LEDA LEME TALIB

**Biomarcadores na doença de Alzheimer: GSK3B e PLA₂ na resposta aos
inibidores de colinesterase**

Tese apresentada à Faculdade de
Medicina da Universidade de São Paulo
para obtenção do título de doutor em
Ciências

Programa de: **Psiquiatria**

Orientador: **Wagner Farid Gattaz**

São Paulo

2014

Dados Internacionais de Catalogação na Publicação (CIP)

Preparada pela Biblioteca da
Faculdade de Medicina da Universidade de São Paulo

©reprodução autorizada pelo autor

Talib, Leda Leme

Biomarcadores na doença de Alzheimer : GSK3B e PLA₂ na resposta aos
inibidores de colinesterase / Leda Leme Talib. -- São Paulo, 2014.

Tese(doutorado)--Faculdade de Medicina da Universidade de São Paulo.
Programa de Psiquiatria.

Orientador: Wagner Farid Gattaz.

Descritores: 1.Doença de Alzheimer 2.Fosfolipases A₂ 3.Quinase 3 da
glicogênio sintase 4.Plaquetas 5.Inibidores da colinesterase 6.Donepezil

USP/FM/DBD-124/14

LEDA LEME TALIB

**Biomarcadores na doença de Alzheimer: GSK3B e PLA₂ na resposta aos
inibidores de colinesterase**

Tese apresentada à Faculdade de
Medicina da Universidade de São Paulo
para obtenção do título de doutor em
Ciências

Programa de: **Psiquiatria**

Orientador: **Wagner Farid Gattaz**

São Paulo

2014

Esta tese está de acordo com as seguintes normas em vigor no momento desta publicação:

Referências: adaptado de *International Committee of Medical Journal Editors* (Vancouver).

Universidade de São Paulo. Faculdade de Medicina. Divisão de Biblioteca e Documentação. *Guia de apresentação de dissertações, teses e monografias*. Elaborado por Anneliese Carneiro da Cunha, Maria Julia de A. L. Freddi, Maria F. Crestana Marinalva de Souza Aragão, Suely Campos Cardoso, Valéria Vilhena. 3ª ed. São Paulo: Divisão de Biblioteca e Documentação; 2011.

Abreviatura dos títulos dos periódicos de acordo com *List of Journals in indexed Medicus*.

Dedico este trabalho ao meu marido Jerry,
meus filhos Pedro Augusto e Mariah e aos
meus Pais (*in memoriam*).

AGRADECIMENTOS

Agradeço primeiramente a Deus por me amparar nos momentos difíceis, me dar força interior para superar as dificuldades, mostrar os caminhos nas horas incertas e me suprir em todas as necessidades.

Agradeço ao Prof Wagner Farid Gattaz, meu orientador, por todas as oportunidades e principalmente por ter me transformado em uma pesquisadora. O senhor sempre será um exemplo para mim!

Agradeço ao Dr Sergio Hototian, que realizou todas as avaliações dos pacientes. Sem o seu trabalho esse projeto não seria possível;

Agradeço ao Meu marido Jerry e aos meus filhos Pedro Augusto e Mariah, pelo apoio e incentivo constante. Muito obrigada, amo vocês!

Às “minhas filhas” do LIM-27 por toda ajuda, colaboração e companheirismo! (Lanche no Sujinho e Caipirinha no Veloso! “É nós”).

Aos colegas do LIM-27, Sandra, Eliane, Luciana, d. Edivani, Eliza e Daniel por todo o apoio.

Às minhas queridas amigas, Helena, Carolina e Zelinda por acreditarem em mim mais do que eu mesma! Adoro vocês! #amigasparasempre.

Ao Dr Orestes Forlenza e toda equipe clínica do LIM-27 pelo suporte clínico ao meu trabalho!

Agradeço ainda à FAPESP, ABADHS e Capes pelo financiamento deste trabalho.

Meu Deus será que esqueci de alguém? É muito difícil agradecer a todos sem ser piegas! Nada teria sido possível sem a colaboração de cada um de vocês! Cada palavra de incentivo, cada ensaio que realizamos, os que funcionavam e os que não funcionavam também! (Se tudo desse certo não haveria desafio!), das rusgas, das discussões, de aprender e crescer um pouco mais a cada dia. Uma tese configura um estudo acadêmico, mas, sem tudo isso não estaria completa!

Então, muito obrigada a todos!!!

APOIO FINANCEIRO

Esta tese foi desenvolvida com o auxílio financeiro da Coordenação de apoio ao Pessoal de Nível Superior (CAPES) (DS, bolsa de doutorado) e da Associação Beneficente Alzira Denise Hertzog Da Silva (ABADHS).

"Desistir... eu já pensei seriamente nisso, mas nunca me levei realmente a sério; é que tem mais chão nos meus olhos do que o cansaço nas minhas pernas, mais esperança nos meus passos, do que tristeza nos meus ombros, mais estrada no meu coração do que medo na minha cabeça." — Cora Coralina.

RESUMO

Talib LL. Biomarcadores na doença de Alzheimer: GSK3B e PLA2 na resposta aos inibidores de colinesterase [tese]. São Paulo: Faculdade de Medicina. Universidade de São Paulo; 2014.

A Doença de Alzheimer (DA) é uma desordem neurodegenerativa progressiva que causa comprometimento cognitivo e demência. O diagnóstico é baseado em parâmetros clínicos, mas sua confirmação é *post-mortem*, após avaliação patológica durante a autópsia. Os tratamentos disponíveis para a DA são os inibidores da colinesterase (IChEs) e os antagonistas de receptores de N-metil-D-aspartato (NMDA), sendo que os IChEs compõe o principal grupo. Diversos estudos tem mostrado um efeito neuroprotetor dos IChEs, levando a alterações na patogênese da DA.

Avaliar e mensurar essas alterações são papéis atribuídos aos biomarcadores. Neste sentido podemos destacar a fosfolipase A₂ (PLA₂), a principal responsável pelo metabolismo de fosfolípidos de membrana, e que tem sido achada diminuída na DA, assim como a glicogênio sintase-quinase (GSK), responsável pela fosforilação da proteína Tau, que é um dos processos alterados na DA.

O objetivo deste trabalho foi avaliar o efeito do tratamento com IChE, sobre a atividade da PLA₂ e expressão da GSK3B em plaquetas de 30 pacientes com DA após 3 e 6 meses de tratamento. Como grupo controle foram investigados 42 indivíduos idosos sem doença neurodegenerativa.

Encontramos nos pacientes com DA antes do tratamento uma diminuição da atividade da iPLA₂ quando comparada ao grupo controle. Após três e seis meses de tratamento a PLA₂ aumentou, atingindo o nível dos controles. Os pacientes que apresentaram um aumento maior da iPLA₂ após 3 meses de tratamento e melhora cognitiva mais marcante após seis meses de tratamento, avaliados pelo CAMCOG. Antes do tratamento não encontramos diferenças na GSK3B entre pacientes e controles. Após 6 meses de tratamento encontramos uma inativação da GSK3B, medida por um aumento em sua forma fosforilada.

Nossos resultados sugerem que o donepezil apresenta propriedades modificadoras na doença de Alzheimer, e ainda que a medida da atividade da iPLA₂ poderia ser usada como marcador de resposta terapêutica ao donepezil e, possivelmente, a outros IChEs, na doença de Alzheimer.

Descritores: Doença de Alzheimer; Fosfolipase A₂; quinase 3 da glicogênio sintase; Plaquetas; Inibidores da colinesterase; Donepezil.

SUMMARY

Talib LL. Biomarkers in Alzheimers disease: GSK3B and PLA2 in response to cholinesterase inhibitors [thesis]. São Paulo: Faculdade de Medicina. Universidade de São Paulo; 2014.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that causes dementia and cognitive impairment. The Diagnosis is based on clinical parameters, but confirmation is *post-mortem* after pathologic evaluation during autopsy. The treatments available for AD are cholinesterase inhibitors (IChEs) and N-methyl-D-aspartate (NMDA) antagonists. The main group comprises the IChEs. Several studies have shown a neuroprotective effect of IChEs, leading to alterations in the pathogenesis of AD.

Evaluate and measure these changes are assigned to biomarkers. In this regard we can highlight the phospholipase A₂ (PLA₂) the main enzyme in membrane phospholipids metabolism and that has been found decreased in AD as well as Glycogen Synthase kinase (GSK), a major responsible for tau phosphorylation which is one processes altered in AD.

The objective of this study was to evaluate the effect of treatment with IChE on PLA₂ activity and GSK3B expression in platelet of 30 AD patients after 3 and 6 months of treatment. The control group comprised 42 elderly individuals without neurodegenerative disease

We found a decreased iPLA₂ activity in patients with AD before treatment as compared to controls. After 3 and 6 months of treatment, we observed a

significant increase in iPLA₂ activity similar to those found in controls. The patients who showed higher iPLA₂ activity in the first three months were those showing cognitive improvement after six months of treatment, measured by CAMCOG. At baseline we found no difference in GSK3B between patients and controls. After 6 months of treatment we found an inactivation of GSK3B, as measured by an increase in its phosphorylated form.

Our results suggest that donepezil present modifying properties in Alzheimer disease and that iPLA₂ activity measurement could be used as a marker of therapeutic response to donepezil and possibly other IChEs in Alzheimer's disease.

Descriptors: Alzheimer disease; Phospholipase A₂; Glycogen synthase Kinase 3; platelets, Cholinesterase inhibitors; Donepezil.

LISTA DE FIGURAS

Figura 1 – Características fisiopatológicas da doença de Alzheimer.....	22
Figura 2 – Via da acetilcolina e princípio do mecanismo de ação dos AChEs.....	24
Figura 3 – Esquema das placas de beta amiloide.....	25
Figura 4 – Modelo da estrutura tridimensional da enzima colinesterase	27
Figura 5 – Fluxograma que descreve o recrutamento dos pacientes com DA.....	52
Figura 6 – Atividade da iPLA ₂ em plaquetas	63
Figura 7 – Atividade da cPLA ₂ em plaquetas	64
Figura 8 – Atividade da sPLA ₂ em plaquetas	64
Figura 9 – Atividade da PLA ₂ total em plaquetas.	65
Figura 10 – Expressão da GSKtotal.....	66
Figura 11 – Expressão da GSKfosforilada	66
Figura 12 – Razão da GSKfosforilada sobre a GSKtotal.....	67
Figura 13 – Variação dos scores do MEEM nas avaliações longitudinais.	68
Figura 14 – Variação dos scores do CAMCOG na avaliação basal.....	68
Figura 15 – Pacientes que melhoraram no CAMCOG.....	69

LISTA DE TABELAS

Tabela 1 - Parâmetros farmacocinéticos dos inibidores da colinesterase	30
Tabela 2 - Parâmetros farmacodinâmicos dos inibidores da colinesterase	31
Tabela 3 - Descrição da amostra	55
Tabela 4 - Descrição da amostra	56

ABREVIATURAS

A β – peptídeo β -amilóide
Ach – acetilcolina
AChE-R – retro colinesterase
AChE-S – colinesterase sináptica
AChEs – inibidores da colinesterase
APP – proteína precursora do Amilóide
CAMCOG – teste cognitivo de Cambridge
CCL – Comprometimento Cognitivo Leve
CDR – *Clinical Dementia Rating*
cPLA₂ – PLA₂ citosólicas dependentes de Ca²⁺
DA – Doença de Alzheimer
DSM – IV, Manual de Diagnóstico e Estatística de Transtornos Mentais
GSK3 – glicogênio sintase-quinase 3
iPLA₂ – PLA₂ intracelulares independentes de Ca²⁺
LCR – líquido cefalorraquidiano
MEEM – Mini Exame do Estado Mental
NMDA – N-metil-D-aspartato
PAF – fator ativador de plaquetas
PC – fosfatidilcolina
PET – tomografia de emissão de pósitron
PKC – proteína quinase C
PLA₂ – fosfolipase A₂
SNC – sistema nervoso central
sPLA₂ – PLA₂ secretórias ou PLA₂ extracelular dependente de Ca²⁺

SUMÁRIO

1	INTRODUÇÃO	17
1.1	Aspectos Gerais da Doença de Alzheimer	18
1.2	Comprometimento cognitivo leve (CCL)	19
1.3	Características histopatológicas e fisiopatológicas da Doença de Alzheimer: 20	
1.4	Hipótese colinérgica da Doença de Alzheimer	22
1.5	Tratamento farmacológico da DA	24
1.6	Biomarcadores	31
1.6.1	Fosfolipase A ₂ (PLA ₂):	34
1.6.2	Classificação das fosfolipases A ₂	35
1.6.3	Glicogênio Sintase Quinase - GSK3B	44
1.7	PLA ₂ , GSK3B e Doença de Alzheimer	46
2	OBJETIVOS	48
3	MATERIAL E MÉTODOS	50
3.1	Casuística	51
3.2	Avaliação diagnóstica	53
3.3	Desenho	54
3.4	Métodos	56
3.4.1	Separação e lavagem das Plaquetas	56
3.4.2	Determinação de proteína das amostras	57
3.4.3	Determinação da Atividade da PLA ₂	58
3.4.4	Ensaio radio-enzimático	59
3.5	Análise estatística	60
4	RESULTADOS	61
4.2	Atividade da PLA ₂	62
4.3	Concentração de GSK3B	65
4.4	Parâmetros cognitivos e biomarcadores	67
5	DISCUSSÃO	70
6	CONCLUSÃO	77
7	REFÊRENCIAS BIBLIOGRÁFICAS	79
	APÊNDICE A	91
	APÊNDICE B	140

1 INTRODUÇÃO

1.1 Aspectos Gerais da Doença de Alzheimer

A doença de Alzheimer (DA) é uma desordem neurodegenerativa progressiva que causa comprometimento cognitivo e demência, sobretudo em idosos. É a causa mais comum de demência, correspondendo a aproximadamente 50 – 60% dos casos de demência em pessoas com mais de 65 anos (Francis et al., 1999). A prevalência da DA aumenta com a idade, afetando de 1 a 3% da população americana entre 60 e 70 anos, 3 a 12% das pessoas entre 70 e 80 anos, e de 25 a 35% dos idosos acima de 85 anos (Walsh e Selkoe, 2004). Na população brasileira a prevalência da DA é semelhante, afetando 1,6% dos indivíduos entre 65 e 69 anos, 7,9% entre 75 e 80 anos e 38,9% de indivíduos com mais de 85 anos (Herrera et al., 2002).

A duração média da DA é difícil de precisar, pois o início insidioso com queixas de perda de memória ou apatia, pode se confundir com outras condições como depressão, perda de memória associada ao envelhecimento, ou ainda, estes sintomas podem passar despercebidos pelos pacientes ou familiares. De qualquer forma, é possível encontrar pacientes com duração de doença entre um ano e meio e 15 anos (Brucki, 2001). Os custos para a saúde pública com tratamentos para a DA são altos, equivalentes aos custos com o tratamento da *diabetes mellitus*. Já os custos psicológicos e emocionais para os familiares e cuidadores são mais difíceis de serem mensurados e não menos importantes (Francis et al., 1999; Standridge, 2004).

Atualmente o diagnóstico da Doença de Alzheimer é baseado, na maioria dos casos, no DSM – IV, Manual de Diagnóstico e Estatística de Transtornos Mentais – 4ª edição (American Psychiatric Association, 1994). A

comprovação do diagnóstico de demência depende de avaliação objetiva das funções cognitivas e um dos testes de avaliação global mais empregado é o Mini Exame do Estado Mental (MEEM) de Folstein et al. (1975). O MEEM permite avaliar a orientação espacial e temporal, memória de fixação, atenção e cálculo, linguagem, praxias e habilidades construtivas. A limitação do MEEM é a influência da escolaridade. Desta forma, dependendo do nível de escolaridade do paciente, pontos de cortes específicos devem ser utilizados. A associação de outros testes ao MEEM, como testes de memória e fluência verbal, aumentam a sensibilidade do diagnóstico. A investigação complementar da demência inclui exames de sangue, exames de neuroimagem e em algumas situações especiais podem ser realizados exames no líquido, eletroencefalograma e até mesmo biópsia cerebral (Caramelli e Nitrini, 1997).

1.2 Comprometimento cognitivo leve (CCL)

O Comprometimento Cognitivo Leve (CCL) define um estágio transitório entre o envelhecimento normal e a demência, refletindo uma situação clínica onde o indivíduo apresenta queixas de memória e evidências de problemas cognitivos, mas sem evidências de demência. O reconhecimento do CCL é importante na identificação de pessoas com elevado risco de desenvolver demência, que poderiam se beneficiar de estratégias preventivas (Burns e Zaudig, 2002). Contudo, a noção de "um estágio transitório entre o envelhecimento normal e a DA" é difícil de ser estabelecida. Isto porque a DA é caracterizada por uma progressiva disfunção neuronal e os déficits cognitivos

resultantes desta disfunção ocorrem continuamente, começando leve e sutilmente até condições graves, com a doença avançada.

1.3 Características histopatológicas e fisiopatológicas da Doença de Alzheimer:

As principais regiões cerebrais afetadas na DA são córtex e hipocampo. A avaliação de tecido cerebral *post-mortem* de pacientes com DA mostra alterações características (Figura1). Estas alterações são basicamente:

- a) As placas senis, compostas principalmente pelo peptídeo β -amilóide ($A\beta$), formado a partir da proteína precursora do Amilóide (APP), que se agrega formando oligômeros que, por sua vez, se agregam formando fibras que se depositam no parênquima cerebral (Haass, 2004);
- b) Os emaranhados de neurofibrilas intracelulares, agregados da proteína Tau hiperfosforilada, proteína do citoesqueleto celular, que em condições normais, é específica e exclusiva de neurônios. É tradicionalmente descrita como uma proteína associada aos microtúbulos celulares com a finalidade de estabilizá-los. Contudo, ela tem outras funções como ativação da fosfolipase C, regulação da atividade motora dos microtúbulos e transmissão dos sinais de transdução para o citoesqueleto. No cérebro de pacientes com doença de Alzheimer, a proteína Tau hiperfosforilada se acumula intracelularmente na forma de filamentos helicoidais pareados, em vez de se ligar aos microtúbulos, danificando assim sua estrutura. Os

filamentos helicoidais pareados se agregam como emaranhados neurofibrilares (Jenkins e Johnson, 1999; Sanchez et al., 2001; Lovestone, 2001);

- c) Há perda de neurônios piramidais e degenerações sinápticas no hipocampo e neocórtex (Francis et al., 1999; Caramelli, 2001; Racchi e Govoni, 2003). Nos estágios finais da doença, o cérebro apresenta atrofia do hipocampo e córtex (sendo a atrofia mais proeminente nas regiões frontais, temporais e parietais, afetando principalmente as áreas corticais associativas), aumento dos ventrículos, alargamento dos sulcos e erosão dos giros (Caramelli, 2001; Mohs e Haroutunian, 2002; Walsh e Selkoe, 2004). A atrofia está relacionada com a perda neuronal, podendo ocorrer perda de 40 a 50% dos neurônios dos córtices frontais e temporais. A extensão da perda neuronal está relacionada com a progressão da gravidade da doença e o tempo de duração (Mohs e Haroutunian, 2002).

Figura 1 – Características fisiopatológicas da doença de Alzheimer. (fonte: adaptado de <http://imagina65.blogspot.com.br/2009/11/la-zona-cerebro-alzheimer-el-silencio.html>).

1.4 Hipótese colinérgica da Doença de Alzheimer

A disfunção colinérgica é uma das principais alterações fisiopatológicas associadas a DA e já está presente desde as fases iniciais da doença (Beach et al., 1997). Estudos realizados no final de 60 e início dos anos 70 em cérebros de pacientes com DA mostraram que esses pacientes apresentavam um déficit da enzima colina acetiltransferase, que é responsável pela formação da acetilcolina. Além disso, verificou-se que havia também uma redução na captação de colina, redução na liberação de acetilcolina e perda de

parênquima colinérgico no núcleo basal de *Meynert*, confirmando um substancial déficit colinérgico pré-sináptico. Esses achados somados à importância da acetilcolina na memória e aprendizado levaram a hipótese colinérgica da doença de Alzheimer. Esta hipótese postula então, que a degeneração de neurônios colinérgicos e a perda de neurotransmissão colinérgica no córtex cerebral e em outras regiões contribuem significativamente, para o comprometimento das funções cognitivas observadas em pacientes com DA (Francis et al., 1999; Talesa, 2001).

A neurotransmissão colinérgica (Figura 2) desempenha importante papel no metabolismo da APP e, conseqüentemente, na produção do peptídeo β -amilóide. Em modelos animais, a deficiência colinérgica provoca aumento nos níveis intracerebrais do peptídeo β -amilóide 40 e 42 (Roher et al., 2000; Beach et al., 2003). Por outro lado, a ativação de receptores muscarínicos M1 e M3 em neurônios estimula a secreção do fragmento solúvel da APP (sAPP α), diminuindo a produção do peptídeo β -amilóide (Buxbaum et al., 1992; Nitsch et al., 1992). Em suma, a estimulação colinérgica leva ao metabolismo da APP preferencialmente pela via não-amiloidogênica. A hipofunção colinérgica também está envolvida com outro importante mecanismo fisiopatológico associado à DA, a hiperfosforilação da proteína Tau (Fuentealba, et al., 2004). Portanto, a ativação da neurotransmissão colinérgica poderia ter efeitos neuroprotetores contra diversas alterações fisiopatológicas associadas à DA.

Figura 2 – Via da acetilcolina (ACh) e princípio do mecanismo de ação dos inibidores da colinesterase (AChEIs). Colinesterase (AChE), Acetil coenzima A (AcetylCoA), Transportador vesicular de acetilcolina (VACHT) Colina acetiltransferase (ChAT) sistema de alta afinidade de captação de colina (HACU); receptor colinérgico nicotínicos unidade $\alpha 7$ ($\alpha 7$ AChR) e receptores colinérgicos muscarínicos (m_1 AChR). Fonte: Martorama et al., 2010.

1.5 Tratamento farmacológico da DA

Apesar dos recentes avanços no entendimento da patogenia da doença de Alzheimer, o racional terapêutico ainda é baseado nas investigações patológicas e bioquímicas realizadas no cérebro de pacientes com DA. Diversas abordagens terapêuticas vêm sendo investigadas no tratamento da doença de Alzheimer, incluindo antioxidantes, agentes anti-inflamatórios antagonistas de canais de cálcio e agonistas muscarínicos e nicotínicos (Jann

et al, 2002) (Figura 3). Porém, a atenuação do déficit colinérgico constitui-se em uma importante estratégia terapêutica nos pacientes com DA.

Figura 3 – Esquema das placas de beta amiloide e potenciais alvos para intervenção farmacológica. Astrócitos estão intimamente associados com as placas e mediam reações imunológicas por meio de citocinas e interleucinas (A), tornando-se um alvo atrativo para as terapias anti-inflamatórias. APP e colinesterase contribuem para a patogênese da DA por meio de seu papel na formação da placa (B). Inibidores da colinesterase podem agir como modificadores da doença diminuindo a formação e agregação do AB. Receptores glutamatergico NMDA/Glu R (C), Receptores colinérgicos nicotínicos (D) e muscarínicos (E) (nAChR e mAChR) estão presentes nos astrócitos e nos neurônios ao redor das placas. Existem evidências que os inibidores da colinesterase podem restaurar a expressão alterada dos receptores colinérgicos nicotínicos que ocorre no cérebro com DA. Perda de fatores neurotróficos NGF (F) e BDNF (G) têm sido implicada na degeneração dos neurônios colinérgicos do pró encéfalo basal que é característica da DA (adaptado de Nordberg A, 2006).

Atualmente, os tratamentos farmacológicos disponíveis para os sintomas da DA são restritos a dois tipos de fármacos: 1) inibidores da acetilcolinesterase (donepezil, rivastigmina, galantamina e tacrina) e 2) antagonista de receptores de N-metil-D-aspartato (NMDA), como a memantina (Selkoe, 2004).

a) Os Inibidores da colinesterase

Os inibidores das colinesterases (IChE) compõe o principal grupo de medicações usadas para o tratamento sintomático da DA e determinam o aumento da disponibilidade da acetilcolina na fenda sináptica, levando ao aumento da transmissão colinérgica.

Do ponto de vista clínico, seu uso tem por objetivo primário o tratamento dos déficits cognitivos associados a esta doença e, secundariamente, pode auxiliar na melhora do déficit funcional e também na diminuição da incidência de sintomas comportamentais associados a estes quadros (Burns e O'Brien, 2006). Apesar das evidências de ensaios clínicos abertos em pacientes com DA demonstrarem que o uso crônico de IChE diminui a taxa de progressão dos déficits cognitivos e aumenta a preservação do *status* funcional, em relação aos pacientes que não fazem o uso da medicação, ou em relação às curvas projetadas de declínio cognitivo e funcional esperadas nesta doença (López-Pousa et al., 2005). Ainda existem numerosas controvérsias a respeito da significância clínica de seus efeitos terapêuticos e de seus resultados com relação a custo-benefício.

O principal mecanismo de ação dos IChEs é inibir as enzimas acetilcolinesterase e butirilcolinesterase, responsáveis pela clivagem da acetilcolina nas sinapses. Esta ação leva ao acúmulo de acetilcolina ou prolonga seu efeito nas sinapses, o que pode ser equivalente a uma estimulação excessiva dos receptores colinérgicos (muscarínicos e nicotínicos) em todo o sistema nervoso central e periférico (Jann et al, 2002).

A estrutura tridimensional dessas enzimas é bem conhecida. O centro ativo consiste de dois sítios, um aniônico e um esteárico (Figura 4). A acetilcolina se liga a ambos os sítios; já os IChEs ligam-se de uma maneira diferente a um só sítio ou a ambos. Atualmente, os IChEs são farmacologicamente classificados em três grupos baseados na duração da ligação, ou seja, da inibição: a) reversíveis ou de curta-duração (tacriana e donepezil); b) intermediários ou pseudo-reversíveis (Rivastigmina) e c) irreversíveis ou de longa-duração (metrifonato) (Jann et al, 2002).

Figura 4 – Modelo da estrutura tridimensional da enzima colinesterase e sua ação sobre a acetilcolina (adaptado de Jann et al., 2002).

A ação farmacológica dos inibidores de colinesterase vai além da mera inibição da colinesterase, por serem capazes de interferir com outros sistemas neurotransmissores, como os sistemas aminérgicos e glutamatérgicos. Além disso, recentes estudos provam que o tratamento com os inibidores da colinesterase apresentam efeitos neuroprotetores por interferir com os processos patológicos responsáveis pela morte dos neurônios corticais, além de diminuir a progressão da doença (Martorana et al, 2010).

Consequentemente, vale a pena discutir a eficácia dos fármacos usados para o tratamento da DA, frente aos recentes achados dos efeitos da acetilcolina e sua relação com outros sistemas neurotransmissores, bem como sua relevância para a deterioração da cognição.

A inibição da colinesterase pelos IChEs pode ser mensurada no sangue e apesar de mais difícil, também pode ser mensurada por tomografia de emissão de pósitron (PET). Porém no líquido cefalorraquidiano (LCR) essa inibição não é evidente. Estudos com pacientes tratados com tacrina, galantamina e donepezil, ao contrário do que ocorre no sangue, mostraram um aumento da atividade da enzima no LCR em níveis maiores do que antes do tratamento. A Rivastigmina, um IChE com atividade inibitória irreversível é o único que leva a uma diminuição da atividade da colinesterase no LCR (Nordberg, 2006).

A colinesterase é uma enzima que apresenta diversas isoformas moleculares, dentre elas podemos citar a colinesterase sináptica (AChE-S) e a retro colinesterase (AChE-R). A AChE-R tem mostrado efeitos neuroprotetores em ratos transgênicos, enquanto que um excesso de AChE-S aumenta a neurodegeneração. Na verdade a mensuração destas isoformas no LCR de

pacientes tem sido usada para inferir a atividade da enzima no cérebro. Em um estudo com um ano de tratamento com IChEs *versus* pacientes portadores de DA que não receberam tratamento, foi demonstrado que pacientes sem tratamento apresentaram uma diminuição das duas isoformas no LCR, espelhando a perda de neurônios colinérgicos no SNC. Enquanto que pacientes tratados apresentam um aumento das duas isoformas, aumento este atribuído a um mecanismo de *feedback* positivo por meio da regeneração de neurônios e ou receptores colinérgicos, resultado da estimulação dos receptores (Nordberg, 2006).

O uso de IChEs além da inibição da colinesterase, também tem mostrado efeito sobre a expressão de receptores nicotínicos. Na verdade, existe uma correlação positiva entre a melhora na atenção e o aumento do número de receptores nicotínicos, demonstrando que o IChEs tem propriedades modificadoras da doença de Alzheimer.

Diversos estudos (Racchi et al., 2001; Verhoeff, 2005) mostraram o efeito neuroprotetor dos IChEs contra a toxicidade do beta amiloide. Estes estudos evidenciam que os IChEs podem não só reduzir o acúmulo do A β como também, promover o processamento do APP pela via não amiloidogênica, levando a um aumento na formação da APP solúvel. Estes achados representam um novo mecanismo pelo qual os IChES podem alterar a patogênese da DA. Estudos com donepezil sugerem um aumento da atividade de α -secretases promovendo o tráfico destas enzimas (ADAM 20 e ADAM 17) bem como de APP para as membranas. Existe ainda evidências de aumento nos níveis de proteína quinase C (PKC), relacionada ao uso de IChEs que podem estar envolvidas na regulação da APP (Norberg, 2006).

A acetilcolina está envolvida nos processos de aprendizado e memória durante a vigília e também é essencial na consolidação da memória durante o sono, por isso seu aumento causado pelos IChEs promovem melhora das funções cognitivas e funcionais. Este dado salienta um novo aspecto do tratamento com IChEs que é a manutenção do ciclo sono vigília, que leva a uma redução das manifestações de desordens comportamentais e consequentemente do uso de terapias inapropriadas como por exemplo, os neurolépticos (Martorana et al., 2010).

Estas observações corroboraram a hipótese advinda dos estudos pré-clínicos, de que os IChE poderiam ter propriedades modificadoras da história natural da DA. Avaliar e mensurar essas modificações, são papéis atribuídos aos biomarcadores.

As Tabelas 1 e 2 resumizam os aspectos farmacodinâmicos e farmacocinéticos dos principais IChEs em uso para o tratamento da DA.

Tabela 1 - Parâmetros farmacocinéticos dos inibidores da colinesterase.

Medicamento	Biodisponibilidade (%)	T máx (h)	Ligação proteica (%)	T $\frac{1}{2}$ (h)	Metabolismo hepático	Linear/ Não linear
Tacrina	17-37	0,5-3,0	75	1,3-7,0	CYP1A2/CYP2D6	Não linear
Donepezil	100	3,0-5,0	96	60-90	CYP3A4/CYP2D6	Linear
Rivastigmina	40	0,8-17	40	2	Não hepático	Linear
Galantamina	85-100	0,5-1,5	18	5-7	CYP3A4/CYP2D6	Linear

Fonte: Jann et al, 2002

Tabela 2 - Parâmetros farmacodinâmicos dos inibidores da colinesterase.

Medicamento	Inibição da enzima		
	Seletividade	Dose	% inibição
Tacrina	BChE > AChE	160	40 (BChE)
Donepezil	BChE >>> AChE	10	70 (BChE)
Rivastigmina	BChE = AChE	12	62 (BChE) 62(AChE)
Galantamina	BChE >> AChE	NR	NR

Fonte: Jann et al, 2002

1.6 Biomarcadores

A principal característica de um biomarcador é que possa ser objetivamente medido e avaliado como um indicador dos processos biológicos normais, processos patogênicos ou respostas farmacológicas a uma intervenção terapêutica (Cummings, 2009). Para ser útil na prática clínica, a matriz de obtenção dos biomarcadores precisa ser facilmente obtida em um processo seguro e tolerável pelo paciente. Além disso, os métodos laboratoriais devem ser estáveis, confiáveis e econômicos (Di Lucca et al., 2005).

