the Aβ1-42 residues. Aβ1-40 form is the major species secreted by cells and is mainly deposited in cerebral vessels resulting in amyloid angiopathy (AA), in particular, accumulation of the Aβ in the brain parenchyma and cerebral microvasculature (Iwatsubo et al., 1994; Gravina et al., 1995). Aβ1-42 is predominantly found in amyloid plaques and appears to be the initially deposited form (Iwatsubo et al., 1996; Crouch et al., 2008). This is in agreement with results showing that Aβ1-42 polymerizes more rapidly than Aβ1-40 into oligomers (Bitan et al., 2003) and fibrils (Jarrett et al., 1993) in vitro.

Aβ originates by proteolytic processing from a larger precursor, the amyloid precursor protein (APP), which is an integral transmembrane cell surface protein present as numerous alternatively spliced isoforms derived from a single gene localised on human chromosome 21 (Goldgaber et al., 2006; Tanzi et al., 1987). This protein is one of the most abundant proteins present in central nervous system
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(CNS) but it is also ubiquitously expressed in peripheral tissues such as muscles, epithelial and circulating cells.

3.2 Platelets as brain delegates—Amyloid precursor protein forms in platelets

Although a number of cell types express APP, the precise cellular origin of the Aβ deposited in the AD brain and cerebral blood vessels has not been identified. Neurons, astrocytes and microglia are known to express APP and to produce soluble Aβ. Therefore all these cells are possible sources of Aβ in amyloid deposits. In addition, since the various blood and endothelial cells express APP and plasma exhibits immunoreactivity, the circulatory system has also been proposed as a potential source of Aβ. Among the different peripheral cells expressing APP isoforms, platelets are particularly interesting since they show concentrations of three major APP isoforms 770, 751 and 695 amino acid residues equivalent to those found in the brain (Van Nostrand et al., 1990; Schlossmacher et al., 1992; Cattabeni et al., 2004).

Moreover, it has been proposed that platelets contain all the enzymatic machinery to produce all APP metabolites, Aβ and soluble amyloid precursor protein (APPs), which can be stored in α-granules and are released upon platelet activation (Li et al., 1995). Platelets and neurons have numerous similar features: like neurons, platelets store and release neurotransmitters and also express appropriate neurotransmitter transporters and some neuron-related proteins such as NMDA receptors (Da Prada et al., 1988; Morrell et al., 2008). Based on these observations, it is likely that the majority of APP and its metabolites found in blood plasma may derive from platelets and activated lymphocytes and monocytes. Therefore, although unlikely to be the source or to contribute to cerebral amyloid deposition, platelet-associated APP forms provide an easily accessible source of
human material to study APP biochemistry and metabolism both in physiological
and pathological conditions and helps to formulate the leading working hypothesis
in AD, the so-called “amyloid cascade hypothesis” (Hardy 2006; Lee et al., 2007;
Marcello et al., 2008). In addition, these studies may provide evidences for a
parallelism between APP metabolism occurring in peripheral cells and in neuronal
compartment. On this line, different authors reported abnormalities in the platelets'
physiology and function in AD (Zubenko et al., 1987; Davies et al., 1988; Blass and
Gibson 1992; Matsushima et al., 1995; Ferrarese et al., 2000; Zoia et al., 2004;
Casoli et al., 2008). Thus, the appropriateness to use platelets, as a cell mirroring
some neurochemical processes, finds its rationale to identify platelets as brain
delegates, an ideal cell to study the pathogenic mechanism related to AD associated
to the amyloid cascade.

