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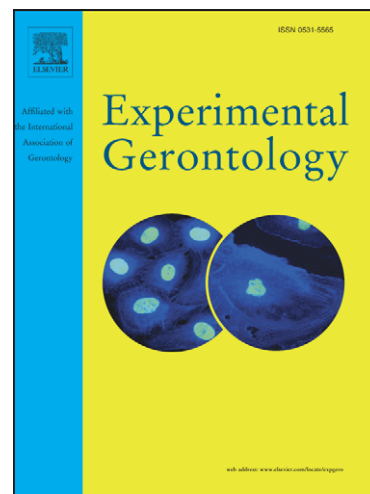
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**Blood cell markers in Alzheimer Disease: Amyloid Precursor Protein form ratio in  
platelets**

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*Running title:* APP in platelets to early AD diagnosis

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**ABSTRACT**

A correct clinical diagnosis in the early stage of Alzheimer disease (AD) is mandatory given the current available treatment with acetylcholine esterase inhibitors. Moreover, an early to preclinical diagnosis would allow to identify patients eligible for future disease-modifying therapies.

In the last ten years, we have focused our attention on peripheral markers, evaluating the role of platelet Amyloid Precursor Protein (APP) forms as a reliable tool for AD diagnosis since preclinical stages. APP is the key player in AD pathogenesis, and platelets contain all the enzymatic machinery to its processing, thus being the ideal candidate where to study AD pathogenetic mechanisms.

In this review, we summarise the published data regarding the usefulness of platelet APP form ratio in the diagnosis of early AD. Approaches combining APP form ratio along with neuroimaging markers show the promise to accurately identify AD, even in the pre-symptomatic stage.

## INTRODUCTION

Few public health problems have captured the attention of the biomedical and lay communities as Alzheimer Disease has (AD). AD represents the most common neurodegenerative disease worldwide accounting for 60% to 70% of cases of progressive cognitive impairment in elderly patients (Cummings and Cole, 2002)

A prompt and early diagnosis is mandatory for many reasons. Firstly, the introduction of acetylcholine esterase inhibitors as symptomatic treatment has highlighted the importance of diagnostic markers for AD (Cacabelos, 2008). Such compounds will probably be most effective in the earlier stages of the disease, before neurodegeneration is too severe and widespread. The increased general knowledge of AD in the population and the awareness of the possibilities for drug treatment have also made patients seek medical advice early in the disease, when the characteristic clinical picture of dementia is rather subtle and barely noticeable. Secondly, in the last years it has been progressively characterised the preclinical phase of AD, and the term of Mild Cognitive Impairment (MCI) defines an heterogeneous population at higher risk to develop AD over time. An early identification of individuals with preclinical AD among MCI is crucial to prompt interventions.

Thus, the search of relevant biomarkers of AD in living patients has been an active part of clinical research for the last decade. As in other field of medicine, biomarkers have been developed to diagnose, prognose, and monitor disease progression. The assumption for such enterprise is that a biomarker provides at least an indirect link to the disease process or ideally directly relates to the primary mechanism of the disease.

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In the last few years, our group has focused the attention on the development of a peripheral biomarker in platelets, evaluating one of the key players of AD pathogenesis, namely the forms of Amyloid Precursor Protein (APP).

In this review, we summarise the usefulness of this biomarker for the diagnosis of AD since the early disease stages, both as single tool and in combination with neuroimaging markers.

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## PLATELETS AS A LABORATORY TO STUDY ALZHEIMER DISEASE

The pathological hallmark of AD is the presence of senile plaques defined by the progressive deposition of beta-amyloid in the parenchyma and cerebral microvasculature. Abeta originates by proteolytic processing from a larger precursor, the APP (Tanzi et al., 1987), an integral transmembrane cell-surface protein present as numerous alternatively spliced isoforms derived from a single gene localized on human chromosome 21 (Selkoe et al., 1988).

This protein is expressed in central nervous system, but it is also ubiquitously expressed in several splice variants in peripheral tissues such as muscles cells, epithelial and circulating cells. Among the different peripheral cells expressing APP forms, platelets are particularly interesting since they show concentrations of the different forms equivalent to those found in brain (Bush et al., 1990).

Some differences between these two cellular populations are nevertheless present: the isoforms 695, lacking the Kunitz Proteinase Inhibitor (KPI) domain, is the most abundant in neuronal tissue, whereas its expression is nearly undetectable in platelets where the major isoforms is APP 770 (Tanzi et al., 1987). The function of APP is yet unknown, but several hypotheses have been put forward a possible physiological role in blood coagulation (Van Nostrand et al., 1990) of APP in platelets. It is known that APP containing KPI domain is highly homologous to Protein Nexin II and it inhibits the activity of the blood coagulation factors IXa, Xa, Xia (Van Nostrand et al., 1992). It has been recently demonstrated that APP metabolism in platelets is regulated by thrombin and prostaglandine 2 (Smirnov et al., in press), but the relationship with AD is still to be defined. It can be hypothesized that APP metabolism might contribute to both the

1 circulating amyloid pool and AD pathology within the brain and its vasculature through  
2 the blood brain barrier (Roher et al., 2009).

