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TOPIC HIGHLIGHT

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Platelet biomarkers in Alzheimer's disease

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Abstract

The search for diagnostic and prognostic markers in Alzheimer's disease (AD) has been an area of active research in the last decades. Biochemical markers are correlates of intracerebral changes that can be identified in biological fluids, namely: peripheral blood (total blood, red and white blood cells, platelets, plasma and serum), saliva, urine and cerebrospinal fluid. An important feature of a biomarker is that it can be measured objectively and evaluated as (1) an indicator of disease mechanisms (markers of core pathogenic processes or the expression of downstream effects of these processes), or (2) biochemical responses to pharmacological or therapeutic intervention, which can be indicative of disease modification. Platelets have been used in neuropharmacological models since the mid-fifties, as they share several homeostatic functions with neurons, such as accumulation and release of neurotransmitters, responsiveness to variations in calcium concentration, and expression of membrane-bound compounds. Recent studies have shown that platelets also express several components related to the pathogenesis of AD, in particular to the amyloid cascade and the regulation of oxidative stress: thus they can be used in the search for biomarkers of the disease process. For instance, platelets are the most important source of circulating forms of the amyloid precursor protein and other important proteins such as Tau and glycogen synthase kinase-3B. Moreover, platelets express enzymes involved in membrane homeostasis (e.g., phospholipase A_2), and markers of the inflammatory process and oxidative stress. In this review we summarize the available literature and discuss evidence concerning the potential use of platelet markers in AD.

Key words: Alzheimer's disease; Biomarkers; Platelets; Amyloid precursor protein; Glycogen synthase kinase-3B; Phospholipase A_2

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INTRODUCTION

The search for diagnostic and prognostic markers in Alzheimer's disease (AD) has been an area of active research in the last two decades. The pathological changes in AD are the result of several biological processes related to the abnormal metabolism of amyloid precursor protein (APP), regulation of Tau phosphorylation, induction of oxidative stress and inflammatory cascades, and deregulation of lipid metabolism affecting membrane homeostasis. Biochemical markers are correlates of intracerebral changes that can be identified in biological

fluids. An important feature of a biomarker is is that it can be objectively measured and evaluated as an indicator of pathophysiological mechanisms, or of biochemical responses to pharmacological or therapeutic intervention[1]. A good biomarker should be a sensitive diagnostic tool to detect early changes of the disease, and specific enough to differentiate AD from conditions that may clinically overlap, such as cognitive changes in normal aging, cognitive and behavioral syndromes secondary to affective disorders, dementia due to other etiologies and pseudodementia. Ideally, the diagnostic accuracy of biomarkers should be confirmed by neuropathological studies. In addition, to be clinically useful as diagnostic aid, the biological samples in which the biomarker is to be measured should easy to obtain in a safe and tolerable procedure, and the laboratory methods must be reliable, stable, and cost-effective[2].

Significant advances have been made in the recent past with the development of technologies addressing cerebrospinal fluid (CSF) biomarkers in AD. The identification of an 'AD signature' in the CSF has proven accurate for the early diagnosis of AD[3]. However, the specificity of this finding still requires additional confirmation, and there are important variations in measurements between and within laboratories that prevent the immediate use of CSF biomarkers in clinical practice[4]. Preferentially, biomarkers to be used in the clinical setting and in epidemiological studies should be obtainable in the periphery, i.e., in samples of blood or urine. In spite of the considerable amount of research invested worldwide in the search for good peripheral biomarkers of AD, to date no such markers have proved accurate enough to safely differentiate cases of AD from non-cases[5,6]. On the other hand, many candidate peripheral markers for AD have been shown to be correlated with core clinical aspects of the disease, providing complementary information on distinct aspects of its pathophysiology and natural history.