3.3 Structure and function of Amyloid precursor protein and its derivatives

The discovery of APP as the source of Aβ and other APP derivatives has led
to an extensive characterization of the protein. The amyloid precursor protein is a
Class I transmembrane glycoprotein expressed in almost all the tissues in the body.
The APP gene maps to chromosome 21q21. Multiple isoforms exist by alternative
splicing of a 19-exon gene: exons 1–13, 13a, and 14–18 (Yoshikai et al., 1990). The
predominant transcripts are APP695 (exons 1–6, 9–18), APP751 (exons 1–7, 9–18),
and APP770 (Figure 3.1).
APP closely resembles a cell surface receptor comprising a signal peptide sequence, a large extramembranous amino terminal region, a single transmembrane domain and a small carboxy terminal tail with the large amino terminal region (the ectodomain) projecting from the cell surface or into the lumens of intracellular vesicles (for example, the endoplasmic reticulum, Golgibodies, trans-golgi network and endosomes) and the short carboxy-terminal region projecting into the cytoplasm. The general structure of the APP protein includes a number of folding and functional domains; each of them has putative relevance for the pathogenesis of AD.
Starting from the amino terminal, a short 17 amino acid residue, the signal peptide, controls the correct topography of APP across cell membranes and secretion from the endoplasmic reticulum. A cysteine rich exon of 170 amino acid follows the signal peptide. This domain comprehends the first heparin binding site and a high affinity zinc-binding domain on exon five. The zinc domain is responsible for the conformation of APP structure suggesting that environmental factors may act directly on APP protein processing. A stretch of 100 amino acid residue is then present in exons 5 and 6, forming the negatively charged region. In the two splice variants APP 770 and 751 a Kunitz protease inhibitor KPI domain exists in exon 7. This domain is capable of regulating extracellular enzymes binding to and inactivating serine proteases. Another alternatively spliced exon is present in APP 770 following the KPI domain, this is homologous to the OX-2 antigen and it is related to neurons and T cells (Kitaguchi et al., 1988; Weidemann et al., 1989). A second heparin binding domain is then present in exon 9, upon which interaction occurs with cell surface heparin sulphate proteoglycans, basal lamina and extracellular matrix. Nearer to the transmembrane region, two N-linked carbohydrate attachment sites are found on exons 13 and 14 (Pahlsson et al., 1992). The Aβ is finally found on exons 16 and 17 and it is followed by a short intracellular cytoplasmic domain, comprised by 46 amino acid residues and possibly involved in signal transduction processes through association with G proteins. From the numerous functional domains described above, a number of physiological functions have been proposed for APP and its major metabolites. Indeed, the secreted form of amyloid precursor protein (APPs) can function as a growth factor and/or a neurotrophic factor, depending on the type of target cells (Van Nostrand et al., 1989, 1990, 1991, 1992). In addition, APP has been suggested to be crucial in regulating intracellular calcium concentrations (Mattson et al., 1992, 1993, 1994). Furthermore, considering the finding that APP undergoes fast anterograde axonal transport, it has
been postulated that APP is an important factor involved in neuronal cell homeostasis and in the maintenance or stability of synaptic structures and function (Koo et al., 1990). Several hypotheses have been put forward on the possible physiological role of APP in the blood. It is now known that soluble APP containing the KPI domain is highly homologous to protease Nexin II and it inhibits the activity of the blood coagulation factors (Van Nostrand et al., 1992).

3.4 Proteolytic processing of APP – Amyloid cascade hypothesis

Proteolytic processing of APP is a normal physiological process. It is normally processed in a secretory pathway by the three protease enzymes (designated as α-, β- and γ-secretases). α-secretase cleaves APP within the amyloid β-domain, β-secretase cleaves APP at the aminoterminus of the amyloid β domain and γ secretase cleaves APP at the carboxyl-terminus of the amyloid β domain. There are two major APP processing pathways: the amyloidogenic and the non amyloidogenic pathways (Figure 3.2).
Figure 3.2  Schematic descriptions of the APP non-amyloidogenic or amyloidogenic pathways and generated products. (Adapted from Checler J. Neurochem. 1995; 65(4): 1431-44).
A major route of APP processing is via $\alpha$-secretase pathway, the non amyloidogenic pathway: $\alpha$-secretase activity has been localized either in the trans golgi network (TGN) or in the plasma membrane, which cleaves between the carboxy terminal side of residues 16 and 17 of the A\(\beta\) sequence, generating 83 residue carboxy terminal fragment (CT83). Recently, two different candidates, members of the disintegrin and metalloproteases ADAM family have been proposed for $\alpha$-secretase activity: ADAM10 (Lammich et al., 1999; Kojro et al., 2005; Deuss et al., 2008) and tumor necrosis factor converting enzyme TACE (Buxbaum et al., 1998; Deuss et al., 2008). After $\alpha$-secretase releases the bulk of APP, the remaining carboxy terminal fragment (CT83) undergoes proteolysis within their plasma membrane domain regulated by intramembrane proteolysis (RIP) by $\gamma$ secretase. It is executed by a set of proteins including presenilin 1 and 2, nicastrin, Pen-2 and Aph-1 (Francis et al., 2002; Edbauer et al., 2003; Takasugi et al., 2003; Nalivaeva et al., 2008). The site of action of $\gamma$-secretase is unusual, it occurs within the hydrophobic milieu of the lipid bilayer. The end-product of non-amyloidogenic processing of APP is the p3 peptide, a truncated variant of A\(\beta\) that cannot form plaques.