A procura por marcadores de diagnóstico e prognóstico da doença de Alzheimer tem sido o foco de diversos estudos nas últimas duas décadas. Para a DA, um biomarcador deve ser uma ferramenta diagnóstica sensível que detecte precocemente alterações cognitivas e biológicas; deve ser capaz de diferenciar DA do envelhecimento normal, de desordens afetivas, de pseudo-demências e também de outras demências como a Demência Vascular, Demência com corpúsculos de Lewy, entre outras. Esta ferramenta deve ser confiável, simples e facilmente aplicável (Di Lucca et al., 2005). Apesar de

nenhum biomarcador ter sido descrito e comprovado, há diversos candidatos a marcadores de DA que já foram correlacionados com características clínicas da doença, oferecendo informações complementares de diferentes aspectos da patofisiologia e história natural da doença.

As alterações bioquímicas e patológicas da DA são resultado de processos celulares como o metabolismo da proteína precursora do amiloide, fosforilação da Tau, de cascatas inflamatórias, estresse oxidativo e alterações homeostáticas da membrana, afetadas pelo metabolismo lipídico. Apesar de muitas pesquisas na busca de um marcador, até o presente momento não existem marcadores bioquímicos capazes de auxiliar com precisão o diagnóstico da DA na prática clínica (Padovani et al., 2001; Irizarry, 2004).

Recentemente houve avanços significativos no desenvolvimento de tecnologias para busca de biomarcadores de DA em líquido. A identificação de uma “assinatura DA” se mostrou eficiente para o diagnóstico precoce da doença (Hansson et al., 2006); porém, a especificidade desse achado ainda requer confirmação adicional, e há variações importantes nas análises *inter* e *intra*-laboratoriais que impedem o uso imediato dos biomarcadores líquidos na prática clínica (Mattsson et al., 2011).

Porém, para serem úteis à prática clínica os biomarcadores devem ser preferencialmente obtidos na periferia: sangue ou urina.

Das células do sangue, uma das matrizes mais estudadas são as plaquetas. Plaquetas e neurônios têm algumas funções homeostáticas em comum como, por exemplo, acumulação e síntese de neurotransmissores, resposta a variações de concentração de cálcio e expressão de compostos ligados à membrana (receptores e enzimas) (Haouari e Rosado, 2009).

A membrana celular das plaquetas contém diversos tipos de receptores de membrana que recebem estímulos externos ativando ou inibindo resposta celular (Holmsen, 1991). As plaquetas contém três tipos de grânulos: grânulos densos, α -grânulos e lisossomos. Estes grânulos e seus conteúdos são liberados após a ativação plaquetária que ocorre em resposta a estímulos fisiológicos (trombina, colágeno, adenosina difosfato (ADP), epinefrina, vasopressina e serotonina) ou não fisiológicos (Barry et al., 2003).

A possibilidade de usar as plaquetas como modelo neurofarmacológico surgiu em meados de 1950 com a descoberta que plaquetas acumulam serotonina (Barradas and Mikhailidis, 1993). Outros neurotransmissores também foram isolados das plaquetas como GABA, glutamato, aspartato e glicina, assim como seus transportadores e receptores. Desde então as plaquetas têm sido extensivamente utilizadas em estudos farmacológicos com o objetivo de identificar o mecanismo de ação de fármacos no sistema nervoso central (SNC).

Estudos mostraram que as plaquetas podem ser usadas como modelo periférico de vias metabólicas associadas à patogênese da DA, particularmente as vias da beta-amilóide e estresse oxidativo. As plaquetas são a fonte mais importante de APP circulante (Padovani et al., 2001) e outras importantes moléculas já relacionadas com a fisiopatologia da DA como, por exemplo, a proteína Tau (Neumann et al., 2011) e a glicogênio sintase-quinase 3 (GSK3) (Barry et al., 2003). Além disso, as plaquetas têm enzimas de clivagem da APP que são biologicamente ativas e respondem a estímulos sistêmicos. Portanto, as plaquetas parecem ser uma promissora matriz para se observar o

regulamento sistêmico do metabolismo da APP, na busca de marcadores da patologia e efeitos de intervenções farmacológicas.

Neste sentido, estudar as vias de sinalização desses biomarcadores, pode permitir o acesso a outros marcadores biológicos. Neste cenário podemos destacar a fosfolipase A_2 (PLA_2), a principal enzima responsável pelo metabolismo de fosfolípides de membrana (comprovadamente alterada na DA) e a glicogênio sintase-quinase (GSK), uma das principais Tau quinases, presente em vários sistemas biológicos, que são responsáveis pela regulação de diversas funções, participando de processos homeostáticos, que estariam alterados na DA.

1.6.1 Fosfolipase A_2 (PLA_2):

A fosfolipase A_2 (PLA_2) compreende uma superfamília de enzimas que catalisam a hidrólise da posição *sn*-2 dos fosfolípides de membranas celulares – plasmáticas e subcelulares, levando a produção de um ácido graxo livre e lisofosfolípides. Esta reação é de particular importância se o ácido graxo esterificado for o ácido araquidônico que é precursor de eicosanóides (Fenton et al., 2000; Kudo e Murakami, 2002). Embora o ácido araquidônico possa ser liberado dos fosfolípides por processos que não envolvam a PLA_2 , em muitas células a PLA_2 é o primeiro sistema enzimático que regula a sua liberação. O ácido araquidônico, assim como as prostaglandinas que deles resultam e os lisofosfolípides liberados pela PLA_2 , são importantes mediadores na

transmissão e no processamento de sinais neuronais (Piomelli, 1993; Bazan et al., 1993), modulando canais iônicos, atuando como segundos mensageiros, regulando a expressão gênica além de alterar a liberação e captação de neurotransmissores (Lautens et al., 1998).

1.6.2 Classificação das fosfolipases A₂

Cerca de vinte distintas PLA₂ de mamíferos foram identificadas e caracterizadas. Elas estão divididas em diversos grupos dependendo da sua estrutura, propriedade enzimática, localização e função celular: (Six e Dennis, 2000; Winstead et al., 2000; Kudo e Murakami, 2002; Chakraborti, 2003; Farooqui et al., 2004). Todavia, baseado nas propriedades biológicas, pode-se utilizar uma classificação simplificada dos três principais grupos: as PLA₂ secretórias ou PLA₂ extracelular dependente de Ca²⁺ (sPLA₂); PLA₂ citosólicas dependentes de Ca²⁺ (cPLA₂) e as PLA₂ intracelulares independentes de Ca²⁺ (iPLA₂). Existem ainda as PAF (fator ativador de plaquetas) acetil hidrolases e as PLA₂ Plasmalógeno-seletivas. Todas serão descritas mais detalhadamente a seguir.

a) PLA₂ extracelular dependente de Ca²⁺ (sPLA₂)

Subdividida em diversos grupos: PLA₂GIB, PLA₂GIIA, PLA₂GIID, PLA₂GIIE e PLA₂GIIF, PLA₂GIII, PLA₂GV, PLA₂GX, PLA₂GXII e PLA₂GXIII.

As PLA₂ extracelulares ou secretadas são muito semelhantes. Essas enzimas são caracterizadas por baixo peso molecular (12 – 18 KDa), um domínio catalítico e um domínio fortemente ligado ao íon Ca⁺² (*cálcio loop*), requerimento de Ca²⁺ em concentrações milimolares e por não apresentar nenhuma seletividade ao ácido graxo da posição *sn*-2. As sPLA₂ possuem ligações dissulfetos e um resíduo de histidina como grupo catalítico. A hidrólise do substrato é precedida pela ativação e orientação de uma molécula de água através de uma ponte de hidrogênio com o sítio ativo histidina. Junto à histidina existe um resíduo de aspartato, que juntamente com o sítio de ligação ao cálcio retêm o íon. Membranas ricas em esfingomielina dificultam a penetração da sPLA₂. A presença de fosfolípidos oxidáveis nas vesículas facilita a reação das sPLA₂. Oxidação dos fosfolípidos altera o estado fisiológico das membranas, o que afeta a suscetibilidade dos resíduos de ácidos graxos oxigenados e não oxigenados para o ataque da sPLA₂. As sPLA₂ estão envolvidas em vários processos biológicos tais como modificação na geração de eicosanóides, inflamação e defesa do hospedeiro (Kudo e Murakami, 2002).

No cérebro, as sPLA₂ estão presentes em todas as regiões, apresentando maior atividade na medula oblongata, hipocampo e córtex e, menor atividade no tálamo e hipotálamo (Farooqui et al., 2004).

b) PLA₂ intracelular citosólica dependente de Ca²⁺ (PLA₂ grupo IV, PLA₂GIV ou cPLA₂)

Subdividida em três grupos: PLA₂GIVA, PLA₂GIVB e PLA₂GIVC, com peso molecular de 85, 110 e 60 KDa, respectivamente, a PLA₂GIVA é a melhor caracterizada até o momento. Essa PLA₂ consiste de dois domínios funcionalmente distintos: (1) domínio de ligação a lípides de membrana dependente de Ca²⁺ no terminal amino (N-terminal), ou domínio C2 (138 aminoácidos), contendo os resíduos de ligação de Ca²⁺ e de ligação a membranas; e (2) domínio catalítico independente de Ca²⁺ no terminal carboxil (C-terminal), contendo os resíduos do sítio ativo (Nalefski et al., 1998). É a única PLA₂ que mostra seletividade significativa para fosfolípidos que possuem ácido araquidônico na posição *sn*-2, sendo o grau de insaturação, a posição da dupla ligação e o tamanho da cadeia do ácido graxo na posição *sn*-2 os responsáveis pela especificidade da enzima ao substrato. Concentrações micromolares de Ca²⁺ ativam a PLA₂GIVA, onde o íon permite o deslocamento da enzima até a vesícula de fosfolípide para a hidrólise interfacial (Kudo e Murakami, 2002). A PLA₂GIVA, por estar presente no citosol, precisa de algo que lhe dê acesso aos substratos fosfolipídicos, e é devido a isso que há a necessidade de íons Ca²⁺. O aumento na concentração interna desses íons ocorre pela mobilização de Ca²⁺ armazenado internamente e/ou pelo influxo de Ca²⁺ do espaço extracelular via canais operados por voltagem ou por receptores (Hirabayashi e Shimizu, 2000). O domínio C2 isolado da PLA₂GIVA humana mostrou uma seletividade para a ligação às vesículas de fosfolípidos com cabeça polar com características hidrofóbicas (cabeça polar neutra), como

fosfatidilcolina (PC) e fosfatidiletanolamina (PE) (Nalefski e Falke, 1996; Nalefski et al., 1998).

As PLA₂GIVA são expressas na maioria das células e tecidos, incluindo plaquetas. Sem dúvida, a PLA₂GIVA desempenha um papel essencial na liberação do ácido araquidônico dos fosfolípides suprindo as cascatas das ciclo-oxigenases e das lipoxigenases para a produção de eicosanóides, além de regular a produção de mediadores derivados de lisofosfolípides como o fator ativador de plaquetas (PAF). Algumas linhas de pesquisa têm associado a PLA₂GIVA com a regulação de apoptose, por mecanismos pouco conhecidos. Presumivelmente certos produtos da PLA₂GIVA, como eicosanóides ou lisofosfolípides, devem secundariamente afetar a viabilidade celular e a sua proliferação (Kudo e Murakami, 2002).

A PLA₂GIVB também está localizada no citosol, porém, na presença de íons Ca²⁺ desloca-se para as membranas. A liberação de ácido araquidônico do substrato *Phosphatidylcholine, L- α -1 palmitoyl-2-arachidonyl-[arachidonyl-1-¹⁴C]* (PC14C) pela PLA₂GIVB foi estritamente dependente de Ca²⁺, uma vez que não foi detectada atividade da enzima na presença do quelante de Ca²⁺, etileno glicol-bis-(b-amino-etil éter) (EGTA) (Song et al., 1999). Adicionalmente, foi observada uma diminuição da atividade desta PLA₂ de 5-10 vezes na presença de outro quelante de Ca²⁺, tetra acetato de etilenodiamina (EDTA), em relação à atividade observada na presença de Ca²⁺ 1,0 mM (Pickard et al., 1999). Tomados em conjunto, os achados descritos sugerem que a PLA₂GIVB pode responder a agonistas que mobilizam Ca²⁺ de maneira similar à PLA₂GIVA. A sensibilidade da PLA₂GIVB ao Ca²⁺ é provavelmente devida a

uma região na sua sequência de aminoácidos que possui uma semelhança ao domínio C2 da PLA₂GIVA (Song et al., 1999).

A PLA₂GIVC não contém o domínio C2 (Underwood et al., 1998; Pickard et al., 1999) o qual é importante para a ligação dependente de Ca²⁺ da PLA₂GIVA às membranas (Nalefski et al., 1994), indicando que a ativação da PLA₂GIVC é regulada por um mecanismo diferente daquele da PLA₂GIVA, bem como da PLA₂GIVB. A investigação da presença da PLA₂GIVC humana no sobrenadante ou no “pellet” de membranas de células de ovário de *hamster* chinês (CHO) ou de células de insetos Sf9 super expressando a enzima encontrou que a PLA₂GIVC localiza-se na fração de membranas (Underwood et al., 1998; Stewart et al., 2002). A determinação do requerimento de Ca²⁺ para a atividade da PLA₂GIVC em lisados de células COS que super expressam a enzima, incubados com o substrato PC14C, na ausência Ca²⁺ ou na presença de Ca²⁺ 10 mM, mostrou que a atividade da enzima não é afetada por Ca²⁺ (Underwood et al., 1998). Além disso, a atividade da PLA₂GIVC não foi afetada ao quelar o Ca²⁺ por EDTA (Pickard et al., 1999). Esses achados indicam que a PLA₂GIVC é uma enzima independente de Ca²⁺ e é fortemente ligada às membranas. Porém, pertence ao grupo de enzimas dependentes de Ca²⁺ devido a sua grande semelhança estrutural com as PLA₂ desse grupo, principalmente seu domínio catalítico, que é bem semelhante ao da PLA₂GIVA (Underwood et al., 1998).

Em membranas neuronais e em outros sistemas celulares, a ativação da PLA₂GIV e a consequente liberação de ácido araquidônico podem ocorrer por meio de diferentes mecanismos de ligações com receptores dopaminérgicos, serotoninérgicos, glutamatérgicos, citosinas e de fator de crescimento. Por

meio dessas ligações às diferentes cascatas de sinalizações a PLA₂GIV é uma importante PLA₂ para liberação rápida de ácido araquidônico nas células além de modular diversos processos intracelulares (Farooqui et al., 2004; Sun, et al., 2004).

Um novo grupo de PLA₂GIV foi descrito, a PLA₂GIVD, porém ela é expressa exclusivamente em epitélio escamoso estratificado (pele) e está marcadamente aumentada em casos de psoríase. Esta enzima apresenta grande similaridade e níveis de atividade próximos às PLA₂GIVB. Ela não mostra especificidade ao ácido araquidônico na posição *sn*-2 do substrato, mas sim ao ácido linolêico. Assim como as PLA₂GIVB, as PLA₂GIVD apresentam atividade dependente de Ca²⁺ (Sun et al., 2004).

c) PLA₂ intracelular independente de Ca²⁺ (PLA₂GVI ou iPLA₂): São subdivididas em PLA₂GVIA e PLA₂GVIB.

Essas enzimas são caracterizadas por peso molecular em torno de 80 kDa e por hidrolisar os ácidos graxos da posição *sn*-2 dos fosfolípides com a seguinte ordem de preferência: ácido linolêico > ácido palmítico > ácido olêico > ácido araquidônico. A PLA₂GVI tem uma única sequência de aminoácidos contendo uma lípase e sete ou oito repetições *ankyrin* (Kudo e Murakami 2002; Farooqui et al., 2004).

Embora a PLA₂GVI não tenha muita preferência em clivar fosfolípides com ácido araquidônico na posição *sn*-2, está diretamente envolvida na

regulação das reações de remodelamento de ácidos graxos nos fosfolípidos de membranas celulares, então deve influenciar a distribuição subcelular de ácido araquidônico entre os diferentes compartimentos, bem como a quantidade relativa de ácido graxo presente em cada compartimento celular (Balsinde et al., 1999).

A atividade da PLA₂GVIA foi determinada no sobrenadante ou no “pellet” de membranas de células COS que super expressam a enzima. PLA₂GVIA foi incubada com ambos os substratos PC¹⁴C e fosfatidiletanolamina marcada com ¹⁴C (PE14C), mostrando níveis de atividade similares na fração citosólica de células controles e de células que super expressam a PLA₂GVIA, enquanto que sua atividade na fração de membranas de células super expressando a enzima foi 5,5 vezes maior do que aquela em células controle. Foi observada uma correlação entre a atividade aumentada da PLA₂GVIA e a presença maior da enzima na fração de membranas, indicando que a PLA₂GVIA é uma proteína ligada a membranas (Larsson et al., 1999). A determinação do requerimento de Ca²⁺ para a atividade da PLA₂GVIA humana em lisados de células COS que super expressam a enzima, incubados com ambos os substratos PC¹⁴C e PE¹⁴C, na ausência de Ca²⁺ ou na presença de Ca²⁺ 5,0 mM, mostrou que a atividade da enzima foi significativamente maior na ausência de Ca²⁺ (Larsson et al., 1998).

Na determinação da atividade da PLA₂GVIB no sobrenadante ou no “pellet” de membranas de células Sf9 ou de células COS que super expressam a enzima, foram observados níveis similares tanto na fração citosólica de células controles quanto na de células que super expressam a PLA₂GVIB. A atividade dessa enzima foi detectada predominantemente na fração de

membranas. A determinação do requerimento de Ca^{2+} para a atividade da PLA_2GVIB humana no “pellet” de membranas de células Sf9 super expressando a enzima, incubado com fosfatidilcolina sintética como substrato, na ausência ou na presença de Ca^{2+} (variando de 0-10 mM), mostrou que a atividade da PLA_2GVIB foi independente de Ca^{2+} (Mancuso et al., 2000; Tanaka et al., 2000).

d) PAF-acetilhidrolases (plasmáticas): Subdivididas em PLA_2GVII (PLA_2GVIIA e $\text{PLA}_2\text{GVIIIB}$) e PLA_2GVIII ($\text{PLA}_2\text{GVIIIA}$ e $\text{PLA}_2\text{GVIIIB}$).

Essas enzimas exibem um substrato específico, são as chamadas PAF (fator ativador de plaquetas) acetilhidrolases (PAF-AH) que hidrolisam e inativam o mediador lipídico PAF. A degradação desses fosfolípides bioativos pela PAF-AH pode levar a alteração da reação inflamatória e arteriosclerose (Farooqui et al., 1999; Kudo e Murakami, 2002).

e) PLA_2 Plasmalógeno seletiva: PlsEtn-PLA_2

Essas enzimas são caracterizadas por peso molecular em torno de 39 kDa. Elas hidrolisam ácido araquidônico e DHA da posição *sn*-2 dos plasmalógenos, um tipo especial de fosfolípides com um vinil éster ligado à

posição *sn*-1 do glicerol, liberando um lisoplasmalógeno. DHA é metabolizado pelas ciclo-oxigenases e lipoxigenases liberando docosanóides (resolvins, decosatrienos e neuroprotectinas), os quais possuem propriedades antiinflamatórias e neuroprotetoras (Farooqui e Horrocks, 2006). As PLA₂ plasmalógeno seletivas diferem das demais PLA₂ intracelulares não somente no peso molecular, mas também, nas propriedades cinéticas, especificidade ao substrato e resposta aos inibidores. (Farooqui e Horrocks, 2003; 2006). As PlsEts-PLA₂ localizam-se no citosol e apresentam atividade independente de Ca²⁺ porém, não são inibidas pelo Bromoenol lactone (BEL), potente inibidor das PLA₂GVI. Essas enzimas são inibidas por ATP (adenosina trifosfato) em concentrações milimolares, ou seja, concentrações intracelulares normais, diferentemente das PLA₂GVI que são ativadas na presença do nucleotídeo (Farooqui, 2004; 2001).

Assim, alterações na atividade da PLA₂ podem ser relevantes para o surgimento e para o curso de diferentes doenças neuropsiquiátricas. De fato, distúrbios da PLA₂ já foram descritos em pacientes com epilepsia do lobo temporal (Simonato, 1993; Gattaz et al., 2011), esquizofrenia (Gattaz et al., 1987; Gattaz et al., 1995a; Ross et al., 1999; Tavares et al., 2003), e doença de Alzheimer (Gattaz et al., 1995b; Ross et al., 1998).

Estudos de Gattaz et al. (1996), mostram uma correlação entre a diminuição da PLA₂ e o número de placas senis no cérebro de pacientes com DA. Além disso, sugere-se uma relação entre a diminuição da PLA₂ e a gravidade clínica da DA, visto que a redução da atividade da enzima correlacionava-se com início da doença e com óbitos mais precoces. Neste estudo também foi mostrado que em plaquetas de pacientes com DA a

atividade da PLA₂ está reduzida quando comparada com controles sadios e controles psiquiátricos, sugerindo assim uma especificidade do achado para a DA. A redução da atividade da enzima em plaquetas também se correlacionava com a gravidade da doença.

Também foi investigada a atividade da PLA₂ em pacientes com a DA, comparados com pacientes com Comprometimento Cognitivo Leve (CCL) e controles sadios (Gattaz et al., 2004). A atividade da PLA₂ estava reduzida em pacientes com DA comparadas com controles e com CCL. A diminuição da PLA₂ também estava correlacionada com um pior rendimento cognitivo.

A PLA₂ influencia o processamento e a secreção da APP. Uma ativação da PLA₂ aumenta a liberação da APP solúvel no meio extracelular (Emmerling et al., 1993, Cho et al., 2006). Como o aumento da secreção da α -APPs diminui a formação do A β , é razoável supormos que uma redução da PLA₂ diminui a secreção da APP, contribuindo assim, para um aumento da produção do A β .

1.6.3 Glicogênio Sintase Quinase - GSK3B

A glicogênio sintase quinase 3 (GSK3) foi descrita inicialmente como uma enzima que fosforila a enzima glicogênio sintase que, por sua vez, tem papel fundamental na síntese do glicogênio. Atualmente sabe-se, que apesar do nome restritivo, a GSK3 exerce diferentes funções no metabolismo celular que vão desde a sobrevivência celular, metabolismo e processamento de proteínas, até processos cognitivos (Grimes e Jope, 2001; Jope e Johnson,

2004; Jope e Roh, 2006). Na célula, influencia a arquitetura e a plasticidade, sendo fundamental em mecanismos como: divisão, proliferação, diferenciação e adesão celular (Grimes e Jope, 2001; Jope e Roh, 2006; Peineau et al., 2008). Por ter um papel de regulação de alguns fatores de transcrição, regula também a expressão gênica e está envolvida com apoptose e sobrevivência celular (Grimes e Jope, 2001; Planel et al., 2002; Li et al., 2004, 2007).

A enzima tem duas isoformas principais: GSK3A (51 KDa) e GSK3B (46 KDa), que são codificadas de maneiras diferentes, porém reguladas de forma semelhante (Frame et al., 2001; Fuentealba et al., 2004). A GSK3B é a isoforma mais abundante no organismo, altamente expressa no sistema nervoso central, tendo fundamental importância na homeostase cerebral. O principal mecanismo de regulação da GSK é a fosforilação da enzima (Li et al., 2004; Jope e Roh, 2006). No caso da GSK3B, a fosforilação da porção tyr216 (tirosina 216) é necessária para sua atividade básica e altos níveis de fosforilação resultam em ativação nas células em repouso. Um segundo nível de fosforilação, agora na porção ser9 (serina 9), por diversas quinases, leva à inativação da GSK3B, mesmo que a porção tyr216 esteja também fosforilada. Caso haja uma defosforilação da porção ser9 a enzima será ativada (Peineau et al., 2008). A fosforilação da porção serina pode ser mediada por diferentes quinases, ou seja, diversos sistemas de sinalização convergem no sentido de controlar a atividade da GSK3B, contribuindo para a regulação de suas funções celulares específicas (Li et al., 2006). A ativação da GSK3B pode ser uma condição prejudicial à plasticidade, estrutura e sobrevivência neural, portanto, a inativação da enzima pode ser relacionada à neuroproteção (Li et al., 2004).

A enzima GSK3B tem papel central na DA, já que a sua atividade está associada a diversos aspectos da fisiopatologia desta doença (Hooper et al., 2008). O aumento da atividade da enzima GSK3B leva a maior produção da proteína β -amilóide e também a fosforilação proteína Tau (Li et al., 2004; Rametti et al., 2008). Por outro lado, a sua inibição implica em menor produção da proteína β -amilóide, reduzindo assim a neurotoxicidade associada a esta proteína (Koh et al., 2008).

Em vista do papel fundamental destas enzimas na formação do β -amilóide, podemos dizer que tanto a PLA₂, como a GSK3B, poderiam servir como marcadores periféricos para a DA em si, ou como preditores de sua gravidade, como por exemplo, DA de início precoce.

1.7 PLA₂, GSK3B e Doença de Alzheimer

Blusztajn et al, (1987) sublinharam o significado do metabolismo dos fosfolípidos para a síntese do neurotransmissor acetilcolina. O metabolismo da fosfatidilcolina (PC) da membrana contribui para a síntese da acetilcolina (Blusztajn e Wurtman 1983). A PC é o substrato natural da PLA₂. Uma redução da PLA₂ resulta em uma redução do metabolismo da PC, podendo contribuir assim para um déficit colinérgico. Esse déficit, primariamente resultante de uma degeneração de neurônios colinérgicos, é discutido como aspecto central do distúrbio cognitivo na DA.

Existem evidências em estudos pré-clínicos demonstrando que a ativação de receptores muscarínicos leva à defosforilação da proteína Tau em

um processo que depende da inibição da atividade da GSK3B (Forlenza et al., 2000). Portanto, um dos mecanismos pelo qual os IChE poderiam exercer sua atividade modificadora da história natural da DA seria por meio da modulação da atividade desta enzima. Porém, até o momento, não há dados na literatura acerca de estudos clínicos que avaliam a relação entre o tratamento com IChE e a atividade da GSK3B em pacientes com DA.

Embora o papel de GSK3 na fisiopatologia de certas doenças neuropsiquiátricas permaneça desconhecido, sugere-se seu potencial como alvo para diferentes fármacos (Beaulieu, 2007). De fato, Forlenza et al. (2000) encontraram uma diminuição da fosforilação da proteína tau, via GSK3 β , ao usar agonistas de receptores muscarínicos em culturas de células.

2 OBJETIVOS

- 1) Avaliar o efeito do tratamento com IChE sobre a atividade da PLA₂ e a expressão da GSK3B em pacientes portadores de DA leve e moderada.
- 2) Estudar a correlação entre resposta terapêutica aos IChE e alterações das atividades da GSK3B e da PLA₂ durante o tratamento com IChE.

3 MATERIAL E MÉTODOS

3.1 Casuística

Os pacientes e controles para este estudo foram recrutados no ambulatório de Psiquiatria Geriátrica do Laboratório de Neurociências – LIM 27, Departamento e Instituto de Psiquiatria, USP. Foram recrutados cinquenta e sete pacientes com diagnóstico de doença de Alzheimer (DA) de acordo com os critérios diagnósticos do NINCDS-ADRDA (McKann et al., 1984) em estágio leve a moderado. Nove pacientes foram excluídos na primeira fase do estudo devido à presença de outras comorbidades, como deficiência visual grave, quadros depressivos, demência fronto-temporal, demência mista e quadro de DA grave. Quarenta e oito pacientes realizaram a coleta de sangue na avaliação basal, 35 realizaram a segunda coleta (3 meses após o início do tratamento) e 30 pacientes completaram as 3 visitas (6 meses após o início do tratamento) (Figura 5). Dezesete pacientes descontinuaram o estudo por não adesão ao tratamento, uma paciente foi internada pela família.

Figura 5 - Fluxograma que descreve o recrutamento dos pacientes com DA. T1 e T2 = 3 e 6 meses após o início do tratamento, respectivamente.

Quarenta e dois idosos sem evidência de transtornos cognitivos ou psiquiátricos foram incluídos como grupo controle.

Os pacientes com DA não faziam uso de IChEs no momento da avaliação diagnóstica inicial.

Para determinação da atividade da PLA_2 no T0 (*baseline*) foram avaliados 38 controles e 48 pacientes com doença de Alzheimer. Para a determinação no *baseline* (T0) da concentração da GSK3B total e GSK3B fosforilada foram avaliados 48 pacientes com DA e 28 controles saudáveis. Nos tempos T1 e T2 foram avaliados 30 pacientes que completaram o estudo e 28 controles saudáveis.

3.2 Avaliação diagnóstica

Todos os pacientes e controles tiveram avaliação neuropsiquiátrica completa e avaliação neuropsicológica antes do estabelecimento do diagnóstico cognitivo. Todos os indivíduos incluídos neste trabalho foram avaliados pela escala *Clinical Dementia Rating* (CDR) para determinar a gravidade dos déficits cognitivos e funcionais. Todos os pacientes com DA foram classificados como CDR 1 ou 2. Todos os sujeitos no grupo controle foram classificados como CDR 0.

O teste cognitivo de Cambridge (CAMCOG) e o Mini Exame do Estado Mental (MEEM) foram usados como medida de desempenho cognitivo global. A pontuação em ambos os testes não foi usada para o estabelecimento do diagnóstico dos pacientes. Ambos os testes foram usados para a avaliação da resposta ao tratamento com IChEs.

Como critério de melhora durante o tratamento adotamos o aumento maior ou igual a um ponto nos scores do MEEM e CAMCOG.

Todos os indivíduos incluídos no estudo realizaram exames laboratoriais de rotina (hemograma completo, função tireoidiana, função renal e hepática, perfil lipídico, dosagem de ácido fólico e vitamina B12 e sorologia para sífilis) e de imagem (tomografia computadorizada ou ressonância magnética) para afastar outras possíveis causas de demência.

Os pacientes com DA recrutados não estavam em tratamento com IChE nem tinham feito uso dos mesmos.

Os critérios de exclusão deste estudo foram:

- 1) Deficiências sensoriais e intelectuais que os impedissem de desempenhar adequadamente os testes necessários para avaliação das funções cognitivas.
- 2) Quadro demencial pré-existente.
- 3) Doenças clínicas ou neurológicas com impacto sobre a capacidade cognitiva.
- 4) Diagnóstico de doenças neurodegenerativas do Sistema Nervoso Central (ex. Doença de Parkinson).
- 5) Evidência clínica prévia de Ataque Isquêmico Transitório ou Acidente Vascular Cerebral.
- 6) Pacientes clinicamente instáveis ou com saúde frágil no momento da avaliação inicial.
- 7) Outros transtornos psiquiátricos maiores, tais como esquizofrenia, transtorno bipolar.
- 8) História ou evidências de alcoolismo ou abuso de substâncias depressoras do sistema nervoso central.
- 9) Uso recente ou atual de medicamentos com propriedades anticolinérgicas.
- 10) Recusa em assinar o termo de consentimento livre e esclarecido.

3.3 Desenho

Após a inclusão no estudo, os pacientes iniciaram tratamento com Donepezil 5 mg/dia, aumentando para 10 mg/dia após 1 mês de tratamento.

Neste estudo prospectivo de 6 meses de seguimento, foram feitas avaliações nos seguintes pontos:

- Avaliação basal (T0).
- Avaliação de 3 meses (T1).
- Avaliação de 6 meses (T2).
- A coleta de sangue para determinação da atividade da PLA₂, e expressão da GSK3B em plaquetas foi feita na avaliação basal e repetida longitudinalmente, após 3 e 6 meses.

Não houve diferença estatística quanto à idade e o sexo, entre os pacientes com DA (n = 48) e o grupo controle. A escolaridade foi avaliada quanto aos anos de estudo e o grupo DA apresentou escolaridade mais baixa (6 anos), diferindo significativamente do grupo controle, com média de 14 anos (p= 0,001). Quanto aos parâmetros cognitivos, como esperado e por definição, o grupo DA foi significativamente diferente em relação ao grupo controle nos resultados de CAMCOG e MEEM (p< 0,0001) tanto na avaliação basal quanto no grupo que completou os seis meses do estudo (n = 30) (Tabelas 3 e 4).