The prospect of using platelets in neuropharmacological models began in the mid-fifties with the discovery that platelets accumulate the neurotransmitter serotonin[7]. Thereafter, several other neurotransmitters have been isolated in platelets, namely GABA, glutamate, aspartate, and glycine, along with their transporters and receptors. Since then, platelets have been extensively used in pharmacological studies addressing the mechanism of action of centrally active drugs. Platelets and neurons share several homeostatic functions, such as the accumulation and release of neurotransmitters, responsiveness to variations in calcium concentration, and expression of membrane-bound compounds (e.g., receptors and enzymes)[8]. The platelet membrane contains several types of surface receptors that respond to external stimuli yielding activating or inhibitory responses[9] and thus modulating the main homeostatic function of thrombocytes, which is to halt the loss of blood from injured vessels. Platelets contain three types of granules: dense granules, α-granules and lysosomes. The granules and their contents are released upon platelet activation, which occurs in response to physiological stimuli, e.g., thrombin, collagen, ADP, epinephrine, vasopressin and serotonin. There are also non-physiological stimuli such as divalent cationic substances and phorbol esters. Therefore, platelets require careful handling because of the possibility of activation through experimentation. The potential for interference must be controlled during preparation of aliquots, by using protease and phosphatase inhibitors and preserving membrane integrity, depending on the type of determination that is intended, in order to preserve the resting state of these molecules[10].

Studies have shown that platelets may be used as peripheral models for the analysis of metabolic pathways related to the pathogenesis of AD, in particular to the amyloid cascade and the regulation of oxidative stress. Platelets are the most important source of circulating forms of APP[5] and other important pathophysiological components of AD, such as microtubule-associated protein Tau[11] and glycogen synthase kinase-3B (GSK3B)[10]. In addition, platelets contain APP-cleaving enzymes, which are biologically active and responsive to systemic stimuli., Using platelets to address the systemic regulation of APP metabolism, offers many possibilities in the search for surrogate markers of intracerebral pathology and effects of pharmacological interventions.

AMYLOID-RELATED MARKERS IN PLATELETS

Amyloid-β (Aβ) peptides originate from the proteolytic cleavage of APP, an integral membrane protein with a large extracellular domain, a membrane-spanning fragment, and a short intracellular, C-terminal tail. APP metabolism can follow different routes resulting either in an amyloidogenic or a non-amyloidogenic processing, in which three different proteolytic enzymes take part (APP secretases α, β and γ). The first (α-secretase) cleaves APP in the extracellular domain and determines the release of its N-terminal peptide (secreted APP, or sAPPα) into the extracellular space. Two enzymes belonging to the ADAM family (disintegrins and metalloproteases) have α-secretase activity: ADAM 10 and ADAM 17 (or TACE, for tumor necrosis factor α converting enzyme)[12,13]. This proteolytic cleavage occurs within the sequence of aminoacids corresponding to Aβ, and therefore precludes the formation of amyloid fragments. This constitutes the secretory or non-amyloidogenic pathway of APP processing. Conversely, enzymes with β-secretase activity (e.g., BACE-1) cleave APP at the amino terminus of the Aβ domain, yielding intact Aβ sequences within the C-terminal residue of APP. A subsequent cleavage of APP at the carboxyl terminus of Aβ sequence by γ-secretase releases Aβ peptides of 40 (Aβ40) or 42 amino acids (Aβ42).

Tang *et al*[14] provided evidence that the amyloidogenic pathway is up-regulated in platelets of patients with AD, having found increased levels of Aβ, increased immunoreactivity of BACE1, and decreased immunoreactivity of ADAM10. Therefore, the modifications of the expres-

sion of APP-cleaving enzymes affecting the Aβ content in platelets are similar to those observed in the AD brain. The extent to which this metabolic pathway is related to platelet function is unknown. Anyway, the activation of platelets by thrombin, collagen, or calcium ionophores releases Aβ40[15], suggesting that the amyloidogenic cleavage of APP from platelet membranes is subsequent to the secretion of α-granules.

It is likely that secreted Aβ40 peptides contribute to the amyloid burden in the vascular walls of the AD brain. In fact, a recent study indicated that soluble Aβ can be internalized into lysosomes by the cerebrovascular smooth muscle cells, yielding Aβ deposits within the extracellular space of the vessel wall upon degeneration of smooth muscle cells. This is supposedly an important mechanism leading to cerebral amyloid angiopathy in AD[16].