An alternative pathway, the amyloidogenic pathway involves proteolytic processing of APP by $\beta$ and $\gamma$-secretases, a process which occurs constitutively also in normal metabolism (Haass et al., 1992). $\beta$-secretase was identified as transmembrane aspartic protease and known as beta site APP cleaving enzyme BACE (Cole et al., 2008) and its activity is mainly confined to the endoplasmic reticulum and the endosomal lysosomal system. $\beta$-secretase cleaves APP at the amino terminal of the A\(\beta\) sequence. This releases a large amino-terminal part of APP and forms a 12 kDa carboxy terminal fragment of 99 amino acids (CT99). The cleavage of this CT99 fragment by the $\gamma$-secretase generates the 4 kDa A\(\beta\). The $\gamma$-secretase can generate $\beta$-amyloid peptides ending at different residues, valine or...
alanine42 (Aβ40 and Aβ42), which have been implicated in the pathogenesis of AD (Selkoe et al., 1990). Recently, an additional cleavage site in the APP transmembrane domain was discovered. This cleavage has been named as ε-cleavage and occurs mainly after position 49 in CT99 (Kakuda et al., 2006). The carboxy terminal fragment formed by ε-cleavage is called the APP intracellular domain (AICD).

The amyloidogenic pathway of APP processing appears to be the preferred pathway in neurons while the non amyloidogenic pathway is mainly found in other cell types (Vetrivel and Thinakaran 2006). For the last decade two major hypothesis on the cause of AD have been proposed: the “Amyloid cascade hypothesis” states that the neurodegenerative process is a series of events triggered by the abnormal processing of APP (Hardy et al., 2006) and the “neuronal cytoskeletal degeneration hypothesis” (Braak et al., 1991) which proposes that cytoskeletal changes are the triggering events.

3.4.1 APP mutations increase the production of Aβ1-42 peptides

There is growing evidence suggesting that Aβ1-42 is the initiating species in AD pathogenesis (McGowan et al., 2006). So far there are sixteen known missense mutations in APP gene and they have been found to increase Aβ production or disturb the normal proteolytical processing of APP by subtly different mechanisms. APP mutations at codons 716/717 (London mutation) lead to increased production of Aβ42 (Eckman et al., 1997), mutations at codons 670/671 (Swedish mutation) increase Aβ42 and Aβ40 production (Scheuner et al., 1996), the APP 692 flemish mutation and the arctic mutation (Nilsberth et al., 2001) alter APP metabolism and subsequently increase the production and accumulation of Aβ. In Down syndrome (trisomy 21), the over expression of chromosome 21-linked genes, including the
APP gene, is related to the development of the neuropathological changes similar to AD at the age of 40 years (Iwatsubo et al., 1995). The vast majority of all known familiar AD mutations are found in gene coding Presenilin 1 and 2 (Scheuner et al., 1996; Bernardi et al., 2008). To date over 140 mutations in the presenilins have been reported to cause autosomal dominant familial Alzheimer’s disease (FAD) in several hundred families worldwide. Mutations in both these genes increase the production of $\beta_1^{-42}$ by increasing $\gamma$-secretase cleavage of APP (Lemere et al., 1996; Lippa et al., 2000; Neve 2008).