Tabela 3 - Descrição da amostra e distribuição dos indivíduos envolvidos no estudo no baseline.

		Diagnóstico		P
		Controles (n=42)	DA (n=48)	
Gênero	Masculino	12	13	0,653
	Feminino	30	35	
Idade		71,92(6,6)	72,25(7,4)	0,510
Escolaridade		13,3 (5,1)	6,06 (3,5)	0,001
MEEM		28,9 (1,6)	17,9 (4,3)	0,0001
CAMCOG		96,9 (6,2)	54,1 (15,6)	0,0001

Tabela 4 - Descrição da amostra e distribuição dos pacientes que completaram o estudo e controles.

		Diagnóstico		P
		Controles (n=42)	DA (n=30)	
Gênero	Masculino	12	11	0,718
	Feminino	30	19	
Idade		71,92(6,6)	74,11(6,7)	0,293
Escolaridade		13,3 (5,1)	5,8 (3,3)	0,001
MEEM		28,9 (1,6)	18,9 (5,3)	0,0001
CAMCOG		96,9 (6,2)	56,2 (16,6)	0,0001

3.4 Métodos

3.4.1 Separação e lavagem das Plaquetas

Foram coletados 40 mL de sangue em quatro tubos com capacidade de 10 mL, contendo anticoagulante citrato de sódio 0,106 mol/L. A cada tubo foi adicionado 1 mL de ACD-NH. Os tubos foram homogeneizados delicadamente por inversão e centrifugados durante 15 minutos a 1600 rpm a 20 °C. A seguir, os sobrenadantes (Plasma Rico em Plaquetas - PRP) foram transferidos para outro tubo com capacidade de 50 mL (tipo falcon) e o pH ajustado para 6,5 com ACD-NH. O PRP foi, então, transferido para 4 tubos de poliestireno (4 a 5 mL/tubo) e centrifugados durante 10 minutos a 2400 rpm à 20 °C. O sobrenadante foi removido cuidadosamente por inversão e adicionou-se ao *pellet* 2,5 mL de solução de lavagem.

Após descanso de 10 minutos, as plaquetas foram homogeneizadas cuidadosamente com uma pipeta Pasteur até total diluição e então foram

adicionados mais 2,5 mL de solução de lavagem. A mistura foi centrifugada por 8 minutos a 2400 rpm, o sobrenadante foi removido cuidadosamente, e o *pellet* ressuspenso com 0,5 mL de tris-sacarose. As plaquetas em tris-sacarose foram armazenadas em 4 criotubos com 0,5 mL e mantidas em freezer -70°C .

3.4.2 Determinação de proteína das amostras

Após o preparo da amostra, uma alíquota de 50 μL foi destinada a determinação de proteínas totais. As proteínas foram quantificadas utilizando o kit *Bio-Rad DC Protein assay* (*Bio-Rad Hercules*) que é um ensaio colorimétrico para a determinação de proteínas, baseado no método de *Lowry* (*Lowry et al.*, 1951). O ensaio consiste na reação de proteínas com uma solução alcalina de tartarato de cobre (Reagente A) e com o reagente de Folin (*1,2-naphthoquinone-4-sulfonate*) (Reagente B). São duas etapas que levam ao aparecimento da cor cuja absorbância foi lida a 680 nm (*Peterson*, 1979). A determinação da concentração de proteínas totais foi obtida por meio de uma curva padrão, empregando-se albumina de soro bovino (*bovine serum albumin* ou BSA; *Sigma-Aldrich*) nas concentrações: 0,1 mg/mL, 0,2 mg/mL, 0,5 mg/mL, 1,0 mg/mL e 1,5 mg/mL.

3.4.3 Determinação da Atividade da PLA₂

O método utilizado para determinar a atividade da PLA₂ foi o rádio-enzimático, descrito previamente (Flesch et al., 1985; Gattaz et al., 1995a) e modificado por Talib e colaboradores (2012). Os substratos da enzima utilizados foram o L- α -1-palmitoil-2-araquidonil-fosfatidilcolina marcada com [1-¹⁴C] na cauda araquidonil na posição *sn*-2 (araquidonil-1-¹⁴C-PC) (PerkinElmer, Boston, MA) e o *L*-3-Phosphatidylcholine, 1-palmitoyl-2-[1-¹⁴C]palmitoyl. Neste ensaio, o [1-¹⁴C]-ácido graxo, ligado à posição *sn*-2 da fosfatidilcolina, é clivado pela PLA₂ e extraído. A radioatividade do [1-¹⁴C]-ácido graxo liberado é medida em um contador de cintilação líquida e utilizada para calcular a atividade da PLA₂.

Esse método pode ser utilizado para diversas amostras, como homogenato de tecido cerebral, cultura de células ou plaquetas. Para o preparo da amostra é imprescindível utilizar um tampão de lise (tris-sacarose) para romper as membranas e, assim, permitir a incorporação do substrato marcado aos fragmentos de membrana. É necessário também adicionar um tampão para formar lipossomos (Tris-HCl 50 mM) para simular um ambiente celular, unindo os fragmentos de membranas contendo o substrato marcado. Para se formar lipossomos, é importante agitar e sonicar o substrato fosfolipídico em meio aquoso.

3.4.4 Ensaio radio-enzimático

As amostras previamente armazenadas em *freezer* -70°C foram descongeladas e mantidas em gelo. Após determinação dos níveis de proteínas, foi pipetado um volume de cada amostra, necessário para obter uma concentração de proteína de $0,2\text{ mg/mL}$, completamos o volume para $400\text{ }\mu\text{L}$ com Tris-sacarose pH 7,4 (tampão de lise) e $600\text{ }\mu\text{L}$ de Tris 50 mM pH 8,5 (tampão para formar os lipossomos). A amostra referente ao branco foi composta por $400\text{ }\mu\text{L}$ de Tris-Sacarose pH 7,4 e $600\text{ }\mu\text{L}$ de Tris 50 mM. Cada amostra foi analisada em triplicata. Após homogeneização foi pipetado em cada tubo: $100\text{ }\mu\text{L}$ de tampão/ CaCl_2 / EDTA, $50\text{ }\mu\text{L}$ de Tris-HCl 1,0 M, $200\text{ }\mu\text{L}$ da amostra diluída (ou do branco) e $150\text{ }\mu\text{L}$ ($0,06\text{ }\mu\text{Ci}$) do substrato radioativo.

A mistura foi homogeneizada novamente e incubada por 30 min a 37°C em banho-maria sob agitação. Após a incubação, as amostras foram colocadas imediatamente em banho de gelo e foram adicionados $700\text{ }\mu\text{L}$ de solução de parada (isopropanol: HCl), para interromper a reação. Em seguida as amostras foram levadas à temperatura ambiente e após 10 min de repouso, adicionadas de $700\text{ }\mu\text{L}$ de *n*-heptano, para extração do ácido graxo marcado por meio de agitação rigorosa em vórtex por 30 seg. A mistura foi então centrifugada a 4000 rpm por 10 min à temperatura ambiente. Transferiu-se $500\text{ }\mu\text{L}$ da fase orgânica para tubos contendo 60 mg de sílica gel e $300\text{ }\mu\text{L}$ de *n*-heptano para reter os fosfolípidos e centrifugou-se novamente a 4000 rpm por 5 min a temperatura ambiente. Transferiu-se $500\text{ }\mu\text{L}$ do sobrenadante para tubos contendo 6,0 mL de líquido de cintilação e após homogeneização, colocou-se para contar em um

contador de cintilação líquida (Tri-Carb 2100 TR) na seguinte ordem: Branco, 100% (somente o substrato marcado) e amostras. Os resultados foram fornecidos em CPM e, então, corrigidos para pmol/mg de proteína/min.

3.5 Análise estatística

Para análise estatística foi utilizado o programa SPSS para Windows, (versão 14 SPSS, Inc. Chicago, IL). Inicialmente foi realizada a análise descritiva da amostra total. O teste t de *Student* (ou teste de *Mann-Whitney*) foi usado para comparar a diferença entre as variáveis contínuas entre os dois grupos (DA vs. controles) na avaliação inicial. Para verificar a normalidade da amostra foi utilizado o teste de Kolmogorov Smirnov. Para controlar por escolaridade as diferenças da PLA₂ e GSK3B entre controle e DA foi utilizado o teste de regressão logística. O efeito do IChE sobre a atividade da GSK3B e da PLA₂ foi avaliado por meio da análise de Anova de medidas repetidas com correção de Bonferroni para análise de 2 tempos e Huynh-Feldt para todos os tempos juntos. Foram realizadas análises multivariadas para avaliar o efeito do tratamento com IChE sobre a atividade da GSK3B e da PLA₂ e dos parâmetros cognitivos nos pacientes com DA.

4 RESULTADOS

4.2 Atividade da PLA₂

A atividade da iPLA₂ estava reduzida nos pacientes com DA em relação aos controles ($p < 0,001$) (Figura 6a). O tratamento com donepezil aumentou a atividade da iPLA₂ após 3 meses ($p = 0,015$) e após 6 meses (T0-T2 $p = 0,001$ e T1-T2 $p = 0,021$) (Figura 6b). Ao final dos 6 meses de tratamento, não havia diferenças na iPLA₂ entre pacientes e controles (Figura 6c).

Referente aos outros grupos da PLA₂ (cPLA₂ e sPLA₂) não encontramos diferenças entre pacientes e controles nas medidas basais e nem um efeito do donepezil durante os 6 meses de tratamento. (Figuras 7, 8 e 9).

Figura 6 – Atividade da iPLA₂ em plaquetas: (a) avaliação basal de pacientes e controles (b) avaliação dos pacientes longitudinalmente (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente) (c). Controles versus paciente após 6 meses de tratamento. As barras representam as médias ($\pm$ erro padrão).

Figura 7 – Atividade da cPLA₂ em plaquetas do grupo controle e dos pacientes longitudinalmete (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente). As barras representam as médias ($\pm$ erro padrão).

Figura 8 – Atividade da sPLA₂ em plaquetas do grupo controle e dos pacientes longitudinalmete (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente). As barras representam as médias ($\pm$ erro padrão).

Figura 9 – Atividade da PLA₂ total em plaquetas do grupo controle e dos pacientes longitudinalmente (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente) A PLA₂ total representa a somatória da i,c e sPLA₂. As barras representam as médias ($\pm$ erro padrão).

4.3 Concentração de GSK3B

Não encontramos diferenças entre pacientes com DA e controles na avaliação basal para GSK3B total e GSK3B fosforilada (Figuras 10 e 11). Todavia o tratamento de 6 meses com donepezil aumentou a forma fosforilada (inativa) da enzima ($p = 0,02$). Não encontramos diferenças na rGSK entre pacientes e controles, nem observamos um efeito do tratamento na rGSK (Figuras 10, 11 e 12).

Figura 10 – Expressão da GSKtotal nos grupos controles e dos pacientes longitudinalmete (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente). As barras representam as médias ($\pm$ erro padrão).

Figura 11 – Expressão da GSKfosforilada nos grupos controles e dos pacientes longitudinalmete (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente). As barras representam as médias ($\pm$ erro padrão).

Figura 12 – Razão da GSKfosforilada sobre a GSKtotal, inferindo a atividade da GSK3B, nos grupos controles e dos pacientes longitudinalmente (T0 - avaliação basal; T1 e T2 - avaliações de 3 e 6 meses respectivamente). As barras representam as médias ($\pm$ erro padrão).

4.4 Parâmetros cognitivos e biomarcadores

Os pacientes foram submetidos ao MEEM nas três visitas, concomitantemente à coleta de sangue e ao CAMCOG no T0 e T2. As variações no MEEM e no CAMCOG durante o tratamento não foram significantes (Figuras 13 e 14). Para o MEEM 64% dos pacientes tiveram aumento de 1 ponto ou mais no T1 e 46% no T2. Com relação ao CAMCOG 50% dos pacientes apresentaram melhora de pelo menos 1 ponto após 6 meses de tratamento.

Figura 13 – Variação dos scores do MEEM nas avaliações longitudinais.

Figura 14 – Variação dos scores do CAMCOG na avaliação basal e após seis meses de tratamento.

Pacientes que tiveram uma melhora no CAMCOG após 6 meses de tratamento tiveram um aumento mais acentuado de $iPLA_2$ entre o T0 e T1 ($p=0,037$) (Figura 15). Não houve relação entre melhora no MEEM entre o T0, T1 e T2 com mudanças na $iPLA_2$ nestes períodos.

Figura 15 – Pacientes que melhoraram no CAMCOG com seis meses de tratamento foram os que tiveram aumento de $iPLA_2$ mais acentuada no primeiro trimestre de tratamento ($p = 0,037$).

Não encontramos correlações com nenhum dos outros biomarcadores analisados.

5 DISCUSSÃO

O objetivo deste trabalho foi avaliar o efeito do tratamento com donepezil sobre a PLA₂ e a GSK3B, em plaquetas de pacientes com doença de Alzheimer.

PLA₂

Quando comparamos a atividade basal da PLA₂ em plaquetas dos pacientes com DA ao grupo controle, os resultados do presente estudo confirmaram os resultados anteriores de nosso grupo, ou seja, uma diminuição da atividade da PLA₂ em DA quando comparada aos controles (Gattaz et al., 1995b; Gattaz et al., 1996 e Gattaz et al., 2004). Entre as PLA₂, a iPLA₂ é a mais abundante nos neurônios (Forlenza et al., 2007). Valendo salientar que em estudo recente encontramos uma alta correlação entre a atividade de PLA₂ em plaquetas e tecido cerebral, utilizando amostras de tecido e plaquetas de pacientes que foram submetidos à lobectomia para tratar epilepsia resistente à terapia (Talib et al. 2013). Portanto, nossos achados em plaquetas pode refletir a atividade desta enzima no cérebro, ou seja, como marcador de doença visto que a baixa atividade da iPLA₂ tem sido associada a várias alterações relacionados a fisiopatologia da DA.

Em modelos celulares e em animais, a inibição da atividade de iPLA₂ leva a diminuição da formação de LTP (*Long Term Potentiation*) e prejuízo de memória, reduzindo a formação de neurites em neurônios hipocampais, além de, aumentar a produção de β -amilóide e fosforilação da Tau em serina 214 (De Paula et al, 2009) e participar das disfunções colinérgicas e glutamatérgicas observada nas fases iniciais da DA (Forlenza et al., 2002; Forlenza et al., 2007; Schaeffer et al., 2007; Schaeffer et al., 2008; Schaeffer et

al., 2011). Mais recentemente, Smesny et al. (2008) demonstrou pela primeira vez a atividade de PLA₂ intracelular (c e iPLA₂), reduzida no líquido de pacientes com DA, corroborando as conclusões anteriores. Além disso, a estimulação cognitiva em idosos saudáveis assim como em roedores levou a um aumento da atividade da PLA₂ (Talib et al., 2008, Schaeffer et al 2009), o que sugere o envolvimento da PLA₂ em uma das estratégias usadas na reabilitação de pacientes com DA. O tratamento farmacológico com inibidor da colinesterase, também induziu o aumento da iPLA₂ e este aumento também parece estar correlacionado com a melhora clínica.

O uso de inibidores da colinesterase vai além de seus efeitos paliativos. Na verdade, esses agentes vêm sendo considerados como agentes modificadores da doença. Os inibidores da colinesterase atrasam a deterioração cognitiva e funcional em pacientes com DA leve a moderada e sua efetividade é maior quando o tratamento é iniciado o mais cedo possível (Molinuevo et al., 2011). Diversos estudos têm mostrado as ações biológicas destes fármacos, que nem sempre estão correlacionadas com a melhora clínica. Na verdade esses estudos vêm avaliando diferentes preditores da resposta aos IChEs, como a influência do genótipo APOE, metabólitos no líquido, neuroimagem, biomarcadores no líquido (Tau total, Tau fosforilada e Aβ₄₂). Além dos preditores, esses estudos apresentam diversos desenhos como: diferentes períodos de avaliação, utilização de grupos de pacientes recebendo placebo, existência ou não de grupos controles (Lancôt et al., 2003). Todas essas variáveis levam a achados diferentes, não permitindo avaliar consensualmente o papel neuroprotetor atribuído aos IChEs.

As evidências mais convincentes do efeito neuroprotetor dos IChEs se deve não só ao fato de interferir com a síntese do peptídeo β -amilóide, mas também por interferir em mecanismos de morte celular como: toxicidade por glutamato, disfunção mitocondrial e produção de radicais livres (Martorana et al., 2010).

Para este estudo foi escolhido o IChE donepezil, uma piperidina que tem propriedades competitiva e não competitiva, ou seja, reversível. Esse fármaco possui a vantagem de não necessitar de um escalonamento de dose para atingir a dose ideal, iniciando o tratamento com 5 mg/dia, que já é uma dose terapêutica e, rapidamente incrementando para 10 mg/dia, dose máxima recomendada pelas nossas agências de regulamentação. Em estudos clínicos, o donepezil tem mostrado melhora ou redução no declínio cognitivo como também no funcionamento global com efeitos adversos mínimos. Nem todos os pacientes apresentam melhora cognitiva ou comportamental com o uso de inibidores da colinesterase, portanto entender a correlação de respostas, estudando os efeitos biológicos pode ajudar a explicar os mecanismos de respostas desenvolvidos pelos pacientes.

Nossos resultados mostram que os pacientes que melhoraram no CAMCOG após seis meses de tratamento foram os que tiveram aumento mais expressivo de iPLA₂ nos três primeiros meses de tratamento. O fato do aumento da iPLA₂ anteceder a melhora cognitiva está em linha com nosso estudo prospectivo de 4 anos, onde mostramos que pacientes com comprometimento cognitivo leve (CCL) com atividade da iPLA₂ diminuída eram aqueles com maior risco de conversão para DA após 4 anos, e que esta diminuição da atividade da enzima antecedia a deterioração cognitiva global,

visto que não havia diferenças nas avaliações do CAMCOG ou do MEEM dos pacientes com CCL que converteram para DA e dos que não converteram (Gattaz et al., 2013).

Evidências sugerem a presença de um sistema regulatório mútuo entre quinases e fosfatases, sendo que, a maioria dos estudos foca mais na participação de quinases nas vias de sinalização. Neste sentido a proteína fosfatase 2A (PP2A) juntamente com a proteína fosfatase 1A (PP1A) são consideradas as fosfatases dominantes *in vivo* (Moscardó et al., 2006), além de serem as principais responsáveis pela defosforilação da proteína tau e terem sua atividade diminuída na DA. Noh e colaboradores (2013), estudaram o efeito do donepezil sobre a PP2A em neurônios corticais de ratos e verificaram que o efeito neuroprotetor do donepezil é mediado também pela ativação da atividade de PP2A. Moscardó e colaboradores (2006), mostraram que tanto a PP1A quanto a PP2A estão envolvidas na ativação da c e da sPLA₂, de maneira contrária. A inibição da PP1A/PP2A inibem a cPLA₂ mas não a sPLA₂. Não existe nenhum estudo mostrando o efeito destas fosfatases sobre a iPLA₂. No entanto se levarmos em conta que o donepezil aumenta a atividade de PP2A, talvez esse aumento da atividade da iPLA₂ encontrada em nosso estudo também possa ser explicado por esta via. É interessante notar que mudanças da PP2A precedem a morte neuronal em estudos com neurônios corticais de rato (Noh et al., 2013).

GSK3B

Não encontramos diferenças na expressão das formas totais e fosforilada da GSK3B antes do início do tratamento, quando comparadas ao

grupo controle. Um estudo em nosso laboratório, mostrou um aumento da ativação da GSK3B em plaquetas de pacientes com DA (Forlenza et al., 2011) além disso, Hye e colaboradores (2005) encontraram esse aumento em leucócitos, porém ao contrário do nosso estudo, os pacientes avaliados estavam em tratamento com inibidores da colinesterase.

Sabemos que a fosforilação da GSK3B no sítio serina 9 a torna inativa (Li et al., 2004; Joep e Roh, 2006), o que parece ser neuroprotetor (Chin et al., 2005; Nunes et al., 2007). E, ao contrário, a ativação da enzima pela diminuição da fosforilação parece estar relacionada à neurodegeneração (Kaytor e Orr, 2002) e a déficits cognitivos (Nunes et al., 2007). Em trabalho do nosso grupo envolvendo pacientes com DA, constatamos que maiores concentrações de GSK3B fosforilada estavam relacionadas com melhores rendimentos cognitivos (Forlenza et al., 2011).

No presente estudo encontramos um aumento significativo da fosforilação da GSK3B após seis meses de tratamento com donepezil, sendo que a inativação da enzima passou a ser maior do que no grupo controle. Esses resultados estão em linha com estudos que utilizaram inibidores da colinesterase contra a toxicidade induzida pelo A β em neurônios corticais de rato, mostrando que o efeito neuroprotetor do donepezil é mais potente que os outros inibidores (Noh et al., 2009). Noh e colaboradores (2009), sugerem que o efeito do donepezil nas vias de sinalização da GSK3B e AKT (Proteína quinase B) na ausência do A β 42 não é significativo, todavia na presença do peptídeo, o tratamento leva ao aumento da fosforilação da GSK3B e da AKT.

Estudos recentes mostram que a neuroproteção induzida pelos IChEs se deve a estimulação dos receptores nicotínicos, os quais protegem contra a

toxicidade induzida pelo peptídeo A β em estudos *in vitro* e *in vivo*. (Shimohama et al., 2001). Estes mecanismos envolvem a modulação da atividade glutamatérgica bem como a ativação das vias anti apoptóticas Bcl-2 e Bcl-xL. (Bencherif et al., 2010). Além disso, a ativação do receptor também é capaz de influenciar a via amiloidogênica, reduzindo a formação dos monômeros de A β ou reduzindo a produção de A β insolúvel 1-42 e 1-40. (Martorana et al., 2010). O que temos então é que, após estimulação dos receptores nicotínicos pela acetilcolina (aumentada na fenda sináptica após administração do inibidor da AchE) ocorre uma ativação do fosfatidilinositol 3-Kinase (PI3K) que por sua vez ativa por fosforilação a AKT (AKTp). O aumento da AKTp estimula a expressão de Bcl2. Ao contrário, uma hipoestimulação dos receptores nicotínicos, diminui a ativação do PI3K, que por sua vez leva ao aumento da atividade da GSK3B aumentando a fosforilação da proteína tau, levando à morte celular.

Marcadores bioquímicos para a DA devem ser eficientes para diferenciar a DA de outras demências, e também devem ser capazes de detectar precocemente as alterações típicas da doença, possibilitando assim melhores chances de tratamentos. Para tanto necessitamos de matrizes de fácil acesso para a identificação desses biomarcadores. Nossos achados sugerem que a iPLA₂ em plaquetas pode ser útil como marcador periférico para a DA.

6 CONCLUSÃO

Os achados do presente estudo, complementados por dados da literatura, permitem concluir que:

- A atividade diminuída da iPLA₂ em pacientes com DA, confirmando achados de nosso e de outros laboratórios, corroboram com a hipótese de que a iPLA₂ pode ser útil como um marcador periférico para a DA;
- O donepezil aumenta a atividade da iPLA₂ a níveis semelhantes ao dos controles, sugerindo que este medicamento possui propriedades modificadoras da doença por restaurar vias importantes para a fisiopatologia da DA;
- O aumento da atividade da iPLA₂ pelo donepezil antecedeu a melhora no desempenho dos teste cognitivos, sugerindo que a estimulação da enzima pode estar relacionada aos benefícios cognitivos do donepezil;
- Nossos achados sugerem que a atividade da iPLA₂ em plaquetas poderia ser usada como marcador de resposta terapêutica ao donepezil e, possivelmente, a outros IChEs, na doença de Alzheimer.
- O aumento da forma fosforilada de GSK3B pelo donepezil, levando à inativação da enzima, suporta a ação neuroprotetora do donepezil;

7 REFÊRENCIAS BIBLIOGRÁFICAS

Balsinde J, Balboa MA, Insel PA, Dennis EA. Regulation and inhibition of phospholipase A₂. *Annu Rev Pharmacol Toxicol*. 1999; 39:175-89.

Barradas MA, Mikhailidis DP. The use of platelets as models for neurons: possible applications to the investigation of eating disorders. *Biomed & Pharmacother*. 1993;47:11-8.

Barry, FA.; Graham, GJ.; Fry, MJ.; Gibbins, JM.; Regulation of glycogen synthase kinase 3 in humans platelets: a possible role in platelet function? *FEBS Letters* 2003; 553: 173 – 178.

Bazan NG, Zorumski CF & Clarck GD. The activation of phospholipase A₂ and release of arachidonic acid and other lipid mediators at the sinapse: The role of platelet activating factor. *J Lipid Mediators*. 1993; 6: 421-427.

Bazan NG, Colangelo V, Lukiw WJ. Prostaglandins and other lipid mediators in Alzheimer's disease. *Prostaglandins & other Lipid Mediators*. 2002; 68-69: 197 – 210.

Beach TG, Honer WG, Hughes LH. Cholinergic fibre loss associated with diffuse plaques in the non-demented elderly: the preclinical stage of Alzheimer's disease? *Acta Neuropathol*. 1997; 93(2):146-53.

Beach TG, Sue L, Scott S, Layne K, Newell A, Walker D, Baker M, Sahara N, Yen SH, Hutton M, Caselli R, Adler C, Connor D, Sabbagh M. Hippocampal sclerosis dementia with tauopathy, *Brain Pathol*. 2003; 13(3):263-78.

Beaulieu JM. Not only lithium: regulation of glycogen synthase kinase-3 by antipsychotics and serotonergic drugs. *International Journal of Neuropsychopharmacology*. 2007;10:3-6.

Bencherif M, Lippiello PM. Alpha7 neuronal nicotinic receptors: The missing link to understanding Alzheimer's etiopathology? *Med Hypotheses*. 2010;74:281–285.

Berkelman T. Removal of interfering substances in samples prepared for two-dimensional (2-D) electrophoresis. *Methods Mol Biol*. 2008;424:51-62. Review.

Blusztajn JK & Wurtman RJ. Choline and cholinergic neurons. *Science*. 1983; 221: 614-620.

Blusztajn JK, Liscovitch M & Richardson UI. Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line. *Proc Natl Acad Sci USA* 1987; 84: 5474-5477.

Brucki SMD. Curso clínico da Doença de Alzheimer. In: Forlenza OV, Caramelli

P, editores. *Neuropsiquiatria Geriátrica*. São Paulo: Atheneu; 2001. p. 119 – 128.

Burns A, Zaudig M. Mild cognitive impairment in older people. *The Lancet*. 2002; 360: 1963 – 1965.

Burns A, O'Brien J; BAP Dementia Consensus group, Auriacombe S, Ballard C, Broich K, Bullock R, Feldman H, Ford G, Knapp M, McCaddon A, Iliffe S, Jacova C, Jones R, Lennon S, McKeith I, Orgogozo JM, Purandare N, Richardson M, Ritchie C, Thomas A, Warner J, Wilcock G, Wilkinson D. Clinical practice with anti-dementia drugs: a consensus statement from British Association for Psychopharmacology, British Association for Psychopharmacology. *J Psychopharmacol*. 2006; 20(6):732-55. Review.

Buxbaum JD, Oishi M, Chen HI, Pinkas-Kramarski R, Jaffe EA, Gandy SE, Greengard P. Cholinergic agonists and interleukin 1 regulate processing and secretion of the Alzheimer beta/A4 amyloid protein precursor,. *Proc Natl Acad Sci USA*. 1992; 89(21):10075-8.

Caramelli P, Nitrini R. Conduta diagnóstica em demência. In: Forlenza OV, Almeida OP, editores. *Depressão e Demência no Idoso*. São Paulo: Lemos; 1997. 107 – 119.

Caramelli P. Neuropatologia da Doença de Alzheimer. In: Forlenza OV, Caramelli P, editores. *Neuropsiquiatria Geriátrica*. São Paulo: Atheneu; 2001. p. 107 – 118.

Chakraborti S. Phospholipase A2 isoforms: a perspective. *Cell Signal*. 2003;15:637-65.

Chin, PC.; Majdzadeh, N.; D'Mello, SR. Inhibition of GSK3 β is a common event in neuroprotection by different survival factors. *Molecular Brain Research*. 2005;137:193-201.

Cho HW, Kim JH, Choi S, Kim HJ. Phospholipase A₂ is involved in muscarinic receptor-mediated sAPP α release independently of cyclooxygenase or lipoxygenase activity in SH-SY5Y cells. *Neuroscience Letters*. 2006; 397: 214 – 218.

Cummings JL. Defining and labeling disease-modifying treatments for Alzheimer's disease, *Alzheimers Dement*. 2009;5(5):406-18.

Di Lucca M, Grossi E, Borroni B, Zimmermann M, Marcello E, Colciaghi F, Gardoni F, Intraligi M, Padovani A, Buscema M. Artificial neural networks allow the use of simultaneous measurements of Alzheimer Disease markers for early detection of disease. *Journal of Translational Medicine*. 2005; 3:30.

Diaz-Arrastia R. New biochemical markers in Alzheimer Disease [editorial]. *Arch Neurol*. 2001; 58: 354 – 356.

El Haouari M, Rosado JA. Platelet function in hypertension. *Blood Cells Mol Dis*. 2009;42(1):38-43.

Emmerling MR, Moore CJ, Doyle D, Carroll RT, Davis RE. Phospholipase A₂ activation influences the processing and secretion of the amyloid precursor protein. *Biochemical and biophysical Research Communications*. 1993; 197: 292 – 297.

Farooqui AA, Litsky ML, Farooqui T, Horrocks LA. Inhibitors of intracellular phospholipase A₂ activity: Their neurochemical effects and therapeutical importance for neurological disorders. *Brain Res Bull*. 1999; 49: 139 – 153.

Farooqui AA, Horrocks LA, Farooqui T. Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. *Chemistry and Physics of Lipids*. 2000; 106: 1 – 29.

Farooqui AA, Horrocks LA. Plasmalogens: Workhorse Lipids of Membranes in Normal and Injured Neurons and Glia. *The Neuroscientist*. 2001; 7: 232-45.

Farooqui AA, Horrocks LA. Brain phospholipases A₂: a perspective on the history. Prostaglandins, Leucotrienes and Essential Fatty acids 2004; 71: 161-169.

Farooqui AA, Horrocks LA. Phospholipase A₂-Generated Lipid mediators in the Brain: The Good, the Bad, and the Ugly. *The Neuroscientist*. 2006; 12: 245-60.

Fenton, WS, Hibbeln J & Knable M. Essential fatty Acids, Lipid Membrane Abnormalities, and the Diagnosis and Treatment of Schizophrenia. *Biological Psychiatr*. 2000; 47: 8-21.

Flesch I, Schmidt B, Ferber E. Acyl chain specificity and kinetic properties of phospholipase A₁ and A₂ of bone marrow-derived macrophages. *Z Naturforsch (C)*. 1985; 40(5-6): 356-63.

Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res*. 1975; 12 (3): 189 – 198.

Forlenza OV, Spink JM, Dayanandan R, Anderton BH, Olesen OF, Lovestone S. Muscarinic agonists reduce tau phosphorylation in non-neuronal cells via GSK-3 β inhibition and in neurons. *J Neural Transm*. 2000; 107(10):1201-12.

Forlenza OV, Schaeffer EL, Gattaz WF. Phospholipase A₂ activity in rat embryonic brain and in primary cultures of cortical neurons. *J Neural Transm*.

2002;109(5-6):623-31.

Forlenza OV, Mendes CT, Marie SK, Gattaz WF. Inhibition of phospholipase A₂ reduces neurite outgrowth and neuronal viability. *Prostaglandins Leukot Essent Fatty Acids*. 2007; 76:47-55

Forlenza OV, Torres CA, Talib LL, de Paula VJ, Joaquim HP, Diniz BS, Gattaz WF. Increased platelet GSK3B activity in patients with mild cognitive impairment and Alzheimer's disease. *J Psychiatr Res*. 2011;45(2):220-4.