Early studies by Di Luca *et al*[17] and Rosenberg *et al*[18] showed a reduction in the ratio between larger (120 to 130 kDa) and smaller (110 kDa) specimens of sAPP in platelets of patients with AD. The so-called APP ratio was proposed as a method to ascertain the metabolic cleavage of APP in platelets, larger fragments corresponding to sAPP-α and smaller ones to sAPP-β. Therefore, a reduced APP ratio would be indicative of the abnormal APP metabolism in patients with AD, as a consequence of predominant β-secretase activity. Further studies from these groups and others have found interesting correlations between the APP ratio and other biological and clinical features of the disease. For instance, the presence of the APOE*4 allele is associated with a greater reduction in the APP ratio[18]. Positive correlations were observed between the APP ratio and cognitive performance[19], both in clinical[20] and preclinical AD[21]. More recently, Zainaghi *et al*[22] also showed a positive correlation between the APP ratio and scores on cognitive tests.

GLYCOGEN SYNTHASE KINASE AND TAU

GSK3B is a serine-threonine kinase[23,24] involved in the regulation of important intracellular signaling pathways, including cell cycle, gene expression and apoptosis[25-27]. In neurons, GSK3B is a major Tau kinase, critically involved in the maintenance of the stability of microtubules and, therefore, in the preservation of the structure and function of the neuronal cytoskeleton[28]. Studies have shown that GSK3B is overactive in AD, playing a major role in the hyperphosphorylation of Tau[24,29-31]. In experimental models of AD, GSK3B has been shown to hyperphosphorylate Tau, leading to microtubule disassembly and loss of function[28]. Therefore, the deregulation of GSK3B homeostasis has a major impact on the formation of paired helicoidal filaments and neurofibrillary tangles, which are key pathological features of AD. The activation of GSK3B also favors the amyloidogenic cleavage of APP, leading to the overproduction of the Aβ peptide[32]. According to the 'GSK hypothesis of AD', the abnormal activation of GSK3B is associated both with the overproduction of Aβ and may be implicated in the early and primary event in the physiopathology of AD[33]. In animal models, overactive GSK3B has been associated with memory impairment[34]. The phosphorylation of GSK3B at Serine 9 leads to its inactivation[35,36], which may prevent Tau hyperphosphorylation and also have potential neuroprotective effects against Aβ toxicity[37,39].

GSK3B is also expressed in human platelets in higher amounts than in other blood cells. The precise physiological role of platelet GSK3B is uncertain, but there is evidence of its involvement in the regulation of platelet activation, since platelet agonists inhibit both isoforms of the enzyme by phosphorylation at Ser-21 (GSK3A) or Ser-9 (GSK3B)[10]. Platelet GSK is subject to similar regulatory mechanisms as those observed in the brain, such as phosphoinositide 3-kinase and PKB signaling. The expression of total- and Ser-9 phosphorylated platelet GSK3B can be readily determined by Western-blot or ELISA methods, and enzymatic activity can be indirectly estimated by calculating the ratio between active and inactive forms. In a recent study by our group, platelet GSK3B activity was shown to be increased in pre-dementia stages of AD[39,40], in accordance with the findings of Hye *et al*[41] of a marked increase in GSK3B activity in leukocytes of patients with AD and mild cognitive impairment (MCI). We have additionally shown that the expression of Ser-9 phospho-GSK3B, in addition to the GSK3B ratio (i.e., the proportion between phosphorylated and total GSK3B), bears a positive correlation with performance in cognitive tests on patients with amnestic MCI[39,40].

Li *et al*[42] first characterized the expression of the Tau protein in platelets, and further showed that the phosphoepitope thr-212 is dephosphorylated as a consequence of the inhibition of GSK3B in platelet homogenates. A recent study from Neumann *et al*[11] showed that several molecular species of Tau are present in platelets, and AD patients have a different proportion of the various forms of Tau as compared to controls. In fact they found several immunoreactive fractions ranging from approximately 60 to 240 kDa and, notably, that high molecular weight fractions of Tau (110-130 kDa) are increased in AD patients as compared with controls. In addition, the ratio between high molecular weight and low molecular weight (ranging from 66 to 68 kDa) fractions seems to be significantly increased in AD subjects. The oligomers of the high molecular weight form of Tau may correspond to aggregates of the nearly 60 kDa monomer forms which subsequently decrease since they are consumed when higher order oligomers are being formed in AD[11].