3.5 **APP isoforms in platelets as a possible biochemical marker of AD diagnosis**

The last decade has witnessed an avalanche of information about the morphological and biochemical changes that occur in AD. Tremendous efforts have been made in recent years to identify the neuropathological, biochemical and genetic biomarkers of diseases so that the diagnosis could be established in earlier stages. The search of relevant biomarkers of AD in living patients has been an active part of clinical research for the last decade. In the last decade different biochemical parameters have been looked into the blood for these changes unfortunately this has been unsuccessful. Finding a biochemical marker in the peripheral tissue to identify the progressive pathology in AD would be ideal to treat the disease at an early stage.

APP processing abnormalities are believed to be a very early change in AD. Platelets contain many of the APP processing enzymes found in neurons, and secrete small quantities of Aβ (Casoli et al., 2007). Metabolic changes in platelets are well known in AD and they represent an important peripheral source of APP. Platelets synthesize only traces of APP but process large quantities of APP synthesized by their megakaryocyte precursors. Platelets process intact 150 kDa APP to carboxyl truncated 120–130 and 110 kDa APPs. These APPs, similar to protease nexin II,
may undergo further cleavage or be released from activated platelets in alpha granules (Van Nostrand et al., 1990).

Since platelets express concentrations of amyloid precursor protein isoforms equivalent to those found in brain. Recently many authors have tried to identify peripheral markers of AD, focusing mostly on APP. A number of laboratories reported the alterations in APP metabolism/concentration in platelets of AD patients when compared to control subjects and offer the possibility that they may be used as biomarkers in diagnosing AD and follow the patient’s clinical course (Tang et al., 2005). Moreover, the APP altered level in platelet show a positive correlation to the progression of the disease, thus, suggesting that altered APP forms in platelets may be strictly associated with the onset of AD. The mechanism by which the ratio in platelet APP forms is decreased in AD patients is under investigation. Furthermore, APP alteration clearly correlated with scores on the clinical dementia rating (CDR) scale and on the Mini-Mental Status Examination (MMSE) suggests that this value decreases along with the progression of clinical symptoms. These observations defined the frame of the present work, which aimed to investigate the APPr, the ratio of 120–130 to 110 kDa APPs in normal ageing and in probable/possible AD, with the hypothesis that APPr might represent a sensitive marker for AD.

The study was performed on probable/possible AD patients and age matched non demented healthy subjects. A standardized diagnostic protocol was used in selecting the subjects. The study was conducted in accordance with local clinical research regulations and informed consent was obtained from all the participants and caregivers. All participants received a medical, epidemiological and neuropsychological assessment.
3.6 Materials and Methods

3.6.1 Sources of reagents, solvents and fine chemicals

Sodium dodecyl sulphate (SDS), Sodium chloride (NaCl), Potassium chloride (KCl), Ethylene diamine tetra acetic acid (EDTA), Disodium phosphate, Sodium dihydrogen phosphate and other chemicals were purchased from SRL laboratories, India; Methanol was obtained from Ranbaxy, India. Monoclonal antibody 22C11, was purchased from Chemicon, CA, USA. All other chemicals were of analytical grade and double distilled water was used in all experiments.