Frame, S.; Cohen, P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J*. 2001; 359: 1 – 16.

Francis P, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry*. 1999; 66: 137 – 147.

Fuentealba, RA.; Farias, G.; Scheu, J.; Bronfman, M.; Marzolo, MP.; Inestrosa, NC.; Signal transduction during amyloid β peptide neurotoxicity: role in Alzheimer's Disease. *Brain Research Reviews* 2004; 47: 275 – 289.

Gattaz WF, Köllisch M, Thuren T, Virtanen JA, Kinnunen PKJ. Increased plasma phospholipase A₂ activity in schizophrenic patients: reduction after neuroleptic therapy. *Biol Psychiatry*. 1987; 22: 421 – 426.

Gattaz WF, Steudle A, Maras A. Increased platelet phospholipase A₂ in schizophrenia. *Schizophrenia Research*. 1995(a); 16: 1 – 6.

Gattaz WF, Maras A, Cairns NJ, Levy R, Förstl H. Decreased phospholipase A₂ activity in Alzheimer brains. *Biol Psychiatry*. 1995(b); 37: 13 – 17.

Gattaz WF, Cairns NJ, Levy R, Forstl H, Braus DF, Maras A. Decreased phospholipase A₂ activity in the brain and in platelets of patients with Alzheimer's disease. *Eur Arch Psychiatry Clin Neurosci*. 1996; 246: 129 – 131.

Gattaz WF, Forlenza OV, Talib LL, Barbosa NR, Bottino CM. Platelet phospholipase A₂ activity in Alzheimer's disease and mild cognitive impairment. *J Neural Transm*. 2004; 111 (5): 591 – 601.

Gattaz WF, Valente KD, Raposo NR, Vincentiis S, Talib LL. Increased PLA₂ activity in the hippocampus of patients with temporal lobe epilepsy and psychosis. *J Psychiatr Res*. 2011;45(12):1617-20.

Gattaz WF, Talib LL, Schaeffer EL, Diniz BS, Forlenza OV. Low platelet iPLA₂ activity predicts conversion from mild cognitive impairment to Alzheimer's disease: a 4-year follow-up study. *J Neural Transm*. 2014;121(2):193-200.

Grimes CA, Jope RS. The multifaceted roles of glycogen synthase kinase 3B in cellular signalling. *Progress in Neurobiology*. 2001;65:391-426.

Haass C. Take five-Bace and the γ -secretase quartet conduct Alzheimer's amyloid β -peptide generation. *The EMBO Journal*. 2004; 23 (3): 483 – 488.

Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol*. 2006; 5(3):228-34.

Herrera E Jr, Caramelli P, Silveira ASB, Nitrini R. Epidemiologic survey of dementia in a Community-dwelling brazilian population. *Alzheimer Disease and Associated Disorders*. 2002; 16(2): 103 – 108.

Hirabayashi T, Shimizu T. Localization and regulation of cytosolic phospholipase A₂. *Biochim Biophys Acta*. 2000;1488:124-38.

Holmsen H. Signal transducing mechanisms in platelets. *Proc Natl Sci CouncRepub China B*. 1991 Jul;15(3):147-52. Review.

Hooper C, Killick R, Lovestone S. The GSK3 hypothesis of Alzheimer's disease. *J Neurochem*. 2008;104(6):1433-9.

Hye A, Kerr F, Archer N, Foy C, Poppe M, Brown R, Hamilton G, Powell J, Anderton B, Lovestone S. Glycogen synthase kinase-3 is increased in white cells early in Alzheimer's disease. *Neurosci Lett*. 2005; 373(1):1-4.

Irizarry MC. Biomarkers of Alzheimer Disease in plasma. *The Journal of the American Society for Experimental NeuroTherapeutics*. 2004; 1 (2): 226 – 234.

Jann MW, Shirley KL, Small GW. Clinical pharmacokinetics and pharmacodynamics of cholinesterase inhibitors. *Clin Pharmacokinet*. 2002;41(10):719-39. Review.

Jenkins SM, Johnson VW. Modulation of Tau phosphorylation with ion its microtubule-binding domain by cellular thiols. *Journal of Neurochemistry*. 1999; 73: 1843 – 1850.

Jope RS, Johnson GVW. The glamour and gloom of glycogen sintase kinase-3. *Trends in Biochemical Sciences*. 2004; 29: 95:106.

Jope RS, Roh M-S. Glycogen Synthase Kinase-3 (GSK3) in Psychiatric Diseases and Therapeutic Interventions. *Curr Drug Targets*. 2006; 7(11): 1421-1434.

Jope RS, Yuskaitis CJ, Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics, *Neurochem Res.* 2007; 32(4-5):577-95. Review.

Kaytor, MD.; Orr, HT. The GSK3 β signaling cascade and neurodegenerative disease. *Current Opinion in Neurobiology.* 2002;12:275-8.

Koh SH, Noh MY, Kim SH. Amyloid-beta-induced neurotoxicity is reduced by inhibition of glycogen synthase kinase-3. *Brain Res.* 2008;1188:254-62.

Kudo I, Murakami M. Phospholipase A₂ enzymes. *Prostaglandins & others Lipid Mediators.* 2002; 68-69: 3-58.

Lancôt KL, Herrmann N, LouLou MM. Correlates of response to acetylcholinesterase inhibitor therapy in Alzheimer's disease. *J Psychiatry Neurosci.* 2003; 28(1):13-26. Review.

Larsson PKA, Kennedy BP, Claesson HE. The human calcium-independent phospholipase A2 gene: multiple enzymes with distinct properties from a single gene. *Eur J Biochem.* 1999;262:575-85.

Larsson PKA, Claesson HE, Kennedy BP. Multiple splice variants of the human calcium-independent phospholipase A2 and their effect on enzyme activity. *J Biol Chem.* 1998; 273:207-14.

Lautens LL, Chiou XG, Sharp JD, Young III WS, Sprague DL, Ross LS, Felder CC. Cytosolic phospholipase A₂ (cPLA₂) distribution in murine brain and functional studies indicate that cPLA₂ does not participate in muscarinic receptor-mediated signaling in neurons. *Brain Research.* 1998; 809: 18-30.

Li X, Rosborough KM, Friedman AB, Zhu W, Roth KA. Regulation of mouse brain glycogen synthase kinase-3 by atypical antipsychotics. *International Journal of Neuropsychopharmacology.* 2007; 10: 7-19.

Li X, Zhu W, Roh M-S, Friedman AB, Rosborough K, Jope RS. *In vivo* Regulation by Glycogen Synthase Kinase-3B (GSK3B) by Serotonergic Activity in Mouse Brain. *Neuropsychopharmacology* . 2004; 29: 1426-1431.

López-Pousa S, Turon-Estrada A, Garre-Olmo J, Pericot-Nierga I, Lozano-Gallego M, Vilalta-Franch M, Hernández-Ferrándiz M, Morante-Muñoz V, Isern-Vila A, Gelada-Batlle E, Majó-Llopart J. Differential efficacy of treatment with acetylcholinesterase inhibitors in patients with mild and moderate Alzheimer's disease over a 6-month period, *Dement Geriatr Cogn Disord.* 2005;19(4):189-95.

Lovestone AS. Patogênese molecular da Doença de Alzheimer. In: Forlenza

OV, Caramelli P, editores. *Neuropsiquiatria Geriátrica*. São Paulo: Atheneu; 2001. p. 467 – 477.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin-phenol reagents. *J. Biol. Chem.* 1951;193: 265-275.

Mancuso DJ, Jenkins CM, Gross RW. The genomic organization, complete mRNA sequence, cloning, and expression of a novel human intracellular membrane-associated calcium-independent phospholipase A2. *J Biol Chem.* 2000; 275:9937-45.

Martorana A, Esposito Z, Koch G. Beyond the cholinergic hypothesis: do current drugs work in Alzheimer's disease? *CNS Neurosci Ther.* 2010;16(4):235-45. Review.

Mattsson N, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, Bocchio-Chiavetto L, Blankenstein MA, Carrillo MC, Chabot S, Coart E, Chiasserini D, Cutler N, Dahlfors G, Duller S, Fagan AM, Forlenza O, Frisoni GB, Galasko D, Galimberti D, Hampel H, Handberg A, Heneka MT, Herskovits AZ, Herukka SK, Holtzman DM, Humpel C, Hyman BT, Iqbal K, Jucker M, Kaeser SA, Kaiser E, Kapaki E, Kidd D, Klivenyi P, Knudsen CS, Kummer MP, Lui J, Lladó A, Lewczuk P, Li QX, Martins R, Masters C, McAuliffe J, Mercken M, Moghekar A, Molinuevo JL, Montine TJ, Nowatzke W, O'Brien R, Otto M, Paraskevas GP, Parnetti L, Petersen RC, Prvulovic D, de Reus HP, Rissman RA, Scarpini E, Stefani A, Soininen H, Schröder J, Shaw LM, Skinningsrud A, Skrogstad B, Spreer A, Talib L, Teunissen C, Trojanowski JQ, Tumani H, Umek RM, Van Broeck B, Vanderstichele H, Vecsei L, Verbeek MM, Windisch M, Wang J, Zetterberg H, Blennow K. The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers. *Alzheimers Dement.* 2011;7(4):386-395.e6.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology.* 1984;34(7):939-44.

Mohs RC, Haroutunian V. Alzheimer disease: from earliest symptoms to end stage. In: Davis KL, Charney D, Coyle JT, Nemeroff C, editores. *Neuropsychopharmacology: The fifth generation of progress*. American College of Neuropsychopharmacology. 2002; 1189 – 1197.

Molinuevo JL, Berthier ML, Rami L. Donepezil provides greater benefits in mild compared to moderate Alzheimer's disease: implications for early diagnosis and treatment. *Arch Gerontol Geriatr.* 2011;52(1):18-22.

Morris JC. Mild cognitive impairment is early-stage Alzheimer Disease

[editorial]. *Arch Neurol*. 2006; 63 (1): 15 – 16.

Moscardó A, Vallés J, Piñón M, Aznar J, Martínez-Sales V, Santos MT. Regulation of cytosolic PIA2 activity by PP1/PP2A serine/threonine phosphatases in human platelets. *Platelets*. 2006 Sep;17(6):405-15.

Nalefski EA, Sultzman LA, Martin DM, Kriz RW, Towler PS, Knopf JL, Clark JD. Delineation of two functionally distinct domains of cytosolic phospholipase A₂, a regulatory Ca²⁺-dependent lipid-binding domain and a Ca²⁺-independent catalytic domain. *J Biol Chem*. 1994; 269: 18239-49.

Nalefski EA, Falke JJ. The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci*. 1996;5: 2375-90.

Nalefski EA, McDonagh T, Somers W, Seehra J, Falke JJ, Clark JD. Independent folding and ligand specificity of the C2 calcium-dependent lipid binding domain of cytosolic phospholipase A₂. *J Biol Chem*. 1998; 273: 1365-72.

Neumann K, Fariás G, Slachevsky A, Perez P, Maccioni RB. Human platelets tau: a potential peripheral marker for Alzheimer's disease. *J Alzheimers Dis*. 2011;25(1):103-9.

Nitsch RM, Slack BE, Wurtman RJ, Growdon JH. Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors, *Science*. 1992; 9;258(5080):304-7.

Noh MY, Koh SH, Kim Y, Kim HY, Cho GW, Kim SH. Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. *J Neurochem*. 2009;108(5):1116-25.

Noh MY, Koh SH, Kim SM, Maurice T, Ku SK, Kim SH. Neuroprotective effects of donepezil against Aβ₄₂-induced neuronal toxicity are mediated through not only enhancing PP2A activity but also regulating GSK-3β and nAChRs activity. *J Neurochem*. 2013 Nov;127(4):562-74.

Nordberg A. Mechanisms behind the neuroprotective actions of cholinesterase inhibitors in Alzheimer disease. *Alzheimer Dis Assoc Disord*. 2006; 20(2 Suppl 1):S12-8. Review.

Nunes, P.V.; Forlenza, O.V.; Gattaz, W.F.; Lithium and risk for Alzheimer's disease in elderly patients with bipolar disorder. *Br J Psychiatry*. 2007;190:359-60.

Padovani A, Pastorino L, Borroni B, Colciaghi F, Rozzini L, Monastero R, Perez J, Pettenati C, Mussi M, Parrinello G, Cottini E, Lenzi GL, Trabucchi M,

Cattabeni F, Di Luca M. Amyloid precursor protein in platelets. A peripheral marker for the diagnosis of sporadic AD. *Neurology*. 2001; 57: 2243 – 2248.

Peineau S, Bradley C, Taghibiglou C, Doherty A, Bortolotto ZA, Wang YT, Collingridge GL. The role of GSK-3 in synaptic plasticity. *British Journal of Pharmacology*. 2008; 153, S428-S437.

Peterson GL. Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal Biochem*. 1979; 100(2): 201-20.

Pickard RT, Striffler BA, Kramer RM, Sharp JD. Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A₂. *J Biol Chem*. 1999; 274: 8823-31.

Piomelli D. Arachidonic acid in cell signaling. *Current Opinion in Cell Biology*. 1993; 5: 274 – 280.

Planel, E.; Sun, X.; Takashima, A.; Role of GSK-3 β in Alzheimer's disease pathology. *Drug Development Research*. 2002;56:491-510.

Racchi M, Sironi M, Caprera A, König G, Govoni S. Short- and long-term effect of acetylcholinesterase inhibition on the expression and metabolism of the amyloid precursor protein. *Mol Psychiatry*. 2001;6(5):520-8.

Racchi M, Govoni S. The pharmacology of amyloid precursor protein processing. *Experimental Gerontology*. 2003; 38: 145 – 157.

Rametti A, Esclaire F, Yardin C, Cogné N, Terro F. Lithium down-regulates tau in cultured cortical neurons: a possible mechanism of neuroprotection. *Neurosci Lett*. 2008; 21;434(1):93-8.

Roher AE, Baudry J, Chaney MO, Kuo YM, Stine WB, Emmerling MR. Oligomerization and fibril assembly of the amyloid-beta protein, *Biochim Biophys Acta*. 2000; 26:1502(1):31-43. Review.

Ross MB, Moszczynska A, Erlich J, Kish SJ. Phospholipid-metabolizing enzymes in Alzheimer's disease: increased lysophospholipid acyltransferase activity and decreased phospholipase A₂ activity. *Journal of Neurochemistry*. 1998; 70: 786 – 793.

Ross MB, Turenne S, Moszczynska A, Warsh JJ, Kish SJ. Differential alteration of phospholipase A₂ activities in brain of patients with schizophrenia. *Brain Res*. 1999; 821: 698 – 709.

Roth M, Tym E, Mountjoy CQ, Huppert FA, Hendrie H, Verma S, Goddard R. CAMDEX. A standardised instrument for the diagnosis of mental disorder in the elderly with special reference to the early detection of dementia. *Br J*

Psychiatry.

Sanchez MP, Alvarez-Tallada V, Avila J. The microtubule-associated protein tau in neurodegenerative diseases. Tauopathies [abstract]. *Rev Neurol*. 2001; 33 (2): 169 – 177.

Selkoe DJ; American College of Physicians; American Physiological Society. Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann Intern Med*. 2004; 20:140(8):627-38. Review.

Schaeffer EL, Gattaz WF. Requirement of hippocampal phospholipase A2 activity for long-term memory retrieval in rats. *J Neural Transm*. 2007;114:379-385.

Schaeffer EL, Gattaz WF. Cholinergic and glutamatergic alterations beginning at the early stages of Alzheimer disease: participation of the phospholipase A2 enzyme. *Psychopharmacology (Berl)*. 2008;198(1):1-27.

Schaeffer EL, Zorrón Pu L, Gagliotti DA, Gattaz WF. Conditioning training and retrieval increase phospholipase A(2) activity in the cerebral cortex of rats. *J Neural Transm*. 200;116(1):41-50.

Schaeffer EL, Skaf HD, Novaes Bde A, da Silva ER, Martins BA, Joaquim HD, Gattaz WF. Inhibition of phospholipase A₂ in rat brain modifies different membrane fluidity parameters in opposite ways. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011;15: 5(7):1612-7.

Shimohama S, Kihara T. Nicotinic receptor-mediated protection against beta-amyloid neurotoxicity. *Biol Psychiatry*. 2001;49:233–239.

Simonato M. A pathogenetic hypothesis of temporal lobe epilepsy. *Pharmacol Res*. 1993; 27: 217 – 225.

Six DA, Dennis EA. The expanding superfamily of phospholipase A2 enzymes: classification and characterization. *Biochim Biophys Acta*. 2000; 1488: 1-19.

Smesny S, Stein S, Willhardt I, Lasch J, Sauer H. Decreased phospholipase A2 activity in cerebrospinal fluid of patients with dementia. *J Neural Transm*. 2008;115(8):1173-9.

Song C, Chang X -J, Bean KM, Proia MS, Knopf JL, Kriz RW. Molecular characterization of cytosolic phospholipase A2- b. *J Biol Chem*. 1999; 274: 17063-67.

Standridge JB. Pharmacotherapeutic approaches to the treatment of Alzheimer's disease. *Clinical Therapeutics*. 2004; 26 (5): 615 – 630.

Stewart A, Ghosh M, Spencer DM, Leslie CC. Enzymatic properties of human cytosolic phospholipase A₂. *J Biol Chem*. 2002; 277: 29526-36.

Sun GY, Xu J, Jensen MD, Simonyi A. Phospholipase A₂ in the central nervous system: implications for neurodegenerative diseases. *J. Lipid Research*. 2004; 45: 205-13.

Talesa VN. Acetylcholinesterase in Alzheimer's disease. *Mech Ageing Dev*. 2001;122(16):1961-9. Review.

Talib LL, Diniz BS, Zainaghi IA, Forlenza OV, Gattaz WF. A Radioenzymatic assay to identify three groups of phospholipase A(2) in platelets. *Prostaglandins Leukot Essent Fatty Acids*. 2012;86(4-5):149-53.

Tanaka H, Takeya R, Sumimoto H. A novel intracellular membrane-bound calcium independent phospholipase A₂. *Biochem Biophys Res Commun*. 2000; 272: 320-6.

Tavares H, Yacubian J, Talib LL, Barbosa NB, Gattaz WF. Increased phospholipase A₂ activity in schizophrenia with absent response to niacin. *Schizophrenia Res*. 2003; 61: 1 – 6.

Underwood KW, Song C, Kriz RW, Chang XJ, Knopf JL, Lin LL. A novel calcium-independent phospholipase A₂, cPLA₂- that is prenylated and contains homology to cPLA₂. *J Biol Chem*. 1998; 273: 21926-32.

Verhoeff NP. Acetylcholinergic neurotransmission and the beta-amyloid cascade: implications for Alzheimer's disease. *Expert Rev Neurother*. 2005 Mar;5(2):277-84.Review.

Walsh D, Selkoe DJ. Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron*. 2004; 44: 181 – 193.

Winstead MV, Balsinde J, Dennis EA. Calcium-independent phospholipase A₂: structure and function. *Biochim Biophys Acta*. 2000; 1499: 28-39.

APÊNDICE A

Produtividade no período do Doutorado

Foram elaborados seis manuscritos referentes à área de estudo deste projeto:

1) A Radioenzymatic assay to identify three groups of phospholipase A₂ in platelets

Leda L. Talib¹, Breno S. Diniz², Isis A. Zainaghi, Orestes V. Forlenza² and Wagner F. Gattaz²

Status: Publicado na *Prostaglandins, Leucotriens and others fatty acid – PLEFA*

2) Reduced platelet amyloid precursor protein ratio (APP ratio) predicts conversion from mild cognitive impairment to Alzheimer's disease

Leda L. Talib^{1*}; Isis A. Zainaghi^{2*}, Breno S. Diniz³, Wagner F. Gattaz³, Orestes V. Forlenza³

Status: publicado no *Journal of Neural Transmission*

3) Platelet biomarkers in Alzheimer's disease

Leda L. Talib¹, Helena P.G. Joaquim¹, Orestes V. Forlenza²

Status: publicado *World Journal Psychiatry*

4) Correlation bethewen platelets and brain PLA₂ activity

Leda L. Talib, Kette D. Valente, Silvia Vincentiis, Wagner F. Gattaz

Status: Publicado na *Prostaglandins, Leucotriens and others fatty acid – PLEFA*

5) Low platelet iPLA₂ activity predicts conversion from mild cognitive impairment to Alzheimer's disease: a 4-year follow-up study

Wagner F. Gattaz, Leda L. Talib, Evelin L. Schaeffer, Breno S. Diniz, Orestes V. Forlenza

Status: Publicado no *Journal of Neural Transmission*

6) Long-term treatment of Alzheimer's disease with cholinesterase inhibitors restores homeostatic levels of Calcium-independent PLA₂ and GSK3B phosphorylation

Talib L.L., Hototian S., Forlenza, O.V., Gattaz, W.F.

Status: Submetido ao *Journal of Psychiatric Research*

Contents lists available at SciVerse ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

A radioenzymatic assay to identify three groups of phospholipase A₂ in platelets

Leda L. Talib, Breno S. Diniz, Isis A. Zainaghi, Orestes V. Forlenza, Wagner F. Gattaz*

Laboratory of Neuroscience–LIM 27, Department and Institute of Psychiatry, Faculty of Medicine, University of Sao Paulo, Brazil

ARTICLE INFO

Article history:

Received 4 August 2011

Received in revised form

10 February 2012

Accepted 11 February 2012

Keywords:

Phospholipids

PLA₂

Platelets

Neuropsychiatric disorders

Radioenzymatic assays

ABSTRACT

Phospholipases A₂ (PLA₂) are key enzymes in membrane metabolism. The release of fatty acids and lysophospholipids by PLA₂ activates several intra-cellular second messenger cascades that regulate a wide variety of physiological responses. The aim of the present study is to describe a radioenzymatic assay to determine the activity of three main PLA₂ subtypes in platelets, namely extracellular calcium-dependent PLA₂ (sPLA₂) and intracellular calcium-dependent (cPLA₂) and calcium-independent PLA₂ (iPLA₂). The differentiation of these distinct PLA₂ subtypes was based on the enzyme substrate preference (arachidonic acid or palmitoyl acid) and calcium concentration. Our results indicate that this new assay is feasible, precise and specific to measure the activity of the aforementioned subtypes of PLA₂. Therefore, this protocol can be used to investigate modifications of PLA₂ homeostasis in distinct biological models addressing the pathophysiology of many medical and neuropsychiatric disorders such as schizophrenia and Alzheimer's disease.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The phospholipase A₂ (PLA₂) group comprises a super-family of enzymes that catalyze the hydrolysis of sn-2 ester bonds of phospholipids, resulting in the production of free fatty acids and lysophospholipids. PLA₂ enzymes play a major role in many important neuronal functions, such as modulation of membrane fluidity and polarization states, activation of protein kinase C (PKC), stimulation of calcium ion release, activation of several important modulating enzymes, and regulation of gene transcription [1]. Changes in PLA₂ homeostasis have been described in distinct neuropsychiatric disorders, such as schizophrenia [2–9], Alzheimer's disease [10–12], epilepsy [13–18] major depression [19,20], multiple sclerosis [16] and dyslexics [21].

Approximately 20 distinct mammalian PLA₂ enzymes have been identified and characterized. The main enzyme groups described so far are the secreted PLA₂ (sPLA₂), characterized by low molecular weight (12–18 kDa) and calcium-dependent activation at millimolar concentrations; the cytosolic PLA₂ (cPLA₂), with molecular weight ranging from 60 to 110 kDa and also calcium-dependence at micromolar levels; and the intracellular calcium-independent PLA₂ (iPLA₂) [22–25,1]. sPLA₂ and cPLA₂ enzymes selectively hydrolyze

arachidonic acid (AA) at the sn-2 position while iPLA₂ hydrolyzes membrane phospholipids in the following order of preference: linoleic acid; palmitic acid; oleic acid; and arachidonic acid [26].

Distinct methods have been used to determine the PLA₂ activity in different tissues, such as fluorimetric and radioenzymatic assays. Commercial kits for cPLA₂ using ELISA (Cayman Chemical) are also available [27–30]. Although these methods can measure the sPLA₂ and cPLA₂ enzymatic activity, they are not able to specifically measure iPLA₂ activity in platelets. As changes in the activity of specific PLA₂ groups might have distinct roles in the pathogenesis of neuropsychiatric disorders, it is important to develop laboratorial methods that can quantify, with high specificity, the enzymatic activity of distinct PLA₂ subtypes. Therefore, the aim of this study is to describe a new radioenzymatic assay that specifically determines the activity of cPLA₂, sPLA₂ and iPLA₂ in platelets, based on the enzymatic substrate preference and dependence on calcium concentration [31].

2. Materials and methods

2.1. Biological matrix

Platelets were chosen as the biological matrix for this method because they are readily available from peripheral blood, easy to handle provided activation be precluded during experimentation, and express the PLA₂ subtypes of interest to this protocol. In addition, platelets have been extensively studied as a peripheral source of biological markers for many neuropsychiatric disorders [32–35].

* Correspondence to: Laboratório de Neurociências (LIM 27), Departamento de Instituto de Psiquiatria, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Rua Dr. Ovídio Pires de Campos 785 - 3º andar, 05403-010 São Paulo, SP, Brazil. Tel.: +55 11 3069 7283; fax: +55 11 3085 5412.
E-mail address: gattaz@usp.br (W.F. Gattaz).

Samples of fresh blood were obtained from healthy adult volunteers of both genders, through the puncture of a peripheral vein in the forearm. Platelets were isolated by centrifugation at $515 \times G$ for 15 min at $20^\circ C$ with acid citrate dextrose solution (ACD-NIH-formel-A solution: glucose 123.8 mM, tri-sodium citrate 83.9 mM, citric acid 41.3 mM) (1:10v:v). Platelet-rich plasma (PRP) was collected and pH was adjusted to 6.5 by titration with ACD-NIH-formel-A. PRP was centrifuged at $1159 \times G$ for 10 min at $20^\circ C$ and the supernatant was carefully removed. A washing solution (30 mM sodium citrate pH 6.5; 5 mM potassium chloride; 2 mM calcium chloride; 1 mM magnesium chloride; 5 mM glucose; 500 $\mu g/mL$ albumin; 50 $\mu g/mL$ apyrase) was added to the supernatant and incubated for 10 min. The solution was centrifuged at

$1159 \times g$ for additional 8 min at $20^\circ C$ and the pellet was resuspended with tris-sucrose solution. Platelets aliquots were frozen immediately at $-70^\circ C$ until experimentation. Under these conditions, there was no evidence of platelet activation, (data not shown).

2.2. Radioenzymatic assays

All radioactive substrates were processed similarly, i.e., diluted 1:10 in a 1:2 toluene-ethanol solution containing 140 mg/mL of antioxidant butylatedhydroxytoluene (BHT). The radioactive substrate was kept at $-20^\circ C$ until use. Prior to experimentation, the solution was then evaporated under stream of nitrogen (12 μl or 0.06 μCi per sample), resuspended with 150 mL of ice-cold

Fig. 1. Optimal conditions for the determination of PLA₂ activity in platelets: (A) total protein concentration curve; (B) substrate concentration; (C) Time of incubation curve (D) Calcium requirement for cPLA₂ and sPLA₂ activity and (E) inhibitions curves using MAFP (100 μM). All assays were performed using PC-AA-[^{14}C]; bars represent mean values of 3 assays. The featured bars represent the optimal condition for each variable.

0.3 mg/mL BSA solution and homogenized using a sonicator to form liposomes. The count of total radioactivity was standardized for both substrates (120,000 CPM). All assays were conducted with a blank sample. The assays were considered viable when the blank sample values were below 300 CPM.

PLA₂ assays were done in triplicate. Standard curves of PLA₂ activity were constructed in order to standardize the methodology. The curves were based on the substrate concentration (0.03, 0.045, 0.06 and 0.075 μ Ci/mL), protein concentration (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL), Ca²⁺ concentration (100 and 300 nM; 1.0, 3.0, 10, 30 and 100 μ M; 1.0 and 5.0 mM) for sPLA₂ and cPLA₂ assays, and EDTA concentration (500 μ M, 1.0 e 5.0 mM) for iPLA₂ assay and inhibitions curve with MAFP.

2.3. Determination of sPLA₂ and cPLA₂ activity

To determine the enzymatic activity of sPLA₂ and cPLA₂, L- α -1 palmitoyl-2-arachidonyl-phosphatidylcholine (Perkin Elmer Life Science, USA) labeled in position 2 with [¹⁴C] arachidonic acid (PC-AA-[¹⁴C]) was used as enzyme substrate. The assay mixture contained 50 μ l of 1.0 M Tris-HCl buffer pH 7.5, 200 μ l of platelet (0.2 mg of homogenate protein), 150 μ l of PC-AA-[¹⁴C] (0.06 μ Ci). Because calcium significantly influences sPLA₂ and cPLA₂ activity, distinct calcium concentrations were used to determine the optimal ranges to measure sPLA₂ and cPLA₂ activity.

The solution was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 700 μ l isopropanol-hydrochloric acid. The ¹⁴C-labeled AA released by the cleavage of PC-AA-[¹⁴C] by PLA₂ was extracted with *n*-heptane, followed by adsorption of the unbroken phospholipids and the lysophospholipids on 60 mg of silica. The radioactivity of free ¹⁴C-labeled AA was measured in a Tri Carb Liquid Scintillation counter. The results were given in CPM (counts per minute) and converted to picomoles per milligram of protein per minute using the equation: PLA₂ activity = CPM $\times$ F/A $\times$ E $\times$ 2.22 $\times$ B. The blank counts were subtracted from each sample count.

Where: CPM=counts per minute; F=adjustment factor for protein concentration; A=specific activity of radioactive substrate in mCi/mmol; B=incubation time in minutes, E=equipment efficiency.

2.4. Determination of iPLA₂ activity

To determine iPLA₂ activity, we used the L-3 phosphatidylcholine, 1-palmitoyl-2-palmitoyl labeled in position 2 with [¹⁴C]-PC-PA-[¹⁴C]-(Amersham Biosciences, UK) as enzyme substrate in the same conditions reported above, replacing the CaCl₂ by 50 μ l of EDTA.

Because PLA₂GIVC, another enzyme of the cPLA₂ family, is also activated in the absence of calcium, we further investigated whether the presence of this enzyme might confound the measurement of iPLA₂ activity in platelets. Thus, we repeated the procedures in the presence of 500 μ M of bromoenol-lactone (BEL), a specific iPLA₂ inhibitor, which was added to the assay solution. By comparing the results of these two experimental conditions (i.e., with and without BEL), we were able to determine the contribution of iPLA₂ enzymatic activity to the generation of ¹⁴C-labeled PA, and thus ascertain the specificity of this assay to measure iPLA₂ activity [36].

3. Results

3.1. sPLA₂ and cPLA₂ assays

The optimal concentrations of calcium for the measurement of enzymatic activity were 100 μ M for cPLA₂ and 5 mM for sPLA₂. These results are in accordance with previous reports in the

Fig. 2. PLA₂ activity (pMol/mg protein/min) in platelet lysates in the presence of two distinct substrates, i.e., PC-AA-[¹⁴C] and PC-PA-[¹⁴C]. The assays were performed in optimized concentrations of CaCl₂ for cPLA₂ (100 μ M) and sPLA₂ (5 mM), or EDTA for iPLA₂ (5 mM); bars represent mean values of 3 assays.

Fig. 3. Platelet iPLA₂ activity (pMol/mg protein/min) in the presence of the substrate PC-PA-[¹⁴C] and 5 mM EDTA at 37 °C for 30 min. The enzymatic activity was inhibited by 96% $\pm$ 2.3% in the presence of the specific iPLA₂ inhibitor BEL; bars represent mean values of 3 assays.

literature [24] (Fig. 1). The inter- and intra-assay variability for the cPLA₂ protocol was 6.0% and 3.3%, and for the sPLA₂ protocol was 6.8% and 3.8%, respectively.