3  
4 Literature data have demonstrated the existence of different pathways for APP  
5 metabolism, which are in balance in normal aging. One pool of mature precursor is  
6 processed by alpha-secretase (ADAM10). Alpha-secretase (ADAM10) represents the  
7 main protagonist of the physiological APP metabolic pathway. It cleaves on the C-  
8 terminal side of residue 16 of the Abeta sequence, destroying this amyloidogenic  
9 component. It gives rise, instead, to the release of a large soluble N-terminal fragment  
10 which is called sAPPalpha, whereas a 83 aa long C-terminal fragment (CTF83) remains  
11 stuck to the cellular membrane where it, subsequently, undergoes the gamma-secretase  
12 cleavage. On the contrary, an alternative pathway involves the action of beta-secretase  
13 (BACE), a transmembrane aspartic peptidase, and a second cut by gamma-secretase  
14 leading to release of Abeta peptide. Beta-secretase cleaves at the N-terminus of the  
15 Abeta sequence, releasing into the extracellular space a soluble fragment, designated  
16 sAPPbeta, and leaving attached to the cellular membrane a 99 aa long C-terminal  
17 fragment (CTF99) which is then cleaved by gamma-secretase at exactly the C-terminus  
18 of the Abeta domain. This concerted action of beta- and gamma-secretase, therefore,  
19 gives rise to the release of the deleterious, since amyloidogenic, 4 kDa Abeta fragment  
20 into the extracellular space, where it can accumulate (Small and Duff, 2008) (see

21  
22 **Figure 1**). Evidences propose that platelets contain all the enzymatic machinery to  
23 produce alpha and beta-secretases metabolites, being an easily accessible source of  
24 human material where to study APP biochemistry and metabolism both in physiological  
25 or in pathological conditions (Chen et al., 1995).

26  
27 The appropriateness to use platelets finds its rationale in the numerous similar features of  
28 platelets and neurones: platelets store and release neurotransmitters, express appropriate  
29 neurotransmitter transporters and some neurone-related proteins such as NMDA receptors.



On this line, different authors reported abnormalities in platelets' physiology and function  
in AD. Several studies demonstrated platelet abnormalities in AD patients, supporting  
their usefulness in studying pathogenetic mechanisms.

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## PLATELET AMYLOID PRECURSOR PROTEIN AS A DIAGNOSTIC MARKER IN ALZHEIMER DISEASE

In 1996, the preliminary observation that patients affected by sporadic AD showed a significant alteration of the immunoreactivity of the APP forms in platelets prompted several studies to assess their diagnostic value (Di Luca et al., 1996; Rosenberg et al., 1997; Di Luca et al., 1998).

The evaluation of APP forms was conducted by Western Blot analysis with monoclonal antibody 22C11 raised against the N-terminal domain of APP, therefore recognising the three APP forms with apparent molecular weight of 130, 110, and 106 kDa.

It has been clearly evident that AD patients had a reduced ratio between the upper (130 kDa) and the lower (106 to 110 kDa) immunoreactivity bands, when compared with healthy age-matched controls and patients with non-AD dementia (see **Figure 2**).

In an extensive study performed on 85 patients with AD and 95 control subjects, the accuracy value of APP form ratio was further tested (Padovani et al., 2001b). The control group included a subgroup of non demented subjects (n=52) who were either healthy individuals (normal volunteers, spouses or caregivers) or neurologic nondemented subjects, and a subgroup of patients affected by dementia non fulfilling AD criteria (n=43). In this study, both young (age<60 years old) and old (age≥60 years-old) nondemented controls were considered separately.

AD patients showed a mean APP form ratio of  $0.35 \pm 0.18$ , whilst each control group had a mean ratio  $>0.83$  (standard deviation range: 0.28-0.38).

A cut-off level of platelet APP form ratio lower or equal to 0.57 resulted in a sensitivity of 88.2% and a specificity of 89.4%, with an area under the curve (AUC) of the receiver operating curve (ROC) analysis of 0.945.