INFLAMATORY FACTORS

Cumulative evidence indicates that AD is associated with a chronic inflammatory response in the central nervous system. Activated microglial cells participate in the formation of senile plaques, with increased expression of many inflammatory markers[43]. Aβ deposits contain proteins of

the complement pathway and the interaction between aggregated Aβ42 with C1q is thought to initiate microglial activation. In addition, activated microglial cells synthesize inflammatory mediators by means of the activation of cyclo-oxygenases (COX) 1 and 2. Both activated microglia and reactive astrocytes that surround the senile plaques produce acute-phase proteins, complement components, prostaglandis and cytokines[44].

Given that brain inflammation triggers signaling pathways involving sympathetic and neuroendocrine systems[44], most of the parameters indicative of this response can be found in peripheral blood, including platelets. Platelet granules contain nucleotides, amines, coagulation factors, enzymes and inflammatory mediators. Chemokines, interleukins and adhesive proteins are among the most potent inflammatory signaling molecules secreted by platelets, which also synthesize and secrete prostaglandins produced by the enzymes COX-1 and 2[44]. In addition, secreted phospholipase A_2 (PLA_2) and other subtypes are highly expressed in platelets[41], and play a critical role in the regulation of the inflammatory response[45].

PLA_2 abnormalities have been consistently found in AD[46], with important implications in membrane homeostasis and the inflammatory response. The involvement of platelet PLA_2 in AD will be discussed in detail in a following section of this article. Other important platelet markers of the inflammatory response that have been associated with AD are COX enzymes, adhesion molecules and chemokines. A study conducted by Bermejo *et al*[47] showed elevated expression of COX-2 in platelets of patients with MCI and AD as compared to controls. Platelet endothelial cell adhesion molecule-1 and intracellular adhesion molecule-1 are involved in the inflammatory process associated with endothelium dysfunction; these proteins are present both in plasma and platelets, and were found to be increased in AD. The chemokines Regulated on Activation, Normal T Cells Expressed and Secreted and MIP-1α, present in α-granules, were found to be increased in AD patients. Clusterin, a complement-related protein, is also over-expressed in the hippocampus and CSF of AD patients, and is present on the platelet surface[44]. Clusterin mediates the transport of Aβ across the blood brain barrier, co-localizes with several complement factors in cerebrovascular amyloid deposits, and also binds Aβ and prevents its fibrillization[48].

FREE RADICALS AND MARKERS OF OXIDATIVE STRESS

Free radicals are atoms, molecules or groups of atoms bearing an unpaired electron occupying an outer orbit, such as those found in the superoxide anion, hydroxyl radical and nitric oxide. There are also, reactive compounds that have no unpaired electron in the outer layer; these substances are more broadly classified as reactive oxygen species (ROS) or reactive nitrogen species (RNS). The imbalance between the production of ROS/RNS and removal by antioxidant defense systems is called oxidative stress. Oxidative stress is a cellular or physiological condition of high concentration of ROS/RNS causing irreversible cell damage and death[49]. Increasing evidence suggests that oxidative stress that is normally associated with aging is a prominent and early feature of AD and plays a role in its pathogenesis. The respiratory process of activated microglia is the most important source of oxygen free radicals in the central nervous system, where superoxide ions are abundantly generated. These radicals are translocated to the cell membrane and released from microglia into the extracellular space in response to cell injury or nociceptive stimuli. Evidence of free radical production in the AD brain includes the presence of 4-hydroxynonenal, malondialdehyde, 3-nitrotyrosine and oxidized proteins[50].

Very few investigators have addressed markers of oxidative stress in platelets. Marcourakis *et al*[51] studied the content of thiobarbituric acid-reactive substances in platelets from AD patients, and found evidence of increased lipid peroxidation, NOS and Na^+, K^+-ATPase activity, as compared to controls. In addition, increased platelet NOS activity was higher in carriers of the APOE epsilon-4 allele as compared to non-carriers.