3.2. iPLA₂ assays

The optimal concentration of EDTA for the measurement of iPLA₂ activity was 5 mM (Fig. 2), with an inter- and intra-assay variability of 5.2% and 1.8%, respectively. To determine the potential participation of PLA₂GIVC in the calcium-independent cleavage of the substrate, the experiments repeated in the presence of BEL yielded a 96% (SD 2.3%) reduction in radioactive count (Fig. 3), thus confirming the specificity of the present protocol to determine iPLA₂ activity. The overall results obtained with the distinct variables controlled for in the present protocol, such as substrate type, protein count, Ca²⁺ and EDTA concentrations, are listed in Table 1.

4. Discussion

In this work, we developed a new radioenzymatic assay to determine the activity of three main PLA₂ subtypes in platelets, namely cPLA₂, sPLA₂ and iPLA₂. Our results indicate that the present

Table 1
Variables controlled for in the development of the radioenzymatic assay.

Variable	sPLA ₂	cPLA ₂	iPLA ₂
Fatty acid sn2 position	arachidonyl	arachidonyl	Palmitoyl
Substrate concentration	0.06 µCi or 1.035 nmol	0.06 µCi or 1.035 nmol	0.06 µCi or 1.035 nmol
Protein concentration	0.2 mg/mL	0.2 mg/mL	0.2 mg/mL
Calcium concentration	5 mM	100 µM	–
EDTA concentration	–	–	5 mM

assay is feasible, precise and specific to estimate the activity of these enzymes. Previous studies have consistently demonstrated that PLA₂ is expressed in platelets [37–40] but to the best of our knowledge, no study so far has described a method to discriminate iPLA₂ activity from other PLA₂ subtypes in platelets. Therefore, we provide evidence that iPLA₂ activity can be reliably determined in platelets, given the fact that the potential involvement of PLA₂GIVC, which is another calcium-independent PLA₂ subtype, did not affect the results of the present experiments.

Platelets share many features in common with neurons, such as the presence of monoaminergic membrane receptors and its intra-cellular machinery; they have been used as a major source of peripheral biological matrix to study pathophysiological changes in several neuropsychiatric disorders, such as major depression and Alzheimer's disease [32,33,41]. As iPLA₂ is the main enzyme group in neuronal cells, comprising 70% of the PLA₂ activity in the brain, the detection and quantification of this subtype in platelets, as provided by the present study, may be a useful tool to address modifications of membrane phospholipid metabolism in neuropsychiatric disorders.

Financial sources

This work was supported by grants from the Associação Beneficente Alzira Denise Hertzog da Silva (ABADHS) and from FAPESP (projects no° 02/12633-7 and 2009/52825-8).

Conflict of interest declaration

The authors report no conflict of interest in the preparation of this manuscript.

References

- [1] A.A. Farooqui, L.A. Horrocks, Brain phospholipases A₂: a perspective on the history, prostaglandins, Leukotrienes and Essential Fatty Acids 71 (2004) 161–169.
- [2] W.F. Gattaz, M. Köllisch, T. Thuren, J.A. Virtanen, P.K.J. Kinnunen, Increased plasma phospholipase A₂ activity in schizophrenic patients: reduction after neuroleptics therapy, *Biol. Psychiatry* 22 (1987) 421–426.
- [3] W.F. Gattaz, C.K. Hübner, T.J. Nevalainen, T. Thuren, P.K.J. Kinnunen, Increased serum phospholipase A₂ activity in schizophrenia: a replication study, *Biol. Psychiatry* 28 (1990) 495–501.
- [4] W.F. Gattaz, A. Steudle, A. Maras, Increased platelet phospholipase A₂ in schizophrenia, *Schizophr. Res.* 16 (1995) 1–6.
- [5] M. Noponen, M. Sanfilippo, K. Samanich, H. Ryer, G. Ko, B. Angrist, A. Volkin, J. Rotrosen, Elevated PLA₂ activity in schizophrenics and other psychiatric patients, *Biol. Psychiatry* 34 (1993) 641–649.
- [6] B.M. Ross, C. Hudson, J. Erlich, J.J. Warsh, S.J. Kish, Increased phospholipid breakdown in schizophrenia, *Arch. Gen. Psychiatry* 54 (1997) 487–494.
- [7] B.M. Ross, S. Turenne, A. Moszczynska, J.J. Warsh, S.J. Kish, Differential alteration of phospholipase A₂ activities in brain of patients with schizophrenia, *Brain Res.* 821 (1999) 407–413.
- [8] J.K. Yao, S. Leonard, R.D. Reddy, Membrane phospholipid abnormalities in postmortem brains from schizophrenic patients, *Schizophr. Res.* 42 (2000) 7–17.
- [9] H. Tavares, J. Yacubian, L.L. Talib, N.R. Barbosa, W.F. Gattaz, Increased phospholipase A₂ activity in schizophrenia with absent response to niacin, *Schizophr. Res.* 61 (2002) 1–6.
- [10] W.F. Gattaz, A. Maras, N.J. Cairns, R. Levy, H. Förstl, Decreased phospholipase A₂ activity in Alzheimer brains, *Biol. Psychiatry* 37 (1995) 13–17.
- [11] W.F. Gattaz, N.J. Cairns, R. Levy, H. Förstl, D.F. Braus, A. Maras, Decreased phospholipase A₂ activity in the brain and in platelets of patients with Alzheimer's disease, *Eur. Arch. Psychiatry Clin. Neurosci.* 246 (1996) 129–131.
- [12] W.F. Gattaz, O.V. Forlenza, L.L. Talib, N.R. Barbosa, C.M. Bottino, Platelet phospholipase A₂ activity in Alzheimer's disease and mild cognitive impairment, *J. Neural Transm.* 111 (2004) 591–601.
- [13] M. Simonato, A pathogenetic hypothesis of temporal lobe epilepsy, *Pharmacol. Res.* 27 (1993) 217–225.
- [14] F. Visioli, E.B. Rodriguez-de-Turco, N.R. Kreisman, N.G. Bazan, Membrane lipid degradation is related to interictal cortical activity in series of seizures, *Metab. Brain Dis.* 9 (1994) 161–170.
- [15] A.A. Farooqui, H.C. Yang, L. Horrocks, Involvement of phospholipase A₂ in neurodegeneration, *Neurochem. Int.* 30 (1996) 517–522.
- [16] G.Z. Feuerstein, Platelet-activating factor: a case for its role in CNS function and brain injury, *J. Lipid Mediators Cell Signalling* 14 (1996) 109–114.
- [17] N.G. Bazan, The neuromessenger platelet-activating factor in plasticity and neurodegeneration, *Prog. Brain Res.* 118 (1998) 281–291.
- [18] N.G. Bazan, V. Colangelo, W.J. Lukiw, Prostaglandins and other lipid mediator in Alzheimer's disease, *Prostag. Other Lipid Mediat* 68–69 (2002) 197–210.
- [19] D.F. Horrobin, C.N. Bennett, Depression and bipolar disorder: relationships to impaired fatty acid and phospholipids metabolism and to diabetes, cardiovascular disease, immunological abnormalities, cancer, ageing and osteoporosis, *Prostag. Leukotr. Ess.* 60 (1999) 217–234.
- [20] D.F. Horrobin, C.N. Bennett, Phospholipid metabolism and the pathophysiology of psychiatry and neurological disorders, in: M. Peet, I. Glen, D.F. Horrobin (Eds.), *Phospholipid Spectrum Disorders in Psychiatry and Neurology*, Marius Press, 2003.
- [21] L.E. MacDonell, F.K. Skinner, P.E. Ward, A.I. Glen, A.C. Glen, D.J. Macdonald, R.M. Boyle, D.F. Horrobin, Increased levels of cytosolic phospholipase A₂ in dyslexics, *Prostag. Leukotr. Ess.* 63 (2000) 37–39.
- [22] D.A. Six, E.A. Dennis, The expanding superfamily of phospholipase A₂ enzymes: classification and characterization, *Biochim. Biophys. Acta* 1488 (2000) 1–19.
- [23] M.V. Winstead, J. Balsinde, E.A. Dennis, Calcium-independent phospholipase A₂: structure and function, *Biochim. Biophys. Acta* 1499 (2000) 28–39.
- [24] I. Kudo, M. Murakami, Phospholipase A₂ enzymes, *Prostag. Other Lipid Mediat* 68–69 (2002) 3–58.
- [25] S. Chakraborti, Phospholipase A₂ isoforms: a perspective, *Cell Signalling* 15 (2003) 637–665.
- [26] A.N. Fonteh, R.J. Harrington, A.F. Huhmer, R.G. Biringier, J.N. Riggins, M.G. Harrington, Identification of disease markers in human cerebrospinal fluid using lipidomic and proteomic methods, *Dis. Markers* 22 (2006) 39–64.
- [27] T. Bayburt, F.Z. Yu, I. Street, F. Ghomashchi, F. Laliberté, H. Perrier, Z. Wang, R. Homan, M.K. Jain, M.H. Gelb, Continuous vesicle-based fluorimetric assays of 14- and 85-kDa phospholipases A₂, *Anal. Biochem.* 232 (1995) 7–23.
- [28] E.I. Kitsioulis, G. Nakos, M.E. Lekka, Differential determination of phospholipase A₂ and PAF-acetylhydrolase in biological fluids using fluorescent substrates, *J. Lipid Res.* 40 (1999) 2346–2356.
- [29] D.J. Macdonald, R.M. Boyle, A.C.B. Glen, M. Ross, A.M. Glen, P.E. Ward, S.B. McKinney, M.A. Peterkin, The investigation of cytosolic phospholipase A₂ using ELISA, *Prostag. Leukotr. Ess.* 70 (2004) 377–381.
- [30] B.M. Ross, B. Hughesb, S.J. Kishb, J.J. Warsh, Serum calcium-independent phospholipase A₂ activity in bipolar affective disorder *Bipolar Disorders* 8 (2006) 265–270.
- [31] H.C. Yang, M. Mosior, B. Ni, E.A. Dennis, Regional distribution, ontogeny, purification and characterization of the Ca²⁺-independent phospholipase A₂ from rat brain, *J. Neurochem.* 73 (1999) 1278–1287.
- [32] R. Kramer, E.F. Roberts, J.V. Manetta, P.A. Hysolp, J.A. Jakubowski, Thrombin-induced phosphorylation and activation of Ca²⁺-sensitive cytosolic phospholipase A₂ in human platelets, *J. Biol. Chem.* 268 (1993) 26796–26804.
- [33] M.J. Owens, C.B. Nemeroff, Role of serotonin in the pathophysiology of depression focus on the serotonin transporter, *Clin. Chem.* 40 (1994) 288–295.
- [34] A.I. Bush, R.E. Tanzi, Alzheimer disease-related abnormalities of amyloid β precursor protein isoforms in the platelet, *Arch. Neurol.* 55 (1998) 1179–1180.
- [35] M. Di Luca, F. Colciaghi, L. Pastorino, B. Borroni, A. Padovani, F. Cattabeni, Platelets as a peripheral district were to study pathogenetic mechanisms of Alzheimer disease: the case of amyloid precursor protein, *Eur. J. Pharmacol.* 405 (2000) 277–283.
- [36] K.K. Lucas, E.A. Dennis, Distinguishing phospholipase A₂ types in biological samples by employing group-specific assays in presence of inhibitors, *Prostag. Other Lipid Mediat* 77 (2005) 235–248.
- [37] J.D. Clark, L.-L. Lin, R.W. Kriz, C.S. Ramesha, L.A. Sultzman, A.Y. Lin, N. Milona, J.L.A. Knopf, novel arachidonic acid-selective cytosolic PLA₂ contains a Ca₂-dependent translocation domain with homology to PKC and GAP, *Cell* 65 (1991) 1043–1051.
- [38] J.D. Sharp, D.L. White, X.G. Chiou, T. Goodson, G.C. Gamboa, D. McClure, S. Burgett, J. Hoskins, P.L. Skatrud, J.R. Sportsman, G.W. Becker, L.H. Kang,

- E.F. Roberts, R.M. Kramer, Molecular cloning and expression of human Ca_2^+ -sensitive cytosolic phospholipase A_2 , *Biol. C/tern.* 266 (1991) 14850–14853.
- [39] R.J. Mayer, L.A. Marshall, New insights on mammalian phospholipase A_2 (s); comparison of arachidonoyl-selective and -nonselective enzymes, *FASEB J.* 1 (1993) 339–348. Review.
- [40] W.P. Wilkins 3rd, S.E. Barbour, VI Group, phospholipases A_2 : homeostatic phospholipases with significant potential as targets for novel therapeutics, *Curr. Drug Targets* 9 (2008) 683–697. Review.
- [41] I.A. Zainaghi, O.V. Forlenza, W.F. Gattaz, Abnormal APP processing in platelets of patients with Alzheimer's disease: correlations with membrane fluidity and cognitive decline, *Psychopharmacology* 192 (2007) 547–553.

Reduced platelet amyloid precursor protein ratio (APP ratio) predicts conversion from mild cognitive impairment to Alzheimer's disease

Isis A. Zainaghi · Leda L. Talib · Breno S. Diniz ·
Wagner F. Gattaz · Orestes V. Forlenza

Received: 29 December 2011 / Accepted: 16 April 2012 / Published online: 10 May 2012
© Springer-Verlag 2012

Abstract Studies have shown that platelet APP ratio (representing the percentage of 120–130 kDa to 110 kDa isoforms of the amyloid precursor protein) is reduced in patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD). In the present study, we sought to determine if baseline APP ratio predicts the conversion from MCI to AD dementia after 4 years of longitudinal assessment. Fifty-five older adults with varying degrees of cognitive impairment (34 with MCI and 21 with AD) were assessed at baseline and after 4 years. MCI patients were re-classified according to the conversion status upon follow-up: 25 individuals retained the diagnostic status of MCI and were considered as stable cases (MCI–MCI); conversely, in nine cases the diagnosis of dementia due to AD was ascertained. The APP ratio (APPr) was determined by the Western blot method in samples of platelets collected at baseline. We found a significant reduction of APPr in MCI patients who converted to dementia upon follow-up. These individuals had baseline APPr values similar to those of demented AD patients. The overall accuracy of APPr to identify subjects with MCI who will progress to AD was 0.74 ± 0.10 , $p = 0.05$. The cut-off of 1.12 yielded a sensitivity of 75 % and a specificity of 75 %. Platelet APPr may be a surrogate marker of the disease process in AD, with potential implications for the assessment of abnormalities in the APP metabolism in patients with and at risk for dementia. However,

diagnostic accuracy was relatively low. Therefore, studies in larger samples are needed to determine whether APPr may warrant its use as a biomarker to support the early diagnosis of AD.

Keywords Alzheimer's disease · Dementia · Mild cognitive impairment · Amyloid precursor protein · APP ratio

Introduction

In recent years, the search for peripheral biomarkers of Alzheimer's disease (AD) and related disorders has become a major focus of research. Many candidate markers related to the AD pathophysiology have been tested with promising results, e.g., serum and/or plasma concentrations of the amyloid-beta peptide ($A\beta_{42}$) (Seppälä et al. 2010), neurotrophic (Forlenza et al. 2010a) and inflammatory factors (Diniz et al. 2010), and markers of oxidative stress (Pratico et al. 2002). A recent study has concomitantly addressed serum concentrations of several markers of hypoxia, oxidative stress and membrane lipid remodeling with a metabolomics model (Oresic et al. 2011). Therefore, signaling proteins and enzymes related to core pathological cascades of AD may represent potential biomarkers of the AD process provided their measures in the periphery bear correlation with intracerebral pathology (Ray et al. 2007). In studies conducted in our group, we have provided evidence of abnormal enzymatic activity of important components of AD, such as glycogen synthase-kinase (GSK3B) (Forlenza et al. 2011), phospholipase A_2 (Gattaz et al. 2004) and amyloid precursor protein (APP), by means of determining the peripheral correlates of these abnormalities in human platelets.

I. A. Zainaghi and L. L. Talib equally contributed to this study.

I. A. Zainaghi · L. L. Talib · B. S. Diniz ·
W. F. Gattaz · O. V. Forlenza (✉)
Laboratory of Neuroscience, Department and Institute
of Psychiatry, Faculty of Medicine, University of São Paulo,
São Paulo, Brazil
e-mail: forlenza@usp.br

APP is an integral transmembrane protein widely expressed in neuronal and peripheral tissues. There are three major isoforms (APP₆₉₅, APP₇₅₁, and APP₇₇₀), which are produced by the alternative splicing of mRNA (Daly et al. 1998). In neurons, APP is processed by two major, mutually exclusive metabolic routes: the amyloidogenic pathway, in which APP is sequentially cleaved by β - and γ -secretase; or the secretory (non-amyloidogenic) pathway, upon APP cleavage by α - and γ -secretases. Therefore, the APP metabolism generates soluble APP (sAPP) fragments that have different C-termini, and may represent distinct metabolic routes depending on the cleavage by secretases alpha or beta (i.e., sAPPalpha or sAPPbeta), in addition to A β fragments that are released in only when APP is cleaved in the amyloidogenic pathways. Platelets are the main source of APP in the periphery, being responsible for 95 % of the circulating APP (Davies et al. 1997; Li et al. 1998). The enzymatic machinery needed to process APP in neurons (α , β and γ -secretase) is also present in platelets, and recent studies suggest that abnormalities affecting the APP homeostasis in the AD brain may also be represented in platelets (Tang et al. 2006).

In fact, previous studies demonstrated that platelet APP ratio (APPr) (120–130/110 kDa fragments) is altered in patients with AD, reflecting systemic changes in APP metabolism. A significantly lower APPr has been described in patients with AD (Di Luca et al. 1998 and Rosenberg et al. 1997) and with mild cognitive impairment (MCI) (Zainaghi et al. 2007), indicating that the amyloidogenic pathway is upregulated in these patients. One study showed that patients with MCI who progressed to AD upon follow-up had a significantly lower APPr as compared to stable cases of MCI and those who had other (non-AD) dementia outcomes (Borroni et al. 2003). Therefore, platelet APPr may be viewed as peripheral marker of the pathological process in AD that not only supports and facilitates the cross-sectional diagnosis, but also has predictive value.

Therefore, the objectives of the present study were (1) to determine baseline APPr in a longitudinal cohort of older adults with MCI; and (2) to determine if a lower APPr predicts the conversion from MCI to AD-dementia after 4 years of follow-up.

Materials and methods

Participants

A total of 55 older adults with baseline varying degrees of cognitive impairment (34 subjects with MCI and 21 patients with AD) were included in this study and followed-up for 4 years. The baseline characteristics and assessment and diagnostic procedures for these patients

were published elsewhere (Zainaghi et al. 2007; Diniz et al. 2008). In the follow-up, all MCI patients underwent comprehensive clinical and cognitive assessments. The diagnosis of MCI and AD was ascertained by the Mayo Clinic and NINCDS-ADRDA diagnostic criteria, respectively (McKhann et al. 2011; Petersen et al. 2001). Therefore, the MCI patients were divided according to its diagnostic status in the follow-up as: (1) MCI–MCI: patients with MCI who retained the MCI diagnosis in the follow-up ($n = 25$), (2) MCI–AD: patients with MCI who progressed to AD in the follow-up ($n = 9$).

Platelet preparation and Western blotting

Samples of peripheral blood (15 ml) were collected in 0.1 M sodium citrate-coated tubes (Sarsted). Platelet-rich plasma was separated from blood cells, and platelets were collected by centrifugation. Platelet pellets were washed with Tris and resuspended in lysis buffer containing Tris–HCl, ethylene glycol tetraacetic acid, phenylmethanesulfonyl fluoride, and protease inhibitors. Platelet aliquots were stored at -70°C . Protein concentrations were determined in each sample by a modified Lowry method (Bio-Rad DC Protein Assay) before the Western blot assay.

For each sample, 24 μg of protein was separated by electrophoresis in 8 % polyacrylamide gels and transferred to nitrocellulose membranes. After block unspecific bindings, membranes were incubated for 2 h with the primary antibody m22C11 (Chemicon International). Membranes were then incubated for 1 h with goat anti-mouse secondary antibodies labeled with horseradish peroxidase (Sigma), after which the enhanced chemoluminescence (ECL; GE) reagent was poured. Imaging was performed in ChemiimagerTM 4,000 equipment (Alpha Innotech), which captures the chemiluminescent light emission from the reaction of the peroxidase-conjugated antibody with the ECL reagent (GE). Light emission was continuously captured for 15 min, and the densitometry of distinct bands was performed with specific software tools. All measurements were registered as the proportion of the 130- to 110-kDa band densities (130- to 110-kDa ratio) (Fig. 1). An internal control (IC) consisting of a standard suspension of platelets (prepared in-house from a pool of platelets from young healthy volunteers) was added to each gel to control for analytical differences between blots performed in different days (inter-assay variation). The IC reading in proportion to the 130- to 110-kDa ratio was then calculated, yielding the normalized estimate of the 130- to 110-kDa ratio (APPr). Each sample was analyzed in triplicates, and additional blots of the same samples were performed whenever the reading between replicates displayed a variation $>15\%$.

Fig. 1 Representative western blots illustrating bands found in AD, MCI and controls (IC internal control)

Statistical analysis

We carried out non-parametric Kruskal–Wallis analysis to ascertain differences between MCI–MCI and MCI–AD groups in the baseline socio-demographic, cognitive variables and APPr. Exact Fisher test analysis was done to assess differences in the frequency distribution of gender between groups. ROC curve analyses were done to assess the accuracy, sensitivity and specificity of APPr to identify patients with MCI who will progress to AD in the follow-up.

Results

Table 1 summarizes the clinical and cognitive variables and APPr for MCI–MCI and MCI–AD patients. MCI–AD patients were older and had a significantly higher frequency of males as compared to MCI–MCI patients. APPr was significantly reduced in the MCI–AD as compared to MCI–MCI patients after controlling for demographic confounders such as gender and age distribution ($p = 0.017$). The overall accuracy of APPr to identify subjects with MCI who will progress to AD was 0.74 ± 0.10 , $p = 0.05$. ROC curve analyses indicated that the cut-off of 1.12 yielded a sensitivity of 75 % and a specificity of 75 % (Fig. 2).

No significant differences were observed in mean values of APPr between converters and patients with clinically manifest dementia (MCI–AD: 1.01 ± 0.2 ; AD: 1.03 ± 0.9 ,

Fig. 2 ROC curve for APPr for the prediction of Alzheimer's disease in subjects with MCI (cut-off 1.12; sensitivity 75 %; specificity 75 %)

$p = 0.58$). In contrast, all cognitive test scores as expected were significantly lower in patients with AD (MMSE 18.2 ± 2.1 , $p = 0.003$; CAMCOG 60.3 ± 8.1 , $p = 0.001$), in the absence of any significant differences in age ($p = 0.1$).

Discussion

In the present study, we found a significant reduction in values of APPr in patients with MCI who converted to dementia of the AD-type (MCI–AD), as compared to those who remained cognitively stable (MCI–MCI) upon follow-up. Such finding was indistinguishable from that observed in patients with clinically manifest AD dementia. These findings are in agreement with the previous reports in the literature (Borroni et al. 2003). In addition, the cut-off of 1.12 showed a good sensitivity and specificity (75 %) to discriminate between MCI who converted to AD. The present results provide additional evidence that platelet APPr is a potential marker of the pathological process in AD. However, the interpretation of the present results must be made with caution given the small sample size and the relatively low diagnostic accuracy to predict the dementia outcome given the diagnosis of MCI, which therefore, requires replication in larger and independent samples.

Several lines of evidence suggest that platelets contain the cellular machinery necessary for APP processing and that there is a preferential activation of the amyloidogenic pathway in platelets of patients with AD. Patients with AD show increased immunoreactivity of BACE1 and decreased ADAM10 immunoreactivity (Tang et al. 2006); β -secretase activity is also significantly increased in these patients (Holsinger et al. 2002). Such changes are similar to those observed in neurons. Thus, platelets can serve as a

Table 1 Baseline variables according to diagnostic outcome in the follow-up

	Baseline diagnosis—follow-up diagnosis		<i>p</i>
	MCI–MCI	MCI–AD	
Gender			
M/W	5/20	8/1	0.001
Age	70.5 (68.5–72.5)	79.0 (74.7–83.2)	0.001
Years of education	11.3 (9.4–13.17)	10.0 (5.97–14.0)	0.52
MMSE	27.2 (26.4–28.1)	25.1 (21.46–28.8)	0.54
CAMCOG	93.4 (90.7–96.1)	83.7 (72.1–92.2)	0.07
APPr	1.2 (1.1–1.3)	1.01 (0.8–1.2)	0.017

peripheral model to assess changes in the amyloidogenic pathway and to evaluate the effects of drugs targeting the amyloid cascade in patients with AD.

The early and accurate diagnosis of AD is complex and depends upon the assessment of multiple biologic cascades by distinct methods (Forlenza et al. 2010b). Such biomarker panel not only needs to be accurate but also feasible and widely available in the clinical practice (Di Luca et al. 2005). Currently, structural and functional neuroimaging techniques and cerebrospinal fluid biomarkers (amyloid- β_{42} and Tau protein) are the most widely studied (McEvoy and Brewer 2010). Despite their accuracy, they are not widely available in most clinical centers. Therefore, peripheral markers may be useful tools to identify subjects at increased risk to progress to AD who would benefit from more comprehensive assessment. In this scenario, our results suggest that platelet APPr may be a potential candidate for use in routine biomarker assessment to determine the risk of AD in MCI subjects.

The biological meaning of the present findings must also be inferred with caution, since the monoclonal antibody (22C11) used in this study detects both APP and APLP2 (amyloid-beta precursor-like protein 2) at the N-terminus, the latter protein also present in platelets. Indeed APP and APLP2 are homologous transmembrane proteins, with similar molecular weights (~ 87 kDa) and isoelectric properties (~ 4.7) in their native forms. The bands detected using Western blot at 110–130 kDa may represent immature or mature APP isoforms, respectively, depending on post-transcriptional changes to the molecule mostly due to glycosylation and sulfatation of APP residues. Conversely, the maturation of APLP2 depends largely on the binding of glycosaminoglycan groups (Lo et al. 1995), so that the molecular weight of mature APLP2 is reportedly 120 kDa (Slunt et al. 1994). Therefore, subtle Western-blotting differences may have been identified for APP and APLP2. Anyhow, the present methods have been consistently used in the literature addressing APPr in human platelets. Anyhow, we understand that new research is needed to clarify this important issue.

In conclusion, we showed that platelet APPr is significantly reduced in patients with MCI who progress to AD, and this determination has a good diagnostic accuracy for the identification of these subjects. Platelet APPr may be regarded as a surrogate marker and a theragnostic for the assessing the amyloidogenic pathway in patients with and at risk for AD, and the disease-modifying effect of anti-amyloid agents. Further studies in larger cohorts are needed to make definitive statements about the clinical utility of this measure in the diagnostic workup of AD, i.e., whether APPr can be used in addition to, or instead of, more expensive or invasive PET and CSF methods.

Acknowledgments This work was supported by grants from the Associação Beneficente Alzira Denise Hertzog da Silva (ABADHS) and from FAPESP (projects no. 02/12633-7 and 2009/52825-8).

Conflict of interest The authors report no conflict of interest in the preparation of this manuscript.

References

- American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders, 4th edn. American Psychiatric Association, Washington, DC
- Borroni B, Colciaghi F, Caltagirone C, Rozzini L, Broglio L, Cattabeni F, Di Luca M, Padovani A (2003) Platelet amyloid precursor protein abnormalities in mild cognitive impairment predict conversion to dementia of Alzheimer type: a 2-year follow-up study. *Arch Neurol* 60(12):1740–1744
- Daly IVJ, Lahiri DK, Justus DE, Kotwal GJ (1998) Detection of the membrane-retained carboxy-terminal tail containing polypeptides of the amyloid precursor protein in tissue from Alzheimer's disease brain. *Life Science* 63:2121–2131
- Davies TA, Long HJ, Sgro K, Rathbun WH, Mcmenamin ME, Seetoo K, Tibbles H, Billingslea AM, Fine RE, Fishman JB, Levesque CA, Smith SJ, Wells JM, Simons ER (1997) Activated Alzheimer disease platelets retain more beta amyloid precursor protein. *Neurobiol Aging* 18:147–153
- Di Luca M, Pastorino L, Bianchetti A, Perez J, Viagnolo LA, Lenzi GL, Trabucchi M, Cattabeni F, Padovani A (1998) Differential level of platelet amyloid β precursor protein isoforms—an early marker for Alzheimer disease. *Arch Neurol* 55:1195–1200
- Di Luca M, Grossi E, Borroni B, Zimmermann M, Marcello E, Colciaghi F, Gardoni F, Intraligi M, Padovani A, Buscema M (2005) Artificial neural networks allow the use of simultaneous measurements of Alzheimer disease markers for early detection of disease. *J Transl Med* 3:30
- Diniz BS, Nunes PV, Yassuda MS, Pereira FS, Flaks MK, Viola LF et al (2008) Mild cognitive impairment: cognitive screening or neuropsychological assessment? *Rev Bras Psiquiatr* 30(4):316–321
- Diniz BS, Teixeira AL, Ojopi EB, Talib LL, Mendonça VA, Gattaz WF, Forlenza OV (2010) Higher serum sTNFR1 level predicts conversion from mild cognitive impairment to Alzheimer's disease. *J Alzheimers Dis* 22(4):1305–1311
- Forlenza OV, Diniz BS, Teixeira AL, Ojopi EB, Talib LL, Mendonça VA, Izzo G, Gattaz WF (2010a) Effect of brain-derived neurotrophic factor Val66Met polymorphism and serum levels on the progression of mild cognitive impairment. *World J Biol Psychiatry* 11(6):774–780
- Forlenza OV, Diniz BS, Gattaz WF (2010b) Diagnosis and biomarkers of pre-dementia in Alzheimer's disease. *BMC Med* 8:89 (Review)
- Forlenza OV, Torres CA, Talib LL, de Paula VJ, Joaquim HP, Diniz BS, Gattaz WF (2011) Increased platelet GSK3B activity in patients with mild cognitive impairment and Alzheimer's disease. *J Psychiatr Res* 45(2):220–224
- Gattaz WF, Forlenza OV, Talib LL, Barbosa NR, Bottino CM (2004) Platelet phospholipase A(2) activity in Alzheimer's disease and mild cognitive impairment. *J Neural Transm* 111:591–601
- Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G (2002) Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann Neurol* 51(6):783–786
- Li QX, Whyte S, Tanner JE, Evin G, Beyreuther K, Masters CL (1998) Secretion of Alzheimer's disease A β amyloid peptide by activated human platelets. *Lab Invest* 78:461–469

- Lo AC, Thinakaran G, Slunt HH, Sisodia SS (1995) Metabolism of the amyloid precursor-like protein 2 in MDCK cells. Polarized trafficking occurs independent of the chondroitin sulfate glycosaminoglycan chain. *J Biol Chem* 270(21):12641–12645
- McEvoy LK, Brewer JB (2010) Quantitative structural MRI for early detection of Alzheimer's disease. *Expert Rev Neurother* 10(11):1675–1688 (Review)
- McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Kawash CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Ross MN, Scheltens P, Carillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging and the Alzheimer's Association workgroup. *Alzheimer's Dementia* 1–7 (E-pub ahead of print)
- Oresic M, Hyotylainen T, Herukka S-K, Sysi-Aho M, Mattila I, Seppanen-Laakso T, Julkunen V, Gopalacharyulu PV, Hallikainen M, Koikkalainen J, Kivipelto M, Helisalmi S, Lotjonen J, Soininen H (2011) Metabolome in progression to Alzheimer's disease. *Transl Psychiatry* 1:e57. doi:10.1038/tp.2011.55 (E-pub ahead of print)
- Petersen RC, Doody R, Kurz A, Mohs RC, Morris JC, Rabins PV et al (2001) Current concepts in mild cognitive impairment. *Arch Neurol* 58:1985–1992
- Pratico D, Clark CM, Liun F, Rokach J, Lee VY, Trojanowski JQ (2002) Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. *Arch Neurol* 59:972–976
- Ray S, Britschgi M, Herbert C, Takeda-Uchimura Y, Boxer A, Blennow K, Friedman LF, Galasko DR, Jutel M, Karydas A, Kaye JA, Leszek J, Miller BL, Minthon L, Quinn JF, Rabinovici GD, Robinson WH, Sabbagh MN, So YT, Sparks DL, Tabaton M, Tinklenberg J, Yesavage JA, Tibshirani R, Wyss-Coray T (2007) Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nat Med* 13(11):1359–1362
- Rosenberg RN, Baskin F, Fosmire JA, Risser R, Adams P, Svetlik D, Honig LS, Cullun MC, Weiner MF (1997) Altered amyloid protein processing in platelets of patients with Alzheimer disease. *Arch Neurol* 54:139–144
- Seppälä TT, Herukka SK, Hänninen T, Tervo S, Hallikainen M, Soininen H, Pirttilä T (2010) Plasma Aβ42 and Aβ40 as markers of cognitive change in follow-up: a prospective, longitudinal, population-based cohort study. *J Neurol Neurosurg Psychiatry* 81(10):1123–1127
- Slunt HH, Thinakaran G, Von Koch C, Lo AC, Tanzi RE, Sisodia SS (1994) Expression of a ubiquitous, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). *J Biol Chem* 269(4):2637–2644
- Tang K, Hyman LS, Baskin F, Rosenberg RN (2006) Platelet amyloid precursor protein processing: a bio-marker for Alzheimer's disease. *J Neurol Sci* 240:53–58
- Zainaghi IA, Forlenza OV, Gattaz WF (2007) Abnormal APP processing in platelets of patients with Alzheimer's disease: correlations with membrane fluidity and cognitive decline. *Psychopharmacology* 192:547–553

Christian Humpel, Professor, Series Editor

Platelet biomarkers in Alzheimer's disease

Leda L Talib, Helena PG Joaquim, Orestes V Forlenza

Leda L Talib, Helena PG Joaquim, Orestes V Forlenza, Laboratory of Neuroscience (LIM-27), Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo, 05403-010 São Paulo, SP, Brazil

Author contributions: All authors contributed to this manuscript.