1 Reduction of the platelet APP ratio was found to be correlated with disease severity,  
2 measured either with Clinical Dementia Rating Scale or Mini-Mental Status  
3  
4 Examination. The lower the APP form ratio score, the greater the disease severity, thus  
5  
6 suggesting that this value varies according to the progression of clinical symptoms  
7  
8 (Padovani et al., 2001b). Mean APP form ratio of mild AD patients was  $0.45\pm 0.12$ , of  
9  
10 moderate AD was  $0.31\pm 0.17$ , and of severe AD was  $0.22\pm 0.10$ , indicating that this  
11  
12 peripheral biochemical parameter is modified early in the course of the disease as well  
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14 (Padovani et al., 2001a).  
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18 The test was demonstrated to be highly reproducible, with a interassay variability  
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20  $<10\%$ . To confidentially measure the biomarker score, the blood drawing occurred  
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22 while fasting, and the APP ratio for each subject was determined by averaging the  
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24 values from three replications. The main limitation to perform the test is the likely  
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26 interaction between APP metabolism and concurrent pharmacological treatment, i.e.  
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28 cholinergic agents and antiplatelets drugs.  
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33 As it has been demonstrated the helpfulness of this biomarker in mild AD, when  
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35 however the symptoms are already overt, a further step has been to consider the role of  
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37 platelet APP ratio in very early and preclinical stages of AD (Padovani et al., 2002;  
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39 Borroni et al., 2004). To this aim, subjects with MCI were enrolled, and were followed  
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41 up periodically for 2 years, and the progression to dementia was evaluated (Borroni et  
42  
43 al., 2003). Interestingly, MCI individuals who progressed to AD showed a significant  
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45 decrease of baseline platelet APP forms ratio values (mean $\pm$ SD,  $0.36\pm 0.28$ ) when  
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47 compared with stable MCI subjects ( $0.73\pm 0.32$ ,  $P<.01$ ) and patients who developed  
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49 other types of dementia ( $0.83\pm 0.27$ ,  $P=.03$ ) (see **Figure 2** and **Figure 3**). By fixing a  
50  
51 cutoff score of 0.6, the sensitivity of APP ratio marker in detecting preclinical AD was  
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53 83%, while the specificity was 71%. This study suggested that alteration of platelet APP  
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55 forms is an early event in AD, antedating clinical symptoms up to at least two years, and  
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1 that the measurement of APP ratio may be useful for the identification of preclinical AD  
2 in subjects with MCI (Borrioni et al., 2003). This is in line with the general knowledge  
3 that biological changes antedate the onset of symptoms in neurodegenerative disorders.  
4 Furthermore, as platelets contain all the enzyme machinery for APP metabolism, it  
5 would be hypothesized that ADAM10 and BACE changes may also occur in platelets  
6 along with APP form abnormalities. To evaluate this issue, these three parameters were  
7 measured in platelets of mild AD patients and controls.

8 A significant modification of the activity of platelet alpha-secretase (ADAM10) and  
9 beta-secretase (BACE) was observed in platelets of AD patients, which paralleled the  
10 alteration of APP form ratio since the early stages (Colciaghi et al., 2004).

11 Accordingly, the evaluation of the three parameters of platelet machinery, namely APP  
12 form ratio, alpha-secretase and beta-secretase, by using artificial neural networks,  
13 allowed a very good discrimination of AD in early stages, higher than that obtainable  
14 with classical statistical methods (Di Luca et al., 2005).

15 Indeed, these results are in accordance with the recent literature suggesting that the  
16 combination of different markers may achieve higher accuracy values in early AD.

17 In this view, the combination of platelet APP ratio along with neuroimaging markers,  
18 such as brain perfusion SPECT, increased the discriminative power of the analysis in  
19 identifying presymptomatic AD in MCI subjects. The positive predictive value of these  
20 combined markers in identifying preclinical AD was 0.87, and the negative predictive  
21 value was 0.90 (Borrioni et al., 2005).

## CONCLUSIVE REMARKS

Aging population and increasing life expectancy result in an increasing number of patients with dementia. In the vast majority of individuals symptoms begin after a long period of pre-clinical stage, the so called MCI. Literature data have proposed different biochemical marker strictly associated with the key-players of AD pathogenesis.

Among these, platelet APP form ratio has hold the promises to identify preclinical AD among subjects with MCI with high sensitivity and specificity values, as it detects a fundamental feature of AD neuropathology. Moreover, APP ratio biomarker is reliable, simple to perform as compared to more invasive tests, non invasive and inexpensive.

We acknowledge some limitations which prevent its applicability in the wider field:

APP form ratio should be validated in autopsy-confirmed AD and non-AD cases to further confirm its sensitivity and specificity; further, APP metabolism may be affected by concurrent pharmacological treatments.

The interplay of different markers should be considered for further ameliorating diagnostic accuracy to define MCI subjects who will develop dementia, choosing in each subject which markers are better to perform.

## Acknowledgements

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Authors have nothing to disclose

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## Legende to Figures

### **Figure 1. Amyloid Precursor Protein processing.**

Amyloid Precursor Protein (APP), which contains Kunitz Proteinase Inhibitor (KPI), might be processed by alfa-secretase (ADAM10), which precludes formation of amyloid deposits, or by beta-secretase (BACE), leading to release of Abeta peptide.