COATED-PLATELET LEVELS

Coated platelets are a subpopulation of platelets observed on dual-agonist stimulation with thrombin and collagen, differing from ordinary platelets that are activated by a single agonist. The biochemical characteristics of coated platelets include a robust prothrombinase activity, the retention of several procoagulant proteins on the cell surface, and the release of microparticles[52]. Coated platelets retain full-length APP on their surface upon activation[53]. In a cross-sectional study in AD, Prodan *et al*[54,55] reported that the number of coated platelets in inversely correlated with the severity of cognitive decline, i.e., higher levels are found at earlier stages of the dementing process. In a longitudinal study of disease progression in AD, these authors reported that higher counts of coated platelets at baseline predicted a more severe cognitive deterioration over time. In addition, coated platelets are also over-expressed in patients with amnestic MCI as compared to non-amnestic counterparts[54].

PLATELET PHOSPHOLIPASE A_2 ACTIVITY

Platelet PLA_2 enzymes are critically involved in the metabolism of membrane phospholipids. The PLA_2 superfamily is composed of three main groups: calcium-dependent, cytosolic PLA_2; calcium-dependent, secretory PLA_2; and calcium-independent, intra-cellular PLA_2. PLA_2 hydrolyzes fatty-acids located at the sn-2 position of phospholipids, releasing free fatty-acids and lysophospholipids[56,57]. These are substrates for the synthesis of prostaglandins and arachidonic acid, which are important mediators of neuronal transmission and signaling processes. Moreover, PLA_2 activity controls the physico-

chemical properties of neuronal membranes, affecting the function of membrane receptors, and the release and reuptake of neurotransmitters[58,59]. The hydrolysis of membrane phosphatidylcholine releases free choline that is a substrate for the synthesis of acetylcholine, an important neurotransmitter that has been implicated in the pathophysiology of AD. Changes in PLA_2 activity have been demonstrated in various neuropsychiatric disorders such as temporal lobe epilepsy[60], schizophrenia[61-64], and AD[65,66].

Experimental models have shown that PLA_2 influences APP processing and secretion. In neuronal cultures, PLA_2 activation leads to increased release of soluble forms of APP (sAPPα) into the extracellular medium[67,68]; because the secretory and the amyloidogenic pathways of APP metabolism are mutually exclusive, it is reasonable to assume that such effect is accompanied by the inhibition of the production of Aβ. This assumption is supported by early post-mortem studies of the AD brain[69], showing that intra-cerebral PLA_2 activity is negatively correlated with the number of senile plaques and parameters indicative of the clinical severity of dementia.

Similar findings have been reported in platelets from patients with AD. Gattaz *et al*[69] found evidence of decreased platelet PLA_2 activity in patients with AD as compared to healthy controls and patients with other psychiatric disorders. In another study conducted by our group[70], platelet PLA_2 activity was found to be decreased not only in AD patients, but also in patients with MCI, as compared to cognitively unimpaired older adults. In addition, lower PLA_2 activity was found to be correlated with poorer cognitive performance. It is noteworthy that MCI patients are at higher risk of dementia, with an estimated rate of progression from MCI to AD ranging from 10% to 30% per year[71,72]. Because MCI patients have values of PLA_2 activity between those observed in AD and normal controls, it is likely that in such patients the identification of PLA_2 activity in the lower range may be considered a risk marker for future conversion to dementia[71]. This notion is supported by data from an ongoing longitudinal study conducted by our group addressing the distinct PLA_2 isoforms (unpublished observation). Such abnormality is possibly a peripheral correlate of the intracerebral deregulation of membrane phospholipid metabolism, given the fact that *in vivo* spectroscopy (^{31}P-MRS) studies indicated that decreased phospholipid breakdown in the AD brain is correlated with lower platelet PLA_2 activity[73]. In addition to being a candidate marker of the disease process, PLA_2 may also respond to therapeutic interventions. In a recent study, PLA_2 activity was modulated by cognitive training in healthy elders[74].

CONCLUSION

Platelets express several proteins and molecules that have been implicated in the pathophysiology of AD, such as APP and its related metabolic enzymes, distinct isoforms of microtubule-associated protein Tau, several kinases (particularly GSK3B), enzymes involved in membrane homeostasis (namely PLA_2), and markers of the inflammatory process and oxidative stress. Such properties render platelets a useful model to address, peripherally, certain pathological changes that occur in the AD brain. In this paper, we review the relevant literature addressing platelet markers in AD, in the light of the growing need for biomarkers to support the diagnosis of AD.

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