Supported by Associação Beneficente Alzira Denise Hertzog da Silva (ABADHS) and FAPESP, Fundação de Amparo à Pesquisa do Estado de São Paulo (Project 02/13633-7)

Correspondence to: Orestes V Forlenza, MD, PhD, Associate Professor, Laboratory of Neuroscience (LIM-27), Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo, Rua Dr. Ovídio Pires de Campos 785, 05403-010 São Paulo, SP, Brazil. forlenza@usp.br

Telephone: +55-11-26617283

Received: November 20, 2011 Revised: October 23, 2012

Accepted: November 17, 2012

Published online: December 22, 2012

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 A₂), 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.

© 2012 Baishideng. All rights reserved.

Key words: Alzheimer's disease; Biomarkers; Platelets; Amyloid precursor protein; Glycogen synthase kinase-3B; Phospholipase A₂

Peer reviewer: Safa A Elgamal, MBBCh, MSc, PhD, MD, Faculty of Applied Health Sciences, University of Waterloo, 200 University Avenue, West, Waterloo, N2L3G1, Canada

Talib LL, Joaquim HPG, Forlenza OV. Platelet biomarkers in Alzheimer's disease. *World J Psychiatr* 2012; 2(6): 95-101 Available from: URL: <http://www.wjgnet.com/2220-3206/full/v2/i6/95.htm> DOI: <http://dx.doi.org/10.5498/wjp.v2.i6.95>

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,

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 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 pseudo-dementia. 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 be 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 (A β) 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 sAPP α) 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 A β , 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 A β domain, yielding intact A β sequences within the C-terminal residue of APP. A subsequent cleavage of APP at the carboxyl terminus of A β sequence by γ -secretase releases A β peptides of 40 (A β 40) or 42 amino acids (A β 42).

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

sion of APP-cleaving enzymes affecting the A β 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 A β 40^[13], suggesting that the amyloidogenic cleavage of APP from platelet membranes is subsequent to the secretion of α -granules.

It is likely that secreted A β 40 peptides contribute to the amyloid burden in the vascular walls of the AD brain. In fact, a recent study indicated that soluble A β can be internalized into lysosomes by the cerebrovascular smooth muscle cells, yielding A β 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 helical 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 A β peptide^[32]. According to the 'GSK hypothesis of AD', the abnormal activation of GSK3B is associated

both with the overproduction of A β 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 A β 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 amnesic MCI^[39,40].

Li *et al.*^[42] first characterized the expression of the Tau protein in platelets, and further showed that the phosphopeptide 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].

INFLAMMATORY 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]. A β deposits contain proteins of

the complement pathway and the interaction between aggregated A β 42 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, prostaglandins 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 A₂ (PLA₂) and other subtypes are highly expressed in platelets^[41], and play a critical role in the regulation of the inflammatory response^[45].

PLA₂ abnormalities have been consistently found in AD^[46], with important implications in membrane homeostasis and the inflammatory response. The involvement of platelet PLA₂ 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-1 α , 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 A β across the blood brain barrier, co-localizes with several complement factors in cerebrovascular amyloid deposits, and also binds A β 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 orbital, 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 oxi-

dative 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 is 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 amnesic MCI as compared to non-amnesic counterparts^[54].

PLATELET PHOSPHOLIPASE A₂ ACTIVITY

Platelet PLA₂ enzymes are critically involved in the metabolism of membrane phospholipids. The PLA₂ superfamily is composed of three main groups: calcium-dependent, cytosolic PLA₂; calcium-dependent, secretory PLA₂; and calcium-independent, intra-cellular PLA₂. PLA₂ 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, PLA₂ 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 PLA₂ 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 PLA₂ influences APP processing and secretion. In neuronal cultures, PLA₂ activation leads to increased release of soluble forms of APP (sAPP α) 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 A β . This assumption is supported by early post-mortem studies of the AD brain^[69], showing that intra-cerebral PLA₂ 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 PLA₂ 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 PLA₂ 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 PLA₂ 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 PLA₂ activity between those observed in AD and normal controls, it is likely that in such patients the identification of PLA₂ 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 PLA₂ 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 (³¹P-MRS) studies indicated that decreased phospholipid breakdown in the AD brain is correlated with lower platelet PLA₂ activity^[73]. In addition to being a candidate marker of the disease process, PLA₂ may also respond to therapeutic interventions. In a recent study, PLA₂ 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 PLA₂), 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.

REFERENCES

- 1 Cummings JL. Challenges to demonstrating disease-modifying effects in Alzheimer's disease clinical trials. *Alzheimers Dement* 2006; 2: 263-271
- 2 Di Luca M, Grossi E, Borroni B, Zimmermann M, Marcello E, Colciaghi F, Gardoni F, Intraligi M, Padovani A, Buscema M. Artificial neural networks allow the use of simultaneous measurements of Alzheimer disease markers for early detection of the disease. *J Transl Med* 2005; 3: 30
- 3 Hansson O, Zetterberg H, Buchhave P, Londo E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* 2006; 5: 228-234
- 4 Mattsson N, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, Bocchio-Chiavetto L, Blankenstein MA, Carrillo MC, Chalbot S, Coart E, Chiasserini D, Cutler N, Dahlfors G, Duller S, Fagan AM, Forlenza O, Frisoni GB, Galasko D, Galimberti D, Hampel H, Handberg A, Heneka MT, Herskovits AZ, Herukka SK, Holtzman DM, Humpel C, Hyman BT, Iqbal K, Jucker M, Kaeser SA, Kaiser E, Kapaki E, Kidd D, Klivenyi P, Knudsen CS, Kummer MP, Lui J, Lladó A, Lewczuk P, Li QX, Martins R, Masters C, McAuliffe J, Mercken M, Moghekar A, Molinuevo JL, Montine TJ, Nowatzke W, O'Brien R, Otto M, Paraskevas GP, Parnetti L, Petersen RC, Prvulovic D, de Reus HP, Rissman RA, Scarpini E, Stefani A, Soininen H, Schröder J, Shaw LM, Skinningsrud A, Skrogstad B, Spreer A, Talib L, Teunissen C, Trojanowski JQ, Tumani H, Umek RM, Van Broeck B, Vanderstichele H, Vecsei L, Verbeek MM, Windisch M, Zhang J, Zetterberg H, Blennow K. The Alzheimer's Association external quality control program for cerebrospinal fluid biomarkers. *Alzheimers Dement* 2011; 7: 386-395.e6
- 5 Padovani A, Pastorino L, Borroni B, Colciaghi F, Rozzini L, Monastero R, Perez J, Pettenati C, Mussi M, Parrinello G, Cottini E, Lenzi GL, Trabucchi M, Cattabeni F, Di Luca M. Amyloid precursor protein in platelets: a peripheral marker for the diagnosis of sporadic AD. *Neurology* 2001; 57: 2243-2248
- 6 Irizarry MC. Biomarkers of Alzheimer disease in plasma. *NeuroRx* 2004; 1: 226-234
- 7 Barradas MA, Mikhailidis DP. The use of platelets as models for neurons: possible applications to the investigation of eating disorders. *Biomed Pharmacother* 1993; 47: 11-18
- 8 El Haouari M, Rosado JA. Platelet function in hypertension. *Blood Cells Mol Dis* 2009; 42: 38-43
- 9 Holmsen H. Signal transducing mechanisms in platelets. *Proc Natl Sci Coun Repub China B* 1991; 15: 147-152
- 10 Barry FA, Graham GJ, Fry MJ, Gibbins JM. Regulation of glycogen synthase kinase 3 in human platelets: a possible role in platelet function? *FEBS Lett* 2003; 553: 173-178
- 11 Neumann K, Fariás G, Slachevsky A, Perez P, Maccioni RB. Human platelets tau: a potential peripheral marker for Alzheimer's disease. *J Alzheimers Dis* 2011; 25: 103-109
- 12 Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. *J Biol Chem* 1998; 273:

- 27765-27767
- 13 Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci USA* 1999; **96**: 3922-3927
- 14 Tang K, Hynan LS, Baskin F, Rosenberg RN. Platelet amyloid precursor protein processing: a bio-marker for Alzheimer's disease. *J Neurol Sci* 2006; **240**: 53-58
- 15 Casoli T, Di Stefano G, Giorgetti B, Ballesti M, Recchioni R, Moroni F, Marcheselli F, Bernardini G, Fattoretti P, Bertoni-Freddari C. Platelet as a physiological model to investigate apoptotic mechanisms in Alzheimer beta-amyloid peptide production. *Mech Ageing Dev* 2008; **129**: 154-162
- 16 Casoli T, Di Stefano G, Giorgetti B, Grossi Y, Ballesti M, Fattoretti P, Bertoni-Freddari C. Release of beta-amyloid from high-density platelets: implications for Alzheimer's disease pathology. *Ann N Y Acad Sci* 2007; **1096**: 170-178
- 17 Di Luca M, Pastorino L, Cattabeni F, Zanardi R, Scarone S, Racagni G, Smeraldi E, Perez J. Abnormal pattern of platelet APP isoforms in Alzheimer disease and Down syndrome. *Arch Neurol* 1996; **53**: 1162-1166
- 18 Rosenberg RN, Baskin F, Fosmire JA, Risser R, Adams P, Svetlik D, Honig LS, Cullum CM, Weiner MF. Altered amyloid protein processing in platelets of patients with Alzheimer disease. *Arch Neurol* 1997; **54**: 139-144
- 19 Liu Q, Zerbinatti CV, Zhang J, Hoe HS, Wang B, Cole SL, Herz J, Muglia L, Bu G. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron* 2007; **56**: 66-78
- 20 Baskin F, Rosenberg RN, Iyer L, Hynan L, Cullum CM. Platelet APP isoform ratios correlate with declining cognition in AD. *Neurology* 2000; **54**: 1907-1909
- 21 Padovani A, Borroni B, Colciaghi F, Pettenati C, Cottini E, Agosti C, Lenzi GL, Caltagirone C, Trabucchi M, Cattabeni F, Di Luca M. Abnormalities in the pattern of platelet amyloid precursor protein forms in patients with mild cognitive impairment and Alzheimer disease. *Arch Neurol* 2002; **59**: 71-75
- 22 Zainaghi IA, Forlenza OV, Gattaz WF. Abnormal APP processing in platelets of patients with Alzheimer's disease: correlations with membrane fluidity and cognitive decline. *Psychopharmacology (Berl)* 2007; **192**: 547-553
- 23 Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-2 and phosphorylase kinase are the same enzyme. *Eur J Biochem* 1979; **100**: 339-347
- 24 Balaraman Y, Limaye AR, Levey AI, Srinivasan S. Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. *Cell Mol Life Sci* 2006; **63**: 1226-1235
- 25 Jope RS, Roh MS. Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Curr Drug Targets* 2006; **7**: 1421-1434
- 26 Muyliaert D, Kremer A, Jaworski T, Borghgraef P, Devijver H, Croes S, Dewachter I, Van Leuven F. Glycogen synthase kinase-3beta, or a link between amyloid and tau pathology? *Genes Brain Behav* 2008; **7** Suppl 1: 57-66
- 27 Peineau S, Bradley C, Taghibiglou C, Doherty A, Bortolotto ZA, Wang YT, Collingridge GL. The role of GSK-3 in synaptic plasticity. *Br J Pharmacol* 2008; **153** Suppl 1: S428-S437
- 28 Lovestone S, Reynolds CH, Latimer D, Davis DR, Anderton BH, Gallo JM, Hanger D, Mulot S, Marquardt B, Stabel S. Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr Biol* 1994; **4**: 1077-1086
- 29 Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 2001; **359**: 1-16
- 30 Lovestone S, McLoughlin DM. Protein aggregates and dementia: is there a common toxicity? *J Neurol Neurosurg Psychiatry* 2002; **72**: 152-161
- 31 Kar S, Slowikowski SP, Westaway D, Mount HT. Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer's disease. *J Psychiatry Neurosci* 2004; **29**: 427-441
- 32 Rockenstein E, Torrance M, Adame A, Mante M, Bar-on P, Rose JB, Crews L, Masliah E. Neuroprotective effects of regulators of the glycogen synthase kinase-3beta signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein phosphorylation. *J Neurosci* 2007; **27**: 1981-1991
- 33 Hooper C, Killick R, Lovestone S. The GSK3 hypothesis of Alzheimer's disease. *J Neurochem* 2008; **104**: 1433-1439
- 34 Grimes CA, Jope RS. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol* 2001; **65**: 391-426
- 35 Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* 1996; **93**: 8455-8459
- 36 Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 2003; **116**: 1175-1186
- 37 Koh SH, Noh MY, Kim SH. Amyloid-beta-induced neurotoxicity is reduced by inhibition of glycogen synthase kinase-3. *Brain Res* 2008; **1188**: 254-262
- 38 Noble W, Planel E, Zehr C, Olm V, Meyerson J, Suleman F, Gaynor K, Wang L, LaFrancois J, Feinstein B, Burns M, Krishnamurthy P, Wen Y, Bhat R, Lewis J, Dickson D, Duff K. Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. *Proc Natl Acad Sci USA* 2005; **102**: 6990-6995
- 39 Forlenza OV, Diniz BS, Talib LL, Radanovic M, Yassuda MS, Ojopi EB, Gattaz WF. Clinical and biological predictors of Alzheimer's disease in patients with amnesic mild cognitive impairment. *Rev Bras Psiquiatr* 2010; **32**: 216-222
- 40 Forlenza OV, Torres CA, Talib LL, de Paula VJ, Joaquim HP, Diniz BS, Gattaz WF. Increased platelet GSK3B activity in patients with mild cognitive impairment and Alzheimer's disease. *J Psychiatr Res* 2011; **45**: 220-224
- 41 Hye A, Kerr F, Archer N, Foy C, Poppe M, Brown R, Hamilton G, Powell J, Anderton B, Lovestone S. Glycogen synthase kinase-3 is increased in white cells early in Alzheimer's disease. *Neurosci Lett* 2005; **373**: 1-4
- 42 Li D, August S, Woulfe DS. GSK3beta is a negative regulator of platelet function and thrombosis. *Blood* 2008; **111**: 3522-3530
- 43 Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautsch S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. *Neurobiol Aging* 2000; **21**: 383-421
- 44 Casoli T, Di Stefano G, Ballesti M, Solazzi M, Giorgetti B, Fattoretti P. Peripheral inflammatory biomarkers of Alzheimer's disease: the role of platelets. *Biogerontology* 2010; **11**: 627-633
- 45 Medina M, Avila J. Glycogen synthase kinase-3 (GSK-3) inhibitors for the treatment of Alzheimer's disease. *Curr Pharm Des* 2010; **16**: 2790-2798
- 46 Schaeffer EL, Gattaz WF. Cholinergic and glutamatergic alterations beginning at the early stages of Alzheimer disease: participation of the phospholipase A2 enzyme. *Psychopharmacology (Berl)* 2008; **198**: 1-27
- 47 Bermejo P, Martin-Aragón S, Benedi J, Susin C, Felici E, Gil P, Ribera JM, Villar AM. Differences of peripheral inflammatory markers between mild cognitive impairment and Alzheimer's disease. *Immunol Lett* 2008; **117**: 198-202
- 48 Nuutinen T, Suuronen T, Kauppinen A, Salminen A. Clusterin: a forgotten player in Alzheimer's disease. *Brain Res Rev* 2009; **61**: 89-104

- 49 Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002; **82**: 47-95
- 50 Halliday G, Robinson SR, Shepherd C, Kril J. Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms. *Clin Exp Pharmacol Physiol* 2000; **27**: 1-8
- 51 Marcourakis T, Bahia VS, Kawamoto EM, Munhoz CD, Górgão R, Artes R, Kok F, Caramelli P, Nitrini R, Curi R, Scavone C. Apolipoprotein E genotype is related to nitric oxide production in platelets. *Cell Biochem Funct* 2008; **26**: 852-858
- 52 Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost* 2005; **3**: 2185-2192
- 53 Prodan CI, Szasz R, Vincent AS, Ross ED, Dale GL. Coated-platelets retain amyloid precursor protein on their surface. *Platelets* 2006; **17**: 56-60
- 54 Prodan CI, Ross ED, Vincent AS, Dale GL. Coated-platelets are higher in amnesic versus nonamnesic patients with mild cognitive impairment. *Alzheimer Dis Assoc Disord* 2007; **21**: 259-261
- 55 Prodan CI, Ross ED, Vincent AS, Dale GL. Rate of progression in Alzheimer's disease correlates with coated-platelet levels—a longitudinal study. *Transl Res* 2008; **152**: 99-102
- 56 Dennis EA. Diversity of group types, regulation, and function of phospholipase A2. *J Biol Chem* 1994; **269**: 13057-13060
- 57 Dennis EA. The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends Biochem Sci* 1997; **22**: 1-2
- 58 Bazan NG, Zorumski CF, Clark GD. The activation of phospholipase A2 and release of arachidonic acid and other lipid mediators at the synapse: the role of platelet-activating factor. *J Lipid Mediat* 1993; **6**: 421-427
- 59 Piomelli D. Arachidonic acid in cell signaling. *Curr Opin Cell Biol* 1993; **5**: 274-280
- 60 Simonato M. A pathogenetic hypothesis of temporal lobe epilepsy. *Pharmacol Res* 1993; **27**: 217-225
- 61 Gattaz WF, Köllisch M, Thuren T, Virtanen JA, Kinnunen PK. Increased plasma phospholipase-A2 activity in schizophrenic patients: reduction after neuroleptic therapy. *Biol Psychiatry* 1987; **22**: 421-426
- 62 Gattaz WF, Schmitt A, Maras A. Increased platelet phospholipase A2 activity in schizophrenia. *Schizophr Res* 1995; **16**: 1-6
- 63 Ross BM, Turenne S, Moszczynska A, Warsh JJ, Kish SJ. Differential alteration of phospholipase A2 activities in brain of patients with schizophrenia. *Brain Res* 1999; **821**: 407-413
- 64 Tavares H, Yacubian J, Talib LL, Barbosa NR, Gattaz WF. Increased phospholipase A2 activity in schizophrenia with absent response to niacin. *Schizophr Res* 2003; **61**: 1-6
- 65 Ross BM, Moszczynska A, Erlich J, Kish SJ. Phospholipid-metabolizing enzymes in Alzheimer's disease: increased lysophospholipid acyltransferase activity and decreased phospholipase A2 activity. *J Neurochem* 1998; **70**: 786-793
- 66 Gattaz WF, Maras A, Cairns NJ, Levy R, Förstl H. Decreased phospholipase A2 activity in Alzheimer brains. *Biol Psychiatry* 1995; **37**: 13-17
- 67 Emmerling MR, Moore CJ, Doyle PD, Carroll RT, Davis RE. Phospholipase A2 activation influences the processing and secretion of the amyloid precursor protein. *Biochem Biophys Res Commun* 1993; **197**: 292-297
- 68 Cho HW, Kim JH, Choi S, Kim HJ. Phospholipase A2 is involved in muscarinic receptor-mediated sAPPalpha release independently of cyclooxygenase or lipoxygenase activity in SH-SY5Y cells. *Neurosci Lett* 2006; **397**: 214-218
- 69 Gattaz WF, Cairns NJ, Levy R, Förstl H, Braus DF, Maras A. Decreased phospholipase A2 activity in the brain and in platelets of patients with Alzheimer's disease. *Eur Arch Psychiatry Clin Neurosci* 1996; **246**: 129-131
- 70 Gattaz WF, Forlenza OV, Talib LL, Barbosa NR, Bottino CM. Platelet phospholipase A(2) activity in Alzheimer's disease and mild cognitive impairment. *J Neural Transm* 2004; **111**: 591-601
- 71 Forlenza OV, Wacker P, Nunes PV, Yacubian J, Castro CC, Otaduy MC, Gattaz WF. Reduced phospholipid breakdown in Alzheimer's brains: a 31P spectroscopy study. *Psychopharmacology (Berl)* 2005; **180**: 359-365
- 72 Petersen RC, Doody R, Kurz A, Mohs RC, Morris JC, Rabins PV, Ritchie K, Rossor M, Thal L, Winblad B. Current concepts in mild cognitive impairment. *Arch Neurol* 2001; **58**: 1985-1992
- 73 Petersen RC, Stevens JC, Ganguli M, Tangalos EG, Cummings JL, DeKosky ST. Practice parameter: early detection of dementia: mild cognitive impairment (an evidence-based review). Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2001; **56**: 1133-1142
- 74 Talib LL, Yassuda MS, Diniz BS, Forlenza OV, Gattaz WF. Cognitive training increases platelet PLA2 activity in healthy elderly subjects. *Prostaglandins Leukot Essent Fatty Acids* 2008; **78**: 265-269

S- Editor Wang JL L- Editor Hughes D E- Editor Zheng XM

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Short communication

Correlation between platelet and brain PLA₂ activity

Leda L. Talib, Kette D. Valente, Silvia Vincentiis, Wagner F. Gattaz^{*}

Laboratory of Neuroscience-LIM 27, Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo, Brazil

ARTICLE INFO

Article history:

Received 4 April 2013

Received in revised form

26 June 2013

Accepted 2 July 2013

Keywords:

PLA₂

Platelets

Brain tissue

Hippocampus

Neuropsychiatric disorders

Epilepsy

Radioenzymatic assays

ABSTRACT

The phospholipase A₂ (PLA₂) enzymes have been implicated in several neuropsychiatry disorders and activity alterations have been described in brain and platelet. Since brain tissue is not readily available for the measurement of PLA₂ activity, it would be of interest to test directly whether PLA₂ activities in both tissues are correlated. We performed this task assessing PLA₂ activity in platelets and hippocampus collected simultaneously from 19 patients undergoing temporal lobectomy for treatment of refractory epilepsy. Our findings suggest that total PLA₂ activity in platelets may reflect the total activity of the enzyme in the brain ($r_s=0.59$, $p=0.008$). However in our sample no correlations were found between the subgroups of the enzyme in brain and in platelets. This lack of correlations may be due to different effects of drug treatment on the PLA₂ subtypes. In face of the difficulty to obtain brain tissues from living patients, further studies with larger drug-free samples are warranted to clarify whether the use of platelets is a reliable strategy to reflect the subtypes of PLA₂ activity in the brain.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

The phospholipase A₂ (PLA₂) enzymes catalyze the hydrolysis of membrane phospholipids at *sn*-2 position, releasing fatty acids and lysophospholipids, which mediate the transmission and processing of neuronal signals [1,2]. Increased PLA₂ activity has been described in platelets and in brain of schizophrenic and psychotic patients [3–10], whereas reduced enzyme activity was found in platelets and in post mortem brain of patients with Alzheimer's disease [11–13].

Platelets share many similar biologic properties to neurons and have been widely used in the search for biomarkers related to neuropsychiatric disorders [14–16]. There is indirect evidence suggesting that abnormal PLA₂ activity in platelets may reflect the enzyme activity in the brain. Since brain tissue is not readily available for the measurement of PLA₂ activity, it would be of interest to test directly whether PLA₂ activities in both tissues are correlated.

We performed this task in the present study, by assessing PLA₂ activity in platelets and hippocampus collected simultaneously from patients undergoing temporal lobectomy for treatment of refractory epilepsy.

2. Methods

2.1. Subjects

The sample comprised 19 patients (11 male/eight female, mean age 39.9 ± 14.2 years) with Temporal lobe epilepsy–mesial temporal sclerosis (TLE–MTS) who underwent an anterior temporal lobectomy at the Functional Neurosurgery Center, Institute of Psychiatry, University of São Paulo, for treatment of refractory epilepsy. Diagnosis of TLE was done according to ILAE criteria (Commission on Classification and Terminology of the International League Against Epilepsy 1989) [17] based on clinical anamnesis, EEG and video-EEG exams. Diagnosis of MTS was confirmed by 1.5 T MRI. Nine subjects of the study presented psychiatry comorbidity (five with psychoses and four with major depression). Exclusion criteria were comorbidity with alcohol and drug abuse, and with other organic or metabolic disease. Part of this sample (psychotic patients and individuals without psychiatric comorbidity) was described elsewhere [18]. All patients were on anti-epileptic drug (AED) treatment.

All 19 patients included in this study underwent a standard surgical procedure with an anterior temporal lobectomy with hippocampus removal performed with the same protocol and by the same neurosurgeon [19,20].

This study was approved by the local ethics committee of the University of São Paulo.

2.2. Determination of PLA₂ activity

2.2.1. Tissue preparations

The hippocampi and blood samples were collected at the same time during the surgery. The hippocampi were immediately frozen

^{*} Correspondence to: Laboratório de Neurociências (LIM 27), Departamento e Instituto de Psiquiatria, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, Rua Dr. Ovídio Pires de Campos 785-3o andar, 05403-010 São Paulo, SP, Brazil. Tel.: +55 11 3069 7283; fax: +55 11 3085 5412.
E-mail address: gattaz@usp.br (W.F. Gattaz).

at -80°C . Tissue was defrosted to 0°C , homogenized in 20 volume of 5 mM Tris-HCl buffer (pH 7.4) and again stored at -80°C until analysis. Prior to PLA₂ activity determination, total protein levels were determined in the homogenates by the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) modified from the Lowry assay [21].

2.2.2. Platelet preparation

Samples of fresh blood were obtained through the puncture of a peripheral vein in the forearm. Platelets were isolated by centrifugation at 515g for 15 min at 20°C with acid citrate dextrose solution (ACD-NIH-formel-A solution: glucose 123.8 mM, tri-sodium citrate 83.9 mM, citric acid 41.3 mM) (1:10v:v). Platelet-rich plasma (PRP) was collected and pH was adjusted to 6.5 by titration with ACD-NIH-formel-A. PRP was centrifuged at 1159g for 10 min at 20°C and the supernatant was carefully removed. A washing solution (30 mM sodium citrate pH 6.5; 5 mM potassium chloride; 2 mM calcium chloride; 1 mM magnesium chloride; 5 mM glucose; 500 $\mu\text{g}/\text{mL}$ albumin; 50 $\mu\text{g}/\text{mL}$ apyrase) was added to the supernatant and incubated for 10 min. The solution was centrifuged at 1159g for additional 8 min at 20°C and the pellet was re-suspended with tris-sucrose solution. Platelets aliquots were frozen immediately at -70°C until experimentation. Under these conditions, there was no evidence of platelet activation, (data not shown).

2.2.3. Radioactive substrates preparation

The radioactive substrates $\text{l-}\alpha\text{-1}$ palmitoyl-2-arachidonyl-phosphatidylcholine (PC-AA-[^{14}C])—(Perkin Elmer Life Science, USA) and l-3 phosphatidylcholine, 1-palmitoyl-2-palmitoyl-PC-PA-[^{14}C])—(Amersham Biosciences, UK) both labeled in position 2 with [^{14}C], were processed similarly, i.e., diluted 1:10 in a 1:2 toluene-ethanol solution containing 140 mg/mL of antioxidant butylatedhydroxytoluene (BHT). The solution was then evaporated under stream of nitrogen (12 μl or 0.06 μCi per sample), re-suspended with 150 μl of ice-cold 0.3 mg/mL BSA solution and homogenized using a sonicator to form liposomes. The count of total radioactivity was standardized for both substrates (120,000 CPM). All assays were conducted with a blank sample. The assays were considered viable when the blank sample values were below 300 CPM.

2.2.4. Radioenzymatic assays for brain tissue

Aliquots of hippocampus homogenates were used to determine the activity of PLA₂ subtypes (cytosolic cPLA₂, secretory sPLA₂ and calcium-independent iPLA₂) by radioenzymatic assays as described elsewhere (Schaeffer and Gattaz, 2005). Briefly, the substrate used was PC-AA-[^{14}C]. Brain tissue homogenates were diluted to a final protein concentration of 1.5 mg/mL with 50 mM Tris-HCl (pH 8.5 for sPLA₂ and cPLA₂ or pH 7.5 for iPLA₂). The assays contained 100 mM Tris-HCl buffer (pH 8.5 or pH 7.5), 1 μM (for cPLA₂ and iPLA₂) or 2 mM CaCl_2 (for sPLA₂), 100 μM bromoenol lactone (Biomol), (USA), 300 μg of protein from diluted homogenates, and 150 μl of PC-AA-[^{14}C]. After 15 min incubation at 37°C , the reactions were interrupted by the addition of 700 μl isopropanol-hydrochloric acid.

2.2.5. Radioenzymatic assays for platelets

This assay has been previously standardized by our group [22]. Briefly, the assay mixture contained 50 μl of 1.0 M Tris-HCl buffer pH 7.5, 200 μl of platelet (0.2 mg of homogenate protein), 150 μl of PC-AA-[^{14}C]. Calcium concentrations of 5 mM and 100 μM were used to measure sPLA₂ and cPLA₂ activity respectively. To measure iPLA₂ activity were used 5 mM EDTA and 150 μl of PC-PA-[^{14}C] (0.06 μCi). The solution was incubated for 30 min at 37°C and the

reaction was stopped by the addition of 700 μl isopropanol-hydrochloric acid.

For both procedures i.e. hippocampus and platelets samples the ^{14}C -labeled fatty acid released by PLA₂ was extracted with n-heptane, followed by adsorption of the unbroken phospholipids and the lysophospholipids on 60 mg of silica. The radioactivity was measured in a liquid scintillation counter (Tri-Carb 2100 TR; Packard, USA) for calculating PLA₂ activities (pmol/mg protein/min -1). All PLA₂ activities were performed in triplicate. Inter- and intra-assay variation coefficients were below 5%. totalPLA₂ activities were calculated by the sum of the three group activity.

2.3. 3-Statistical analysis

Correlations between enzyme activities were calculated through Spearman Coefficients. All statistical analyses were done with the software Statistical Package for Social Science (SPSS, Chicago, USA), version 14.0 and significance level was set at $p < 0.05$.

3. Results

The correlations were tested with all samples and only in patients without psychiatric comorbidity. Table 1.