3.6.2 Isolation of Platelets

Platelets were prepared by following the previously described protocol (Rosenberg et al., 1997; Diluca et al., 1998, Baskin et al., 2000). Peripheral venous blood sample 10 ml was collected into citric acid dextrose anticoagulant solution (3.8% sodium citrate, 2.7% citric acid, 2% glucose). Platelets were isolated by differential centrifugation according to (Rao et al., 1988). Platelet-rich plasma (PRP) was prepared within 30 minutes after blood collection by centrifuging the blood at 1500 rpm for 10 minutes to remove erythrocytes and the buffy coat fraction. PRP was carefully separated form the blood pellet and transferred to another tube by means of a plastic pipette. PRP was then centrifuged at 2000 rpm for 15 minutes to obtain the platelet pellet. The platelet pellet was washed twice in phosphate buffered saline (PBS) (Containing 135mM NaCl, 5mM KCl, 10mM EDTA, 8mM Na₂PO₄, 2mM NaH₂PO₄ H₂O, pH 7.2) and stored at - 80°C until assay. Platelet volume and number did not differ significantly between the control and patient groups. No erythrocyte or leukocyte contamination was detected in any sample.
3.6.3 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Total platelet proteins were separated in 8% SDS-PAGE. After electrophoresis the proteins were blotted to nitrocellulose membranes in a buffer containing 0.025M Tris–HCl, 0.192M glycine, 20% methanol, pH 8.3 at 230mA for 2 hours and 30 minutes. After blocking with 10% non-fat milk, immunostaining reaction was performed with monoclonal antibody 22C11, (Chemicon, CA, USA; dilution 1:1000) which recognizes the amino terminal domain of APP isoforms of 110 and 120-130 KDa in human platelets (Gardella et al., 1992; Baskin et al., 2003). After 3 hours of incubation the blots were incubated with 1:8000 diluted goat anti mouse horseradish peroxidase conjugated secondary antibody for 1 hour. (Zymed, CA, USA). After incubation with secondary antibody the peroxidase activity was revealed using diaminobenzidine and hydrogen peroxide as a substrate. The antibody recognized three different APP forms of 130, 110 and 106 kDa respectively in each samples.

3.7 Data analysis and Statistical evaluation

Quantitative analysis of the optical density of APP bands was performed by means of imaging densitometer (Bio-Rad, CA, USA). The results were expressed as the ratio (APPr) between the optical density of the upper (130 kDa) and the lower (110–106) APP immunoreactive bands (APPr). The ratio was determined for each individual from two duplications (SD among duplications, <10%). Results were averaged and are expressed as ± SD. Clinical and laboratory results were assessed using paired \( t \) tests. Differences were considered statistically significant at \( P<0.05 \) (2 tailed). Comparisons between groups were performed using factor one-way analysis of variance (ANOVA). Fisher Protected Least Significant Difference (PLSD) test was applied to determine main effects within groups. Statistical analysis was performed using SPSS 14.0 version.
3.8 Results

In this study, we have evaluated a group of subjects including patients affected by sporadic AD at different stages of severity and age matched non demented healthy subjects matched with the other groups for demographic characteristics. Demographic and clinical characteristics of the subjects are shown in chapter I. Table 1.4. 42 probable / possible AD patients were investigated and were compared with 60 age-matched non demented healthy subjects. The study groups did not differ for sex or education. Each patient underwent a clinical and a neuropsychological evaluation including MMSE, DSM IV and analysis of platelet APP isoforms.

The concentration of APP isoforms was evaluated in platelet homogenate of all these subjects by western blot analysis with a monoclonal antibody (22C11) recognizing all three APP isoforms of apparent molecular weight of 130 and 110–106 kDa.Interestingly, in probable/possible AD patients a specific significant decrease in the ratio in optical density between the 130 kDa APP form and the lower (106–110 kDa) forms was found when compared to platelets prepared from control subjects (Figure 3.3).

![Western Blot Analysis](image.png)

**Figure 3.3** Representative Western Blot analysis of platelet APP forms in control subjects and in probable/possible AD patients. APP ratio (APPPr) was calculated as the ratio between the 130 kDa immunoreactive band and the 110 KDa immunoreactive bands. Lane 1: control subject; Lane 2,3 probable/possible AD patients.
APP levels were significantly decreased in probable/possible AD compared with age matched non demented healthy controls ($p < 0.01$) Table 3.1. The reduction in platelet APP ratios correlated with declining Mini-Mental State Examination (MMSE) scores (Folstein et al., 1975), and on the Clinical Dementia Rating Scale in AD patients, thus suggesting that the APP varies according to the progression of clinical symptoms. Similar results were reported by Rosenberg et al., 1997, who also showed that the mean ratio of 120–130 kDa isoform to the 110 kDa APP isoform in AD patients was significantly lower than in control subjects.