See text for details.

### **Figure 2. Representative Western Blot of Amyloid Precursor Protein forms in the different diagnostic groups.**

Western Blot analysis in a representative control subject (CON), in a Alzheimer Disease (AD) patient, and in Mild Cognitive Impairment (MCI) individuals who either progressed (MCI-conv.) or not progressed (MCI-stable) to AD.

### **Figure 3. Mean Amyloid Precursor Protein (APP) form ratio in the previously published diagnostic groups.**

CON: control subjects; AD: Alzheimer Disease patients; MCI-conv.: Mild Cognitive Impairment subjects who converted to AD within 2 years; MCI-stable: Mild Cognitive Impairment subjects who did not convert to AD within 2 years.

Red line: cut-off value.

Figure 1

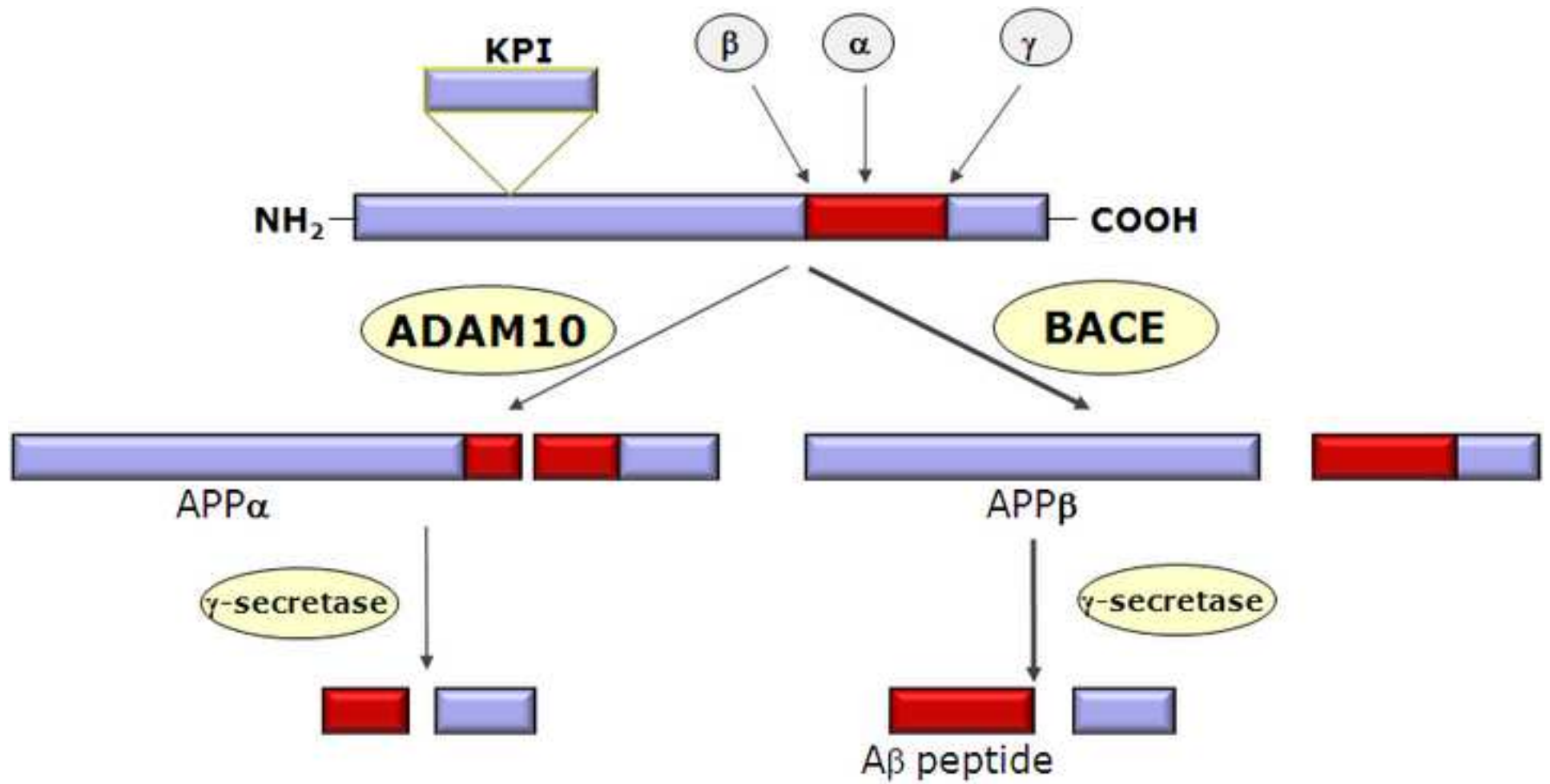


Figure 2