Total PLA₂ (tPLA₂) in the brain was significantly correlated with tPLA₂ in platelets ($r_s = 0.59$, $p = 0.008$). Moreover, tPLA₂ in the brain was also significantly correlated with sPLA₂ and cPLA₂ in platelets ($r_s = 0.60$, $p = 0.007$ and $r_s = 0.57$, $p = 0.01$ respectively)(Fig. 1). No correlations were found among the subtypes iPLA₂, sPLA₂ and cPLA₂ in the brain and in platelets. Table 2.

In the ten subjects without psychiatric comorbidity, the correlation remained significant among tPLA₂ in brain and tPLA₂, sPLA₂ and cPLA₂ in platelets ($r_s = 0.82$, $p = 0.004$; $r_s = 0.87$, $p = 0.001$ and $r_s = 0.84$, $p = 0.002$ respectively). Moreover, cPLA₂ in brain showed a correlation with tPLA₂ in platelets ($r_s = 0.65$, $p = 0.043$). No correlations were found among the subtypes iPLA₂, sPLA₂ and cPLA₂ in the brain and in platelets. No effects of age and sex were found on the correlations.

Table 3 describes the demographic data and PLA₂ group's activities of patients with and without psychiatric comorbidity.

4. Discussion

In this study we tested the correlations between PLA₂ activity in the hippocampus and in platelets of TLE-MTS patients. This is the first investigation in human subjects to correlate PLA₂ activity in platelets and in the brain in vivo, with both collected simultaneously during neurosurgical procedure for therapy-resistant TLE–

Table 1
Correlations between PLA₂ activity in the brain and in platelets in all patients ($n = 19$).

		Platelets			
		iPLA ₂	sPLA ₂	cPLA ₂	tPLA ₂
Brain	iPLA ₂	$r_s = -0.14$ $p = 0.285$	$r_s = 0.38$ $p = 0.112$	$r_s = 0.35$ $p = 0.068$	$r_s = 0.39$ $p = 0.098$
	sPLA ₂	$r_s = -0.21$ $p = 0.191$	$r_s = 0.34$ $p = 0.154$	$r_s = 0.31$ $p = 0.205$	$r_s = 0.30$ $p = 0.22$
	cPLA ₂	$r_s = -0.14$ $p = 0.571$	$r_s = 0.45$ $p = 0.05$	$r_s = 0.42$ $p = 0.072$	$r_s = 0.46$ $p = 0.046$
	tPLA ₂	$r_s = -0.27$ $p = 0.290$	$r_s = 0.60$ $p = 0.007$	$r_s = 0.57$ $p = 0.011$	$r_s = 0.59$ $p = 0.008$

r_s = Spearman's rho correlation coefficient.

Fig. 1. Correlations between sPLA₂ in platelets and tPLA₂ in brain (a) cPLA₂ in Platelets and Total PLA₂ in brain (b) and total PLA₂ in platelets and total PLA₂ in brain and (c) using all sample (n=19).

MTS. We found a positive correlation between total PLA₂ activity in the brain and in platelets, providing evidence that total PLA₂ activity in platelets may be used to reflect total enzyme activity in the brain.

Table 2

Correlations between PLA₂ activity in the brain and in platelets in patients without psychiatry comorbidity (n=10).

		Platelets			
		iPLA ₂	sPLA ₂	cPLA ₂	tPLA ₂
Brain	iPLA ₂	$r_s = -0.01$ $p = 0.986$	$r_s = 0.33$ $p = 0.359$	$r_s = 0.32$ $p = 0.369$	$r_s = 0.21$ $p = 0.555$
	sPLA ₂	$r_s = -0.15$ $p = 0.676$	$r_s = 0.47$ $p = 0.174$	$r_s = 0.49$ $p = 0.15$	$r_s = 0.46$ $p = 0.187$
	cPLA ₂	$r_s = 0.19$ $p = 0.603$	$r_s = 0.54$ $p = 0.108$	$r_s = 0.59$ $p = 0.074$	$r_s = 0.65$ $p = 0.043$
	tPLA ₂	$r_s = 0.02$ $p = 0.96$	$r_s = 0.82$ $p = 0.004$	$r_s = 0.87$ $p = 0.001$	$r_s = 0.84$ $p = 0.002$

r_s = Spearman's rho correlation coefficient.

Table 3

Demographical data and PLA₂ group's activities in platelets and brain samples.

	ELT without psychiatric comorbidity (n=10)	ELT with psychiatric comorbidity (n=9)	p
Gender (%W)	40	44.4	1.0
Age	40.5 ± 17.2	39.3 ± 11.0	0.86
iPLA ₂ (platelets)	1.13 ± 0.68	1.02 ± 1.37	0.24
sPLA ₂ (platelets)	10.73 ± 3.97	11.55 ± 3.70	0.72
cPLA ₂ (platelets)	6.02 ± 2.92	8.04 ± 6.43	0.55
totalPLA ₂ (platelets)	17.89 ± 6.86	20.61 ± 9.12	0.45
iPLA ₂ (brain)	0.20 ± 0.23	0.57 ± 0.15	0.003
sPLA ₂ (brain)	0.38 ± 0.42	0.53 ± 0.63	0.40
cPLA ₂ (brain)	0.62 ± 0.39	0.82 ± 0.45	0.36
totalPLA ₂ (brain)	1.2 ± 0.7	1.9 ± 1.0	0.79

Values are expressed by mean ± SD or frequency. PLA₂ Activity expressed in pMol/mg protein/min.

However, it should be noticed that the correlations among the PLA₂ subtypes in platelets and in the brain were less consistent, and mostly not significant. We are not able to explain this finding in view of this small sample. Our sample comprised patients with epilepsy, and some of them have psychiatric comorbidities that could bias the results. All patients were under an anti-epileptic drug treatment and those with psychiatric comorbidities also were under antidepressants or anti-psychotics drug treatment. Several studies have shown the influence of these drugs on the PLA₂ activity. Valproate has been shown to inhibit fatty-acid metabolism, independent of PLA₂ inhibition in neurons [23,24]; no effect has been reported of carbamazepine on iPLA₂ activity [25]; anti-psychotic drugs (APDs) have been found to reduce PLA₂ activities [26–29]. Smesny et al. (2005 and 2010) detected this effect of APDs on the iPLA₂ form of the enzyme [30,31]. Maybe these drug effects on PLA₂ may have influenced the enzyme activity, which could explain in our study the higher correlation coefficients observed in epileptic patients without psychiatric comorbidity.

The possibility of using platelets in neuropharmacological models began in the mid-fifties with the discovery that platelets accumulate the neurotransmitter serotonin [32]. 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) [33]. Our findings suggest that total PLA₂ activity in platelets may reflect the total activity of the enzyme in the brain. However in our sample no correlations were found between the subgroups of the enzyme in brain and in platelets. This lack of correlations may be due to different effects of

drug treatment on the PLA₂ subtypes. In face of the difficulty to obtain brain tissues from living patients, further studies with larger drug-free samples are warranted to clarify whether the use of platelets is a reliable strategy to reflect the subtypes of PLA₂ activity in the brain.

Financial sources

This work was supported by grants from the Associação Beneficente Alzira Denise Hertzog da Silva (ABADHS) and from FAPESP (project nos 02/12633-7 and 2009/52825-8)

References

- [1] D. Piomelli, Arachidonic acid in cell signaling, *Curr. Opin. Cell. Biol.* 5 (1993) 274–280.
- [2] N.G. Bazan, C.F. Zorumski, G.D. Clark, The activation of phospholipase A2 and release of arachidonic acid and other lipid mediators at the synapse: the role of platelet activating factor, *J. Lipid. Mediat.* 6 (1993) 421–427.
- [3] W.F. Gattaz, M. Köllisch, T. Thuren, J.A. Virtanen, P.K.J. Kinnunen, Increased plasma phospholipase A2 activity in schizophrenic patients: reduction after neuroleptics therapy, *Biol. Psychiatry* 22 (1987) 421–426.
- [4] W.F. Gattaz, C.K. Hübner, T.J. Nevalainen, T. Thuren, P.K.J. Kinnunen, Increased serum phospholipase A2 activity in schizophrenia: a replication study, *Biol. Psychiatry* 28 (1990) 495–501.
- [5] W.F. Gattaz, A. Steudle, A. Maras, Increased platelet phospholipase A2 in schizophrenia, *Schizophr. Res.* 16 (1995) 1–6.
- [6] M. Noponen, M. Sanfilipo, K. Samanich, H. Ryer, G. Ko, B. Angrist, A. Wolkstein, J. Rotrosen, Elevated PLA2 activity in schizophrenics and other psychiatric patients, *Biol. Psychiatry* 34 (1993) 641–649.
- [7] B.M. Ross, C. Hudson, J. Erlich, J.J. Warsh, S.J. Kish, Increased phospholipid breakdown in schizophrenia, *Arch. Gen. Psychiatry* 54 (1997) 487–494.
- [8] B.M. Ross, S. Turenne, A. Moszczynska, J.J. Warsh, S.J. Kish, Differential alteration of phospholipase A2 activities in brain of patients with schizophrenia, *Brain. Res.* 821 (1999) 407–413.
- [9] J.K. Yao, S. Leonard, R.D. Reddy, Membrane phospholipid abnormalities in postmortem brains from schizophrenic patients, *Schizophr. Res.* 42 (2000) 7–17.
- [10] H. Tavares, J. Yacubian, L.L. Talib, N.R. Barbosa, W.F. Gattaz, Increased phospholipase A2 activity in schizophrenia with absent response to niacin, *Schizophr. Res.* 61 (2002) 1–6.
- [11] W.F. Gattaz, A. Maras, N.J. Cairns, R. Levy, H. Förstl, Decreased phospholipase A2 activity in Alzheimer brain, *Biol. Psychiatry* 37 (1995) 13–17.
- [12] W.F. Gattaz, N.J. Cairns, R. Levy, H. Förstl, D.F. Braus, A. Maras, Decreased phospholipase A2 activity in the brain and in platelets of patients with Alzheimer's disease, *Eur. Arch. Psychiatry Clin. Neurosci.* 246 (1996) 129–131.
- [13] W.F. Gattaz, O.V. Forlenza, L.L. Talib, N.R. Barbosa, C.M. Bottino, Platelet phospholipase A(2) activity in Alzheimer's disease and mild cognitive impairment, *J. Neural. Transm.* 111 (2004) 591–601.
- [14] R. Kramer, E.F. Roberts, J.V. Manetta, P.A. Hyslop, J.A. Jakubowski, Trombin-induced phosphorylation and activation of Ca²⁺-sensitive cytosolic phospholipase A2 in human platelets, *J Biol Chem* 268 (1993) 26796–26804.
- [15] M.J. Owens, C.B. Nemeroff, Role of serotonin in the pathophysiology of depression focus on the serotonin transporter, *Clin. Chem* 40 (1994) 288–295.
- [16] I.A. Zainaghi, O.V. Forlenza, W.F. Gattaz, Abnormal APP processing in platelets of patients with Alzheimer's disease: correlations with membrane fluidity and cognitive decline, *Psychopharmacology. (Berl)*, 192 (2007) 547–553.
- [17] Commission on classification and terminology of the international league against epilepsy (ILAE) proposal for classification of epilepsy and epileptic syndromes, *Epilepsia* 30 (1989) pp. 389–399.
- [18] W.F. Gattaz, K.D. Valente, N.R. Raposo, S. Vincentis, L.L. Talib, Increased PLA2 activity in the hippocampus of patients with temporal lobe epilepsy and psychosis, *J. Psychiatr. Res.* 45 (2011) 1617–1620.
- [19] H.T. Wen, A.L. Rhoton Jr., E. de Oliveira, A.C. Cardoso, H. Tedeschi, M. Baccanelli, et al., Microsurgical anatomy of the temporal lobe: part 1: mesial temporal lobe anatomy and its vascular relationships as applied to amygdalohippocampectomy, *Neurosurgery* 45 (1999) 549–591.
- [20] H.T. Wen, A.L. Rhoton Jr., R. Marino Jr., Gray matter overlying anterior basal temporal sulci as an intraoperative landmark for locating the temporal horn in amygdalohippocampectomies, *Neurosurgery* 59 (2006).
- [21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin-phenol reagents, *J Biol Chem* 193 (1951) 265–275.
- [22] L.L. Talib, B.S. Diniz, I.A. Zainaghi, O.V. Forlenza, W.F. Gattaz, A radioenzymatic assay to identify three groups of phospholipase A(2) in platelets, *Prostaglandins. Leukot. Essent. Fatty. Acids*, 86 (2012) 149–153.
- [23] M.C. Chang, M.A. Contreras, T.A. Rosenberger, J.J. Rintala, J.M. Bell, S. I. Rapoport, Chronic valproate treatment decreases the in vivo turnover of arachidonic acid in brain phospholipids: a possible common effect of mood stabilizers, *J. Neurochem* 77 (2001) 796–803.
- [24] F. Bosetti, G.R. Weerasinghe, T.A. Rosenberger, S.I. Rapoport, Valproic acid down-regulates the conversion of arachidonic acid to eicosanoids via cyclooxygenase-1 and -2 in rat brain, *J. Neurochem* 85 (3) (2003) 690–696.
- [25] A.A. Farooqui, W.Y. Ong, L.A. Horrocks, Inhibitors of brain phospholipase A2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders, *Pharmacol. Rev.* 58 (2006) 591–620.
- [26] H.I. Trzeciak, W. Kalacinski, A. Malecki, D. Kokot, Effect of neuroleptics on phospholipase A2 activity in the brain of rats, *Eur. Arch. Psychiatry. Clin. Neurosci.* 245 (1995) 179–182.
- [27] W.F. Gattaz, M. Köllisch, T. Thuren, J.A. Virtanen, P.K.J. Kinnunen, Increased plasma phospholipase A2 activity in schizophrenic patients: reduction after neuroleptics therapy, *Biol. Psychiatry* 22 (1987) 421–426.
- [28] H. Tavares, J. Yacubian, L.L. Talib, N.R. Barbosa, W.F. Gattaz, Increased phospholipase A2 activity in schizophrenia with absent response to niacin, *Schizophr. Res.* 61 (2003) 1–6.
- [29] A. Schmitt, A. Maras, G. Petroianu, D.F. Braus, L. Scheuer, W.F. Gattaz, Effects of antipsychotic treatment on membrane phospholipid metabolism in schizophrenia, *J. Neural. Transm.* 108 (2001) 1081–1091.
- [30] S. Smesny, D. Kinder, I. Willhardt, T. Rosburg, J. Lasch, G. Berger, et al., Increased calcium-independent phospholipase A2 activity in first but not in multipisode chronic schizophrenia, *Biol. Psychiatry* 57 (2005) 399–405.
- [31] S. Smesny, B. Milleit, I. Nenadic, C. Preul, D. Kinder, J. Lasch, et al., Phospholipase A2 activity is associated with structural brain changes in schizophrenia, *Neuroimage* 52 (2010) 1314–1327.
- [32] M.A. Barradas, D.P. Mikhailidis, The use of platelets as models for neurons: possible applications to the investigation of eating disorders, *Biomed. Pharmacother.* 47 (1993) 11–18.
- [33] M. Haouari, J.A. Rosado, Platelet function in hypertension, *Blood. Cells. Mol. Dis.* 42 (2009) 38–43.

Low platelet iPLA₂ activity predicts conversion from mild cognitive impairment to Alzheimer's disease: a 4-year follow-up study

Wagner F. Gattaz · Leda L. Talib ·
Evelin L. Schaeffer · Breno S. Diniz ·
Orestes V. Forlenza

Received: 3 July 2013 / Accepted: 27 August 2013 / Published online: 15 September 2013
© Springer-Verlag Wien 2013

Abstract Reduced phospholipase A₂ (PLA₂) activity has been reported in the brain and in blood cells of patients with Alzheimer's disease (AD). Individuals with mild cognitive impairment (MCI) are at increased risk of developing AD. In the present study, we determined the activity of distinct PLA₂ subgroups (iPLA₂, sPLA₂ and cPLA₂) in older adults with MCI as compared to patients with mild dementia due to AD and to cognitively healthy controls. We investigated whether decreased PLA₂ activity at baseline is associated with the progression of MCI to AD upon follow-up during a period of 4 years. The activity of PLA₂ subgroups was determined in platelets from 169 elderly adults. Progression of MCI to AD was ascertained by standard clinical criteria for AD upon follow-up. At baseline, iPLA₂ activity was significantly decreased ($p = 0.001$) in patients with AD and MCI as compared to controls. Patients with MCI who progressed to AD during follow-up showed significantly lower iPLA₂ activity at baseline as compared to patients with MCI who did not progress to AD ($p = 0.009$). Moreover, subjects from the control group at baseline who progressed to MCI during follow-up had lower sPLA₂ and cPLA₂ ($p = 0.014$ and $p = 0.009$, respectively). Our findings suggest that low platelet iPLA₂ activity may be a risk marker for AD in subjects with MCI. Moreover, low sPLA₂ and cPLA₂ were related to cognitive decline in healthy controls, suggesting a relationship with the very early stages of the disease.

Keywords Phospholipase A₂ · Alzheimer's disease · Mild cognitive impairment

Introduction

Phospholipase A₂ (PLA₂) comprises a super-family of enzymes that have a major role in membrane phospholipid homeostasis. The activity of PLA₂ generates important intracellular signaling molecules and downstream products such as arachidonic acid and choline (Schaloske and Dennis 2006; Burke and Dennis 2009). PLA₂ enzymes are classified into three major groups based on their structure, cellular localization, requirement for Ca²⁺ and substrate specificity. The most studied subtypes are the secreted calcium-dependent PLA₂ (sPLA₂), the cytosolic calcium-dependent PLA₂ (cPLA₂) and the intracellular calcium-independent PLA₂ (iPLA₂) (Dennis 1994). PLA₂ subtypes are involved in distinct biological functions and processes. The subtypes iPLA₂ and cPLA₂ are related to neuronal degeneration and death, whereas, sPLA₂ and cPLA₂ are related to inflammatory processes (Lambeau and Gelb 2008; Schaeffer et al. 2011a). Thus, changes in each of these subtypes may contribute to distinct aspects of several neuropsychiatric disorders including Alzheimer's disease (AD).

Membrane composition is under a dynamic homeostatic control. In animal models and *post-mortem* brain, the inhibition of PLA₂ was associated with reduction in membrane fluidity, therefore, affecting its function (Schaeffer et al. 2011b, 2012). Reduced cPLA₂ and iPLA₂ activity has been associated with mechanisms of neuronal degeneration and memory impairment that may be relevant to the pathogenesis of AD (Sun et al. 2004; Schaeffer et al. 2009a, 2010). In primary cultures of cortical and

W. F. Gattaz (✉) · L. L. Talib · E. L. Schaeffer ·
B. S. Diniz · O. V. Forlenza
Laboratory of Neuroscience (LIM-27), Department and Institute
of Psychiatry, Faculty of Medicine, University of São Paulo,
São Paulo, Brazil
e-mail: gattaz@usp.br

hippocampal neurons, we found that the inhibition of iPLA₂ activity reduced neurite outgrowth and neuronal viability (Forlenza et al. 2007a), and increased the phosphorylation state of Tau (De-Paula et al. 2010). Moreover, we reported that in rodents, iPLA₂ inhibition in the brain disrupted short and long-term memory acquisition and retrieval (Schaeffer and Gattaz 2005, 2007). Conversely, conditioned training in adult rats led to PLA₂ activation in parallel to enhancement of memory performance (Schaeffer et al. 2009b).

In *post-mortem* studies, PLA₂ activity was significantly reduced in the AD brain, and this reduction correlated with the number of neuritic plaques and neurofibrillary tangles (Gattaz et al. 1995, 1996; Talbot et al. 2000). Accordingly, in patients with mild and moderate AD, we found in an *in vivo* ³¹P-spectroscopy study that intracerebral phospholipid breakdown (a surrogate marker of PLA₂ activity) was reduced and correlated with worse cognitive performance (Forlenza et al. 2005). In addition, patients with AD and mild cognitive impairment (MCI) showed significantly lower PLA₂ activity in platelets as compared to controls (Forlenza et al. 2005; Gattaz et al. 2004). Taken together, these findings suggest that reduced PLA₂ activity may contribute to neuropathological and cognitive abnormalities observed in AD. Therefore, in the present study we evaluated, in a longitudinal study with 4 years of follow-up, whether reduced PLA₂ activity at baseline is associated with cognitive decline and with an increased risk of AD in patients with MCI. We confirmed previous findings of reduced platelet PLA₂ activity in patients with AD and found that reduced enzyme activity at baseline increased the risk for cognitive decline during follow-up.

Methods

Sample

One hundred and sixty-nine elderly adults were included in this study, including 44 with mild or moderate AD (mean age $\pm$ standard deviation 74.8 ± 6.5 years), 59 with MCI (71.9 ± 5.8 years) and 66 physically and cognitively healthy controls (67.4 ± 5.4 years). All subjects had a baseline assessment between April 2004 and December 2006. At baseline, both MCI individuals and controls were free of psychoactive drugs. Conversely, AD patients received treatment with cholinesterase inhibitors (rivastigmine, donepezil or galantamine) at therapeutic doses. Current use of any prescription drugs with potential effect on PLA₂ (such as corticosteroids and anti-platelet agents) was regarded as exclusion criteria. Therefore, the presence of medical comorbidities requiring such treatments or

with relevant platelet involvement (e.g., atherosclerosis-related, metabolic syndrome, hypercortisolism, chronic inflammatory diseases, etc.) and neuroimaging-confirmed relevant subcortical cerebrovascular disease were also ruled out. All participants are subjects of an on-going prospective study on aging and cognition carried out at the memory clinic of the Institute of Psychiatry, University of São Paulo, Brazil (Diniz et al. 2008; Forlenza et al. 2010). Local Ethical Committee approved this study and all subjects signed a written informed consent prior to inclusion in the study.

Clinical assessment

All participants underwent standardized clinical and neuropsychological evaluations at baseline and follow-up evaluations. A detailed description of the cognitive assessment and diagnostic procedures can be found elsewhere (Diniz et al. 2008; Forlenza et al. 2010). For the sake of conciseness, we only show the scores in the Cambridge Cognitive Test (CAMCOG) (Roth et al. 1986; Nunes et al. 2008) and the Mini-Mental State Examination (MMSE) (Folstein et al. 1975).

The diagnosis of AD and MCI as well as the characterization of normal cognitive function in the healthy controls was established by consensus at expert multidisciplinary meetings taking into account all available information about current medical history and neuropsychological test scores. The diagnosis of dementia due to AD was made according to the NINCDS-ADRDA diagnostic criteria (McKhann et al. 1984). Diagnosis of MCI was established according to the Mayo Clinic criteria (Petersen et al. 1999): (1) subjective cognitive complaint, preferably corroborated by an informant; (2) objective cognitive impairment in the neuropsychological assessment; (3) preserved global intellectual function; (4) preserved or minimal impairments in activities of daily living; and (5) not demented.

Changes in cognitive status of MCI and control subjects were determined on annual follow-up reassessments over 4 years for MCI subjects and for controls, taking into account all evidence of objective and clinically relevant cognitive decline over time, endorsed by a comprehensive neuropsychological and functional assessment. The characterization of the conversion of MCI to AD considered that former MCI patients, upon reassessment, met the NINCDS-ADRDA diagnostic criteria; otherwise, the diagnoses of MCI or normal cognition were reinforced. Therefore, we reclassified all participants upon follow-up as: (1) MCI-AD, subjects with MCI who progressed to AD; (2) MCI-MCI, subjects with MCI who retained this diagnosis on follow-up; (3) Con-MCI, control subjects who progressed to MCI on follow-up; (4) Con-Con, control

subjects who retained this diagnosis on follow-up. All clinical and diagnostic procedures were done blind to the values of PLA₂ activity (see below).

Determination of PLA₂ Activity

Platelets were isolated from samples of peripheral blood at the baseline visit and at follow-up. Blood samples were centrifuged at 515×*g* for 15 min at 20 °C in acid citrate dextrose solution. Aliquots of platelet-rich plasma (PRP) were re-suspended in a wash solution (sodium citrate 30 mM, pH 6.5, potassium chloride 5 mM, calcium chloride 2 mM, magnesium chloride 1 mM, glucose 5 mM, albumin 500 µg/mL, apyrase 50 µg/mL), centrifuged at 1159×*g* for 8 min at 20 °C, and the pellet was re-suspended in tris-sucrose solution. Platelet aliquots were immediately stored at −70 °C and protein levels were determined by a modified Lowry method prior to experimentation (Bio-Rad, Hercules, California).

PLA₂ activity was determined in platelets by a radio-enzymatic assay in triplicate (Talib et al. 2008, 2012). Accordingly, 14C-labeled fatty acid is preferentially cleaved by PLA₂ at the sn-2 position of the phosphatidylcholine molecule. To access cPLA₂ and sPLA₂ activity, the substrate was L-α-1-palmitoyl-2-arachidonoyl-phosphatidylcholine labeled with [1-14C] at the sn-2 position (New England Nuclear, Boston Massachusetts). For the determination of iPLA₂ activity, the substrate was L-3-phosphatidylcholine, 1-palmitoyl-2- [1-14C]-palmitoyl. Total PLA₂ activity was estimated by the sum of the activities of PLA₂ subgroups. After a 30 min incubation at 37 °C, the reaction was stopped by adding a solution of HCl-isopropanol (1:11.7, v/v), and the [1-14C]-fatty acid released by the enzymatic reaction was extracted with *n*-heptane. The radioactivity of [1-14C]-fatty acid was then measured in a liquid scintillation counter (Tri-Carb 2100TR; Packard, Meriden, CT). PLA₂ activity was calculated in picomoles per milligram of protein per minute (pmol/mg-protein/min). All determinations were done in the same radio-enzymatic procedure. The inter- and intra-assay variability were 6.0 and 3.3 % (cPLA₂), 5.2 and 1.8 % (iPLA₂), 6.8 and 3.8 % (sPLA₂), respectively.

Statistical analysis

Chi-square tests were carried out to assess the distribution of categorical variables across the diagnostic groups at baseline. Multivariate analysis of variance (MANOVA), with type III sum of squares, was done to assess for mean differences in socio-demographic, cognitive variables and PLA₂ activity between diagnostic groups in the baseline assessment. Post-hoc analysis with Tukey test

was done to assess for pairwise differences among diagnostic groups.

Time-dependent Cox regression analysis was done to determine the predictors of progression of cognitive decline in the control group and of progression to AD in subjects with MCI.

MANOVAs were performed to assess whether baseline PLA₂ activity differed according to the cognitive outcome in the MCI (MCI-AD vs. MCI-MCI) and the control group (Con-MCI vs. Con-Con) in the follow-up analysis.

Statistical tests were performed with the Statistical Package for Social Sciences v. 14 for Windows (SPSS Inc., Chicago, IL) and significance was established as $p < 0.05$. When indicated, data are given as mean ± standard deviation.

Results

Baseline assessment

As expected, patients with AD were significantly older, less educated and attained lower scores on cognitive screening tests (CAMCOG and MMSE) as compared to subjects with MCI and controls. There was no significant difference in the gender distribution among groups (Table 1).

After controlling for age, educational level and cognitive performance, we found that AD patients had lower platelet iPLA₂ activity ($F = 7.459$, $df = 2$, $p = 0.001$), higher cPLA₂ ($F = 16.57$, $df = 2$, $p \leq 0.001$) and higher total PLA₂ activity ($F = 3.584$, $df = 2$, $p = 0.039$) as compared to MCI and controls. There was no significant difference in sPLA₂ among the three groups ($F = 1.194$, $df = 2$, $p = 0.3$).

Longitudinal assessment

Follow-up data were available for 50 out of 59 MCI patients (mean follow-up of 51.1 ± 8.2 months) and 58 out of 66 control subjects (mean follow-up of 56.9 ± 19.3 months). The main reasons for discontinuation of follow-up were loss of contact (55 %), unwillingness to perform additional cognitive assessments (35 %), severe and unstable medical condition that precluded cognitive assessment (5 %) and death (5 %).

In the MCI group, during 4 years of follow-up, 18 subjects (36 %) progressed to AD (MCI-AD) and 32 subjects (64 %) remained with the MCI diagnosis (MCI-MCI). In the same period of time, 23 controls (40 %) progressed to MCI (Con-MCI) and 35 controls (60 %) retained normal cognitive function (Con-Con). Patients with MCI who were lost through follow-up had less years of education as compared to those with complete longitudinal data

Table 1 Socio-demographic data, cognitive performance and PLA₂ activity according to diagnostic groups at baseline

	Control (N = 66)	Diagnosis Mild cognitive impairment (N = 59)	Alzheimer's disease (N = 44)	p
Gender (%W)	81.8 %	69 %	75 %	0.27
Age	67.4 ± 5.4	71.9 ± 5.8***	74.8 ± 6.5***#	<0.001
Years of education	14.6 ± 4.2	9.6 ± 4.7***	5.9 ± 4.3***###	<0.001
CAMCOG	99.1 ± 3.2	89.5 ± 7.6***	60.8 ± 17.4***###	<0.001
MMSE	28.2 ± 3.8	27.1 ± 2.5	18.6 ± 5.1***###	<0.001
iPLA ₂	1.69 ± 0.89	1.22 ± 0.77*	1.16 ± 0.75*	0.001
sPLA ₂	16.36 ± 6.82	14.43 ± 7.46	15.28 ± 6.71	0.30
cPLA ₂	7.21 ± 3.13	6.21 ± 2.68	9.92 ± 4.16***###	<0.001
Total PLA ₂	25.27 ± 8.73	21.85 ± 9.69	26.35 ± 8.95#	0.039

Values are expressed by mean ± SD or frequency. One-Way ANOVA followed by Tukey's post-hoc test was performed

Tukey's post-hoc test

* Difference from the control group; # Difference from the mild cognitive impairment group

CAMCOG Cambridge Cognitive Test, MMSE Mini-Mental State Examination, PLA₂ activity (pMol/mg protein/min) controlled for age, years of education and cognitive performance

* $p < 0.05$ compared to control group

** $p < 0.01$ compared to control group

*** $p < 0.001$ compared to control group

$p < 0.05$ compared to MCI group

$p < 0.01$ compared to MCI group

$p < 0.001$ compared to MCI group

($p = 0.001$). No other significant differences were found between these groups regarding age, baseline values of cognitive performance and the activity of distinct PLA₂ subtypes (*data not shown*). Among controls, there were no significant differences between subjects who were lost upon follow-up and those who completed the longitudinal assessment (*data not shown*).

In the MCI group, we found no significant difference in age, years of education and cognitive performance between converter (MCI-AD) and non-converter (MCI-MCI) subjects (Table 2). MCI-AD subjects were more frequently men ($p = 0.04$) as compared to MCI-MCI subjects (Table 2). After controlling for gender, MCI-AD subjects had a significantly lower iPLA₂ activity than MCI-MCI ($F = 7.4$, $df = 1$, $p = 0.009$). In the Cox regression analysis, decreased iPLA₂ activity ($p < 0.001$) and male gender ($p = 0.002$) were the best predictors of progression from MCI to AD. There were no significant differences in cPLA₂ activity ($F = 3.95$, $df = 1$, $p = 0.06$) and sPLA₂ activity ($F = 1.8$, $df = 1$, $p = 0.19$) when comparing the MCI-MCI and MCI-AD groups (Fig. 1).

At baseline, individuals from the control group who converted to MCI (Con-MCI) had significantly lower cPLA₂ activity ($F = 7.3$, $df = 1$, $p = 0.009$), sPLA₂ activity ($F = 6.37$, $df = 1$, $p = 0.014$) and total PLA₂ ($F = 9.05$, $df = 1$, $p = 0.004$) as compared to controls that remained cognitively normal through follow-up (Con-Con). No significant differences in iPLA₂ activity were

observed between these groups ($F = 0.9$, $df = 1$, $p = 0.98$) (Fig. 1). There were no significant differences in age, cognitive performance and gender distribution between Con-MCI and Con-Con subjects (Table 2).