Table 3.1  APP ratios in different groups. $P$ value < 0.01 for probable/possible AD patients versus controls

<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>Probable/Possible AD</th>
<th>Age matched controls</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP ratio, Mean ±SD</td>
<td>0.68±0.37</td>
<td>1.24±0.43</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

3.9 Discussion

The present study demonstrates that APP metabolism is altered in patients affected by sporadic AD at different stages of severity. The probable/possible AD patients showed a specific significant decrease in the APP in optical density between the 130 kDa APP form and the lower (106–110 kDa) forms when compared to platelets prepared from age matched non demented healthy subjects. These findings extend previous reports and confirm that APP metabolic changes are widespread, involving central as well as peripheral tissues, and are rather an early event in the course of the disease (Sevush et al., 1998; Di Luca et al., 2000). Moreover, on the basis of the results derived from different clinical settings, it has claimed that the
measurement of APPr in platelets has the potential to be a useful clinical marker that might strengthen the diagnosis of AD (Padavoni et al., 2002). In the present study this is further substantiated. It has been reported that these findings seems to be very promising for clinical diagnostic purposes with the parallel results of cerebrospinal fluid biomarkers such as protein tau and Aβ 1-42 levels or the combination of the two (Mehta et al., 2000).

The mechanism related to platelet APP alteration and their relations to the pathological changes typically found in AD brain are not known and they are still under investigation. Conversely, it is hypothesised that the observed decrease in platelet APP forms could be due to alteration in the processing of mature platelet APP in AD patients as the RT-PCR experiments demonstrated that AD patients showed the same levels of mRNAs encoding for the three major transcripts APP770, APP751 and APP695 in platelets when compared to both control and non-AD patients and even in the most severe cases of AD. These findings suggest that the observed reduction in the ratio of platelet APP forms cannot be ascribed to a marked alteration in the expression of one of the three transcripts even though APP processing abnormalities are believed to be a very early change in AD in neuronal compartment (Di Luca et al., 2000; Tang et al., 2005).

To acknowledge the use of platelet APPr as a biochemical marker, this study was performed on individuals with relatively little if no confounding co-morbidity, therefore limiting as much as possible ambiguous cases. Nevertheless, some exclusion criteria are still required to avoid as much as possible interfering or confounding factors, such as some pharmacological treatments. Although the study was carefully performed on selected cases, there were still some overlapping ranges of APPr between probable/possible AD, may be for very mild AD and control groups. These findings might have different explanations, including unnoticed
violation of inclusion/exclusion criteria, and platelet sample preparation or storage errors.

Further our data strongly indicate that platelets can be considered as a source of biological material available for the study of biochemical processes occurring in the CNS, as the current knowledge indicates that platelets are the major circulating repository for APP (Van Nostrand et al., 1990, 1991) and they have an effective mechanism to release large quantities of this protein at specific sites throughout the vasculature (Van Nostrand et al., 1990, 1991; Bush et al., 1990; Smith et al., 1990). Thus platelets may be a potential source for the amyloid deposits in meningeal vessels and brain parenchyma. The findings of the present study are in agreement with many other features reported in platelets of AD patients such as abnormal activation (Bush et al., 1998) alterations of phospholipase A and C (Matsushima et al., 1995; Krzystanek et al., 2007) and protein kinase C levels (Matsushima et al., 1994). Hence it is proposed that platelets might be viewed as a source of human biological material mirroring in the peripheral compartment and to study the AD related biochemical process that develop in the CNS.

In conclusion the results of the present study suggest that the platelets APP ratio has the potential to be of clinical usefulness to improve diagnostic accuracy or guide disease-modifying therapy, though not a single definitive test which might be of helpful and adjunctive value in the diagnosis of AD and in tracking the course of illness, also in the early stages when drugs may have the greatest potential of improving symptoms or slowing down the disease process or even preventing the progressive neurodegenerative process.