Discussion

iPLA₂

In the present study, we found in a cross-sectional analysis that patients with AD and MCI showed significantly lower platelet iPLA₂ activity than healthy controls. In the longitudinal analysis, lower iPLA₂ activity at baseline was associated with higher risk of progression from MCI to AD during 4 years of follow-up. The reduction in iPLA₂ activity preceded global cognitive deterioration in subjects with MCI who converted to AD, as at baseline CAMCOG or MMSE scores were not reduced in these subjects as compared to those who did not progress to AD.

Platelets are frequently used as peripheral models for neurons, as they share several membrane receptors and intracellular signal transduction machinery (Bakken et al. 2006; Bianchi et al. 2002). Among the PLA₂ enzymes, iPLA₂ is the most abundant in neurons (Forlenza et al. 2007b). Accordingly, in a recent study we found a high correlation between total PLA₂ activity in platelets and brain tissue from epileptic patients who underwent a

Table 2 Socio-demographic data, cognitive performance and PLA₂ activity according to diagnostic group at follow-up

	MCI-MCI (N = 32)	MCI-AD (N = 18)	Diagnosis on follow-up		Con-MCI (N = 23)	p
			p	Con-Con (N = 35)		
Gender (%W)	78.1 %	50.0 %	0.04	82.9 %	91.3 %	0.31
Age	71.3 ± 5.3	73.6 ± 6.3	0.18	68.29 ± 5.2	67.0 ± 4.9	0.35
Years of education	10.8 ± 4.5	9.8 ± 4.9	0.47	15.5 ± 4.0	13.1 ± 4.3	0.04
CAMCOG	90.6 ± 7.4	89.2 ± 7.7	0.54	99.8 ± 3.1	98.3 ± 3.0	0.08
MMSE	27.28 ± 2.1	27.1 ± 2.8	0.82	28.7 ± 1.7	27.4 ± 5.8	0.26
iPLA ₂	1.37 ± 0.8	0.80 ± 0.47	0.009	1.69 ± 0.88	1.69 ± 1.0	0.98
sPLA ₂	13.0 ± 6.9	16.0 ± 8.9	0.19	17.8 ± 6.7	13.4 ± 6.1	0.014
cPLA ₂	5.6 ± 2.7	7.2 ± 2.8	0.06	8.1 ± 3.5	5.9 ± 2.0	0.009
Total PLA ₂	20.02 ± 9.0	24.07 ± 11.61	0.176	27.60 ± 8.55	21.02 ± 7.56	0.004

Values are expressed by mean ± SD

CAMCOG Cambridge Cognitive Test, MMSE Mini-Mental State Examination, PLA₂ activity (pmol/mg protein/min) controlled for gender

lobectomy to treat therapy resistant epilepsy (Talib et al. 2013). Thus, our finding in platelets may reflect a relationship between low iPLA₂ activity in the brain and the risk for AD.

Low iPLA₂ activity has been associated to many features related to AD pathophysiology. In cellular and animal models, inhibition of iPLA₂ activity leads to decreased LTP formation and memory impairment, reduced neurite outgrowth in hippocampal neurons, increased β -amyloid production and Tau phosphorylation, and may contribute to the cholinergic and glutamatergic dysfunctions observed in early stages of AD (Schaeffer et al. 2011a, b; Schaeffer and Gattaz 2005, 2007, 2008; Forlenza et al. 2002, 2007a). On the other hand, we found in rodents and in humans that memory training and cognitive stimulation via environmental enrichment increase iPLA₂ activity in the brain and in platelets, respectively (Schaeffer et al. 2009b, 2011b).

cPLA₂, sPLA₂, and total PLA₂

Contrasting to the finding of decreased iPLA₂ activity, cPLA₂ activity was increased in our patients with AD at baseline. It should be noticed that all our AD patients were on treatment with cholinesterase inhibitors (ChE-I), which are reported to increase protein kinase C (PKC) (Nordberg 2006). PKC stimulates the production of arachidonic acid through stimulation of cPLA₂ activity (Sun et al. 2004; Farooqui and Horrocks 2004). Thus, in our sample ChE-I treatment may account for increased cPLA₂ activity in AD patients. We also found a significant increase in total PLA₂ activity in the AD group as compared to MCI, probably due to increased cPLA₂ activity. Krzystanek et al. (2007) and Fonteh et al. (2013) also found an increased total PLA₂ activity in platelets and in the CSF of AD patients as compared to controls,

interpreting that these findings may be associated with the up regulation of inflammatory mechanisms in AD; yet, in this case, all AD patients were not receiving treatment with cholinesterase inhibitors.

The activity of sPLA₂ is also related to the stimulation of inflammatory activity via the production and release of arachidonic acid (Heneka et al. 2010). Higher pro-inflammatory activity has been reported in AD and in its prodromal stages (Sun et al. 2010; Diniz et al. 2010; Forlenza et al. 2009). An increase in sPLA₂ has been reported in the cerebrospinal fluid (CSF) of AD patients (Chalbot et al. 2009). However, in the present study in platelets, we did not find changes in sPLA₂ activity in AD patients or a relationship with the progression from MCI to AD.

In our sample we found lower sPLA₂, cPLA₂ and total PLA₂ in controls at baseline who converted to MCI during the follow-up. sPLA₂ and cPLA₂ subtypes are related to inflammatory response, neuronal differentiation and growth, and prevention of apoptosis (Smalheiser et al. 1996; Arioka et al. 2005; Ikeno et al. 2005; Masuda et al. 2005; Forlenza et al. 2007b; Buchhave et al. 2010). Therefore, it is conceivable that reduced activity of both sPLA₂ and cPLA₂ at the pre-clinical stages of AD may render neurons more susceptible to apoptosis and maybe contribute to A β formation through changes in the properties of neuronal membranes (Emmerling et al. 1993, 1996; Nitsch et al. 1997; Cho et al. 2006; Masuda et al. 2008; Schaeffer et al. 2011b). This assumption is in line with the early 31P-spectroscopy study of Pettegrew et al. (1988), who hypothesized that the lowering of phospholipid metabolism in the brain may precede the onset of senile plaques at early stages of AD. In our sample, sPLA₂ and cPLA₂ activities in the MCI-AD group did not differ from controls (Fig. 1), suggesting that with the progression of the disease the enzymes' activities increase to the level

Fig. 1 Activities in platelets of **a** iPLA₂, **b** sPLA₂ and **c** cPLA₂ at baseline in four outcome groups after 4-year follow-up: 35 controls that retained normal cognitive function (Con-Con), 23 controls that progressed to MCI (Con-MCI), 32 MCI that remained with the MCI diagnosis (MCI-MCI) and 18 MCI individuals at baseline that progressed to AD (MCI-AD). PLA₂ activities in pMol/mg protein/min, mean $\pm$ standard error

of controls. This has also been observed in the brains of subjects with AD and discussed as an inflammatory response of reactive astrocytes around to A β -plaques (Stephenson et al. 1996, 1999; Colangelo et al. 2002; Moses et al. 2006). It is worth noticing that both plaques and soluble oligomers of A β may induce the glial cells to produce inflammatory cytokines which, in turn, activate sPLA₂ and cPLA₂ (but not iPLA₂) (Schaeffer et al. 2010).

In summary, our findings suggest that low platelet iPLA₂ activity may be a risk marker for AD in subjects with MCI, whereas, low sPLA₂ and cPLA₂ may be related to the very early stages of the disease. These results stress the importance to study the different subgroups of PLA₂, which may present distinct roles in the pathology of AD. It is conceivable that the inclusion of PLA₂ activities may enhance the predictive power of other biomarkers, such as CSF A β 42 and Tau protein, in the early diagnosis of AD.

References

- Arioka M, Cheon SH, Ikeno Y, Nakashima S, Kitamoto K (2005) A novel neurotrophic role of secretory phospholipases A2 for cerebellar granule neurons. *FEBS Lett* 579:2693–2701
- Bakken AM, Staefler A, Jørgensen HA, Holmsen H (2006) Glycerophospholipid molecular species in platelets and brain tissues—are platelets a good model for neurons? *Platelets* 17(7):484–492
- Bianchi M, Moser C, Lazzarini C, Vecchiato E, Crespi F (2002) Forced swimming test and fluoxetine treatment: in vivo evidence that peripheral 5-HT in rat platelet-rich plasma mirrors cerebral extracellular 5-HT levels, whilst 5-HT in isolated platelets mirrors neuronal 5-HT changes. *Exp Brain Res* 143(2):191–197
- Buchhave P, Zetterberg H, Blennow K, Minthon L, Janciauskiene S, Hansson O (2010) Soluble TNF receptors are associated with A β metabolism and conversion to dementia in subjects with mild cognitive impairment. *Neurobiol Aging* 31(11):1877–1884
- Burke JE, Dennis EA (2009) Phospholipase A2 structure/function, mechanism, and signaling. *J Lipid Res* 50 (Suppl S2):37–42
- Chalbot S, Zetterberg H, Blennow K, Fladby T, Grundke-Iqbal I, Iqbal K (2009) Cerebrospinal fluid secretory Ca²⁺-dependent phospholipase A2 activity is increased in Alzheimer disease. *Clin Chem* 55(12):2171–2179
- Cho HW, Kim JH, Choi S, Kim HJ (2006) Phospholipase A2 is involved in muscarinic receptor-mediated sAPP α release independently of cyclooxygenase or lipoxygenase activity in SHSY5Y cells. *Neurosci Lett* 397:214–218
- Colangelo V, Schurr J, Ball MJ, Pelaez RP, Bazan NG, Lukiw WJ (2002) Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J Neurosci Res* 70:462–473
- Dennis EA (1994) Diversity of group types, regulation, and function of phospholipase A2. *J Biol Chem* 269(18):13057–13060
- De-Paula VJ, Schaeffer EL, Talib LL, Gattaz WF, Forlenza OV (2010) Inhibition of phospholipase A2 increases tau phosphorylation at Ser214 in embryonic rat hippocampal neurons. *Prostaglandins Leukot Essent Fatty Acids* 82:57–60

- Diniz BS, Nunes PV, Yassuda MS, Pereira FS, Flaks MK, Viola LF, Radanovic M, Abreu ID, Borelli DT, Gattaz WF, Forlenza OV (2008) Mild cognitive impairment: cognitive screening or neuropsychological assessment? *Rev Bras Psiquiatr* 30:316–321
- Diniz BS, Teixeira AL, Ojopi EB, Talib LL, Mendonça VA, Gattaz WF, Forlenza OV (2010) Higher serum sTNFR1 level predicts conversion from mild cognitive impairment to Alzheimer's disease. *J Alzheimers Dis* 22(4):1305–1311
- Emmerling MR, Moore CJ, Doyle PD, Carroll RT, Davis RE (1993) Phospholipase A2 activation influences the processing and secretion of the amyloid precursor protein. *Biochem Biophys Res Commun* 197:292–297
- Emmerling MR, Dudley DT, Dyer RD, Carroll RT, Doyle PD, Davis RE (1996) The role of arachidonic acid in the secretion of the amyloid precursor protein (APP). *Ann N Y Acad Sci* 777:310–315
- Farooqui AA, Horrocks LA (2004) Brain phospholipases A2: a perspective on the history. *Prostaglandins, Leucotrienes and Essential Fatty acids* 71:161–169
- Folstein MF, Folstein SE, McHugh PR (1975) Mini-Mental State: a practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 12:189–198
- Fonteh AN, Chiang J, Cipolla M, Hale J, Diallo F, Chirino A, Arakaki X, Harrington MG (2013) Alterations in cerebrospinal fluid glycerophospholipids and phospholipase A2 activity in Alzheimer's disease. *J Lipid Res*. Jul 18 [Epub ahead of print]
- Forlenza OV, Schaeffer EL, Gattaz WF (2002) Phospholipase A2 activity in rat embryonic brain and in primary cultures of cortical neurons. *J Neural Transm* 109(5–6):623–631
- Forlenza OV, Wacker P, Nunes PV, Yacubian J, Castro CC, Otaduy MC, Gattaz WF (2005) Reduced phospholipid breakdown in Alzheimer's brains: a 31P spectroscopy study. *Psychopharmacology* 180(2):359–365
- Forlenza OV, Mendes CT, Marie SK, Gattaz WF (2007a) Inhibition of phospholipase A2 reduces neurite outgrowth and neuronal viability. *Prostaglandins Leukot Essent Fatty Acids* 76:47–55
- Forlenza OV, Schaeffer EL, Gattaz WF (2007b) The role of phospholipase A2 in neuronal homeostasis and memory formation: implications for the pathogenesis of Alzheimer's disease. *J Neural Transm* 114(2):231–238
- Forlenza OV, Diniz BS, Talib LL, Mendonça VA, Ojopi EB, Gattaz WF, Teixeira AL (2009) Increased serum IL-1 β level in Alzheimer's disease and mild cognitive impairment. *Dement Geriatr Cogn Disord* 28(6):507–512
- Forlenza OV, Diniz BS, Talib LL, Radanovic M, Yassuda MS, Ojopi EB, Gattaz WF (2010) Clinical and biological predictors of Alzheimer's disease in patients with amnesic mild cognitive impairment. *Rev Bras Psiquiatr* 32(3):216–222
- Gattaz WF, Maras A, Cairns NJ, Levy R, Förstl H (1995) Decreased phospholipase A2 activity in Alzheimer brains. *Biol Psychiatry* 37:13–17
- Gattaz WF, Cairns NJ, Levy R, Förstl H, Braus DF, Maras A (1996) Decreased phospholipase A2 activity in the brain and in platelets of patients with Alzheimer's disease. *Eur Arch Psychiatry Clin Neurosci* 246(3):129–131
- Gattaz WF, Forlenza OV, Talib LL, Barbosa NR, Bottino CM (2004) Platelet phospholipase A2 activity in Alzheimer's disease and mild cognitive impairment. *Journal of Neural Transmission* 111:591–601
- Heneka MT, O'Banion MK, Terwel D, Kummer MP (2010) Neuroinflammatory processes in Alzheimer's disease. *J Neural Transm* 117(8):919–947
- Ikeno Y, Konno N, Cheon SH, Bolchi A, Ottonello S, Kitamoto K et al (2005) Secretory phospholipases A2 induce neurite outgrowth in PC12 cells through lysophosphatidylcholine generation and activation of G2A receptor. *J Biol Chem* 280:28044–28052
- Krzystanek E, Krzystanek M, Opala G, Trzeciak HI, Siuda J, Małeck A (2007) Platelet phospholipase A2 activity in patients with Alzheimer's disease, vascular dementia and ischemic stroke. *J Neural Transm* 114(8):1033–1039
- Lambeau G, Gelb MH (2008) Biochemistry and physiology of mammalian secreted phospholipases A2. *Annu Rev Biochem* 77:495–520
- Masuda S, Murakami M, Takanezawa Y, Aoki J, Arai H, Ishikawa Y et al (2005) Neuronal expression and neuritogenic action of group X secreted phospholipase A2. *J Biol Chem* 280:23203–23214
- Masuda S, Yamamoto K, Hirabayashi T, Ishikawa Y, Ishii T, Kudo I et al (2008) Human group III secreted phospholipase A2 promotes neuronal outgrowth and survival. *Biochem J* 409:429–438
- McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of department of health and human Services task force on Alzheimer's disease. *Neurology* 34(7):939–944
- Moses GS, Jensen MD, Lue LF, Walker DG, Sun AY, Simonyi A et al (2006) Secretory PLA2-IIA: a new inflammatory factor for Alzheimer's disease. *J Neuroinflammation* 3:28
- Nitsch RM, Deng A, Wurtman RJ, Growdon JH (1997) Metabotropic glutamate receptor subtype mGluR1 α stimulates the secretion of the amyloid β -protein precursor ectodomain. *J Neurochem* 69:704–712
- Nordberg A (2006) Mechanisms behind the neuroprotective actions of cholinesterase inhibitors in Alzheimer disease. *Alzheimer Dis Assoc Disord* 20(2Suppl 1):S12–8. Review
- Nunes PV, Diniz BS, Radanovic M, Abreu ID, Borelli DT, Yassuda MS, Forlenza OV (2008) CAMCOG as a screening tool for diagnosis of mild cognitive impairment and dementia in a Brazilian clinical sample of moderate to high education. *Int J Geriatr Psychiatry* 23:1127–1133
- Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E (1999) Mild Cognitive Impairment: clinical characterization and outcome. *Arch Neurol* 56:303–308
- Pettegrew JW, Moosy J, Withers G, McKeag D, Panchalingam K (1988) 31P nuclear magnetic resonance study of the brain in Alzheimer's disease. *J Neuropathol Exp Neurol* 47:235–248
- Roth M, Tym E, Mountjoy CQ, Huppert FA, Hendrie H, Verma S, Goddard R (1986) CAMDEX: a standardized Instrument for the diagnosis of mental disorders in the elderly with special reference to early detection of dementia. *Br J Psychiatry* 149:698–709
- Schaeffer EL, Gattaz WF (2005) Inhibition of calcium-independent phospholipase A2 activity in rat hippocampus impairs acquisition of short- and long-term memory. *Psychopharmacology* 181:392–400
- Schaeffer EL, Gattaz WF (2007) Requirement of hippocampal phospholipase A2 activity for long-term memory retrieval in rats. *J Neural Transm* 114:379–385
- Schaeffer EL, Gattaz WF (2008) Cholinergic and glutamatergic alterations beginning at the early stages of Alzheimer disease: participation of the phospholipase A2 enzyme. *Psychopharmacology* 198(1):1–27
- Schaeffer EL, Forlenza OV, Gattaz WF (2009a) Phospholipase A2 activation as a therapeutic approach for cognitive enhancement in early-stage Alzheimer disease. *Psychopharmacology* 202(1–3):37–51
- Schaeffer EL, Zorron PuL, Gagliotti DA, Gattaz WF (2009b) Conditioning training and retrieval increase phospholipase

- A(2) activity in the cerebral cortex of rats. *J Neural Transm* 116:41–50
- Schaeffer EL, da Silva ER, Novaes Bde A, Skaf HD, Gattaz WF (2010) Differential roles of phospholipases A2 in neuronal death and neurogenesis: implications for Alzheimer disease. *Prog Neuropsychopharmacol Biol Psychiatry* 34(8):1381–1389
- Schaeffer EL, De-Paula VJ, da Silva ER, de A Novaes B, Skaf HD, Forlenza OV, Gattaz WF (2011a) Inhibition of phospholipase A(2) in rat brain decreases the levels of total Tau protein. *J Neural Transm* 118(9):1273–1279
- Schaeffer EL, Skaf HD, Novaes Bde A, da Silva ER, Martins BA, Joaquim HD, Gattaz WF (2011b) Inhibition of phospholipase A2 in rat brain modifies different membrane fluidity parameters in opposite ways. *Prog Neuropsychopharmacol Biol Psychiatry* 35(7):1612–1617
- Schaeffer EL, Gattaz WF, Eckert GP (2012) Alterations of brain membranes in schizophrenia: impact of phospholipase A(2). *Curr Top Med Chem* 12(21):2314–2323
- Schaloske RH, Dennis EA (2006) The phospholipase A2 superfamily and its group numbering system. *Biochim Biophys Acta* 1761(11):1246–1259
- Smalheiser NR, Dissanayake S, Kapil A (1996) Rapid regulation of neurite outgrowth and retraction by phospholipase A2-derived arachidonic acid and its metabolites. *Brain Res* 721:39–48
- Stephenson DT, Lemere CA, Selkoe DJ, Clemens JA (1996) Cytosolic phospholipase A2 (cPLA2) immunoreactivity is elevated in Alzheimer's disease brain. *Neurobiol Dis* 3:51–63
- Stephenson D, Rash K, Smalstig B, Roberts E, Johnstone E, Sharp J et al (1999) Cytosolic phospholipase A2 is induced in reactive glia following different forms of neurodegeneration. *Glia* 27:110–128
- Sun GY, Xu J, Jensen MD, Simonyi A (2004) Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res* 45(2):205–213
- Sun GY, Shelat PB, Jensen MB, He Y, Sun AY, Simonyi A (2010) Phospholipases A2 and inflammatory responses in the central nervous system. *Neuromolecular Med* 12(2):133–148
- Talbot K, Young RA, Jolly-Tornetta C, Lee VM, Trojanowski JQ, Wolf BA (2000) A frontal variant of Alzheimer's disease exhibits decreased calcium-independent phospholipase A2 activity in the prefrontal cortex. *Neurochem Int* 37(1):17–31
- Talib LL, Yassuda MS, Diniz BS, Forlenza OV, Gattaz WF (2008) Cognitive training increases platelet PLA2 activity in healthy elderly subjects. *Prostaglandins Leukot Essent Fatty Acids* 78:265–269
- Talib LL, Diniz BS, Zainaghi IA, Forlenza OV, Gattaz WF (2012) A radioenzymatic assay to identify three groups of phospholipase A(2) in platelets. *Prostaglandins Leukot Essent Fatty Acids* 86(4–5):149–153
- Talib LL, Valente KD, Vincentiis S, Gattaz WF. (2013) Correlation between platelet and brain PLA(2) activity. *Prostaglandins Leukot Essent Fatty Acids*. doi:10.1016/j.plefa.2013.07.002. [Epub ahead of print]

Long-term treatment of Alzheimer's disease with cholinesterase inhibitors restores homeostatic levels of Calcium-independent PLA2 and GSK3B phosphorylation

Talib L.L., Hototian S., Forlenza, O.V., Gattaz, W.F.

Laboratory of Neuroscience (LIM-27), Department and Institute of Psychiatry, Faculty of Medicine, University of São Paulo.

Address for correspondence:

Wagner F. Gattaz

Instituto de Psiquiatria

Hospital das Clínicas da Faculdade de Medicina da USP

Rua Dr. Ovídio Pires de Campos 785

05403-010 - São Paulo, SP, Brazil.

E-mail: gattaz@usp.br

Acknowledgments:

The present work was supported by FAPESP, Fundação de Amparo à Pesquisa do Estado de São Paulo (grants nº 02/13633-7 and 09/52825-8). The Laboratory of Neuroscience receives financial support from Associação Beneficente Alzira Denise Hertzog da Silva (ABADHS).

Abstract:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that causes dementia and cognitive impairment. The treatments available for AD are cholinesterase inhibitors (IChEs) and N-methyl-D-aspartate (NMDA) antagonists. Several studies have shown a neuroprotective effect of IChEs, especially donepezil, leading to alterations in the pathogenesis of AD. Evaluate and measure these changes are assigned to biomarkers. In this regard we can highlight the phospholipase A₂ (PLA₂) the main enzyme in membrane phospholipids metabolism and that has been found decreased in AD as well as Glycogen Synthase kinase (GSK), a major responsible for tau phosphorylation.

WE evaluate the effect of treatment with IChE on PLA₂ activity and GSK3B expression in platelet of 30 AD patients after 3 and 6 months of treatment. The control group comprised 42 elderly individuals without neurodegenerative disease

Before treatment, we found a decreased iPLA₂ activity in patients with AD as compared to controls. No difference in GSK3B were observed. After 3 and 6 months of treatment, we observed a significant increase in iPLA₂ activity similar to those found in controls. The patients who showed higher iPLA₂ activity in the first three months were those showing cognitive improvement after six months of treatment, measured by CAMCOG. After 6 months of treatment, we found an increase of phosphorylated GSK3B.

Our results suggest that donepezil present modifying properties in Alzheimer disease and that iPLA₂ activity measurement could be used as a marker of therapeutic response to donepezil and possibly other IChEs in Alzheimer's disease.

Introduction:

Acetylcholinesterase inhibitors (AChEI) are used for the treatment of Alzheimer's disease. Evidence from pre-clinical and clinical studies suggest neuroprotective roles for these drugs, especially, donepezil (Leyhe et al. 2009; Shen et al. 2010; Noh et al. 2013). These drugs leads to a delay in progression of brain atrophy, indicating a disease modifying effect by attenuating neuronal death (Hashimoto et al 2005) However, the protective mechanisms of donepezil have not yet been clearly identified.

Phospholipase A2 (PLA2) and glycogen synthase kinase 3B (GSK3B) are enzymes related to pathological cascades of AD and might constitute putative biomarkers of the AD process since their measurement in the periphery bears correlation with intracerebral pathology

PLA2

PLA2 comprises a super-family of enzymes that have a major role in membrane phospholipid homeostasis. The activity of PLA2 generates important intracellular signalling molecules and downstream products such as arachidonic acid and choline (Schaloske & Dennis, 2006; Burke & Dennis, 2009). PLA2 enzymes are classified into three major groups based on their structure, cellular localization, requirement for Ca^{2+} and substrate specificity. The most studied subtypes are the secreted calcium-dependent PLA2 (sPLA2), the cytosolic calcium-dependent PLA2 (cPLA2) and the intracellular calcium-independent PLA2 (iPLA2) (Dennis, 1984). PLA2 subtypes are involved in distinct biological functions and processes. The subtypes iPLA2 and cPLA2 are related to neuronal degeneration and death, whereas, cPLA2 and sPLA2 are related to inflammatory processes (Lambeau & Gelb, 2008; Schaeffer et al., 2011). Thus, changes in each of these subtypes may contribute to distinct aspects of several neuropsychiatric disorders including Alzheimer's disease (AD).

Reduced cPLA2 and iPLA2 activity has been associated with mechanisms of neuronal degeneration and memory impairment that may be relevant to the pathogenesis of AD (Sun

et al., 2004; Schaeffer et al., 2009, 2010). In primary cultures of cortical and hippocampal neurons, we found that the inhibition of iPLA2 activity reduced neurite outgrowth and neuronal viability (Forlenza et al., 2007), and increased the phosphorylation state of Tau (De-Paula et al., 2010). Moreover, we reported that in rodents, iPLA2 inhibition in the brain disrupted short and long-term memory acquisition and retrieval (Schaeffer & Gattaz, 2005, 2007). Conversely, conditioned training in adult rats led to PLA2 activation in parallel to enhancement of memory performance (Schaeffer et al., 2009).

In post-mortem studies, PLA2 activity was significantly reduced in the AD brain, and this reduction correlated with the number of neuritic plaques and neurofibrillary tangles (Gattaz et al., 1995, 1996; Talbot et al., 2000). Accordingly, in patients with mild and moderate AD, we found in an in vivo ³¹P-spectroscopy study that intracerebral phospholipid breakdown (a surrogate marker of PLA2 activity) was reduced and correlated with worse cognitive performance (Forlenza et al., 2005).

GSK3B

GSK3B is a serine-threonine kinase identified in early 1980's as a key enzyme in the regulation of the glycogen synthesis, given its ability to inactivate glycogen synthase by phosphorylation (Embi et al., 1980; Chin et al., 2005; Balaraman et al., 2006). In addition to glucose metabolism, GSK3B is involved in the regulation of critical intracellular signaling pathways, including cell cycle, gene expression and apoptosis (Jope et al., 2007; Peineau et al., 2008; Muylleert et al., 2008). In neurons, GSK3B plays a major role in cytoskeletal organization and remodeling, being thereby involved in mechanisms of synaptic plasticity, neurogenesis and resilience to neuronal injury (Grimes and Jope, 2001; Gould and Manji, 2005; Muylleert et al., 2008; Peineau et al. 2008). The main regulatory mechanism of GSK3B activity is the phosphorylation of the Ser-9 residue, which inhibits the enzymatic activity (Wang et al. 1994). GSK3B is strongly associated with several neuropathological

changes of AD as Tau hiperphosphorilation, neuronal apoptosis and synaptic dysfunction. Increased GSK3B activity was found in postmortem AD brains (Ly et al., 2013).

There are no study about the effect of cholinesterase inhibitor and these enzymes in human peripheral cells. Therefore, in the present study we evaluated the effect of short and long term donepezil treatment on PLA2 and GSK3B in AD patients.

Methods

Sample

Ninety elderly adults were included in this study, including 48 with mild or moderate AD (mean age $\pm$ standard deviation 74.11 ± 6.66 years), and 42 physically and cognitively healthy controls (71.67 ± 6.73 years). The present study was conducted at the Institute of Psychiatry, University of Sao Paulo, Brazil, and participants were middle-income, community-dwelling individuals from the hospital catchment area. Local Ethical Committee approved this study and all subjects signed a written informed consent prior to inclusion in the study.

The diagnosis of dementia due to AD was made according to the NINCDS-ADRDA diagnostic criteria (McKhann et al., 1984). A detailed description of the cognitive assessment and diagnostic procedures can be found elsewhere (Diniz et al., 2008; Forlenza et al., 2010). For the sake of conciseness, we only show the scores in the Cambridge Cognitive Test (CAMCOG) (Roth et al., 1986; Nunes et al., 2008) and the Mini-Mental State Examination (MMSE) (Folstein et al., 1975). In the last 6 months prior to enrolment in the study patients and the control group did not use IChEs or other drugs known effect on enzymes to be studied (eg, neuroleptics, anti-diabetic, lithium, antidepressants, anticonvulsants).

After the completion of baseline assessments, patients were started on anticholinesterase treatment with donepezil (5mg/day) which was delivered by one expert geriatric psychiatrist. Doses were administered orally and adjusted to 10mg/day after 1 month. Patients were monitored monthly and MMSE was evaluated at 3 and 6 month after donepezil treatment. CAMCOG was evaluated 6 months after donepezil treatment.

Laboratory analysis

Platelet preparation

Platelets were isolated from samples of peripheral blood at the baseline visit and at 3 and 6 months of follow-up. Blood samples were centrifuged at 515 x g for 15 minutes at 20 °C in acid citrate dextrose solution. Aliquots of platelet-rich plasma (PRP) were re-suspended in a wash solution (sodium citrate 30mM, pH 6.5, potassium chloride 5 mM, calcium chloride 2 mM, magnesium chloride 1 mM, glucose 5 mM, albumin 500 µg/mL, apyrase 50 µg/mL), centrifuged at 1159 X g for 8 minutes at 20°C, and the pellet was re-suspended in tris-sucrose solution. Platelet aliquots were immediately stored at -70 °C and protein levels were determined by a modified Lowry method prior to experimentation (Bio-Rad, Hercules, California).

Determination of PLA2 Activity

PLA2 activity was determined in platelets by a radio-enzymatic assay in triplicate (Talib et al., 2008, 2012). Accordingly, ¹⁴C-labelled fatty acid is preferentially cleaved by PLA2 at the sn-2 position of the phosphatidylcholine molecule. To access cPLA2 and sPLA2 activity, the substrate was L-α-1-palmitoyl-2-arachidonoyl-phosphatidylcholine labelled with [¹⁴C] at the sn-2 position (New England Nuclear, Boston Massachusetts). For the determination of iPLA2 activity, the substrate was L-3-phosphatidylcholine, 1-

palmitoyl-2- [1-14C]-palmitoyl. TotalPLA2 activity was estimated by the sum of the activities of PLA2 subgroups. After a 30 minute incubation at 37°C, the reaction was stopped by adding a solution of HCl-isopropanol (1:11.7, v/v), and the [1-14C]-fatty acid released by the enzymatic reaction was extracted with n-heptane. The radioactivity of [1-14C]-fatty acid was then measured in a liquid scintillation counter (Tri-Carb 2100TR; Packard, Meriden, CT). PLA2 activity was calculated in picomoles per milligram of protein per minute (pmol/mg protein/min). All determinations were done in the same radio-enzymatic procedure. The inter- and intra-assay variability were 6.0% and 3.3% (cPLA2), 5.2% and 1.8% (iPLA2) and 6.8% and 3.8% (sPLA2), respectively.

Determination of GSK3B

For GSK3B the protein concentration was normalized to 0.1mg/ml with appropriate buffer plus protease inhibitor as provided by the manufacturer (Assay Designs, Inc, MI, USA). Platelets were lysed in RIPA cell buffer modified by the addition of the protease inhibitors PIC (Sigma P8340) and PMSF (Sigma P7626).

The expression of GSK3B was determined at protein level in platelet samples by a specific Enzyme Immunometric Assay (EIA) assessing total- and Ser-9 phosphorylated GSK3B (pGSK3B) (TiterZyme EIA – Assay Designs Inc., MI, USA). The protocol was established according to the manufacturer's instruction. Standard curves were obtained with serial dilutions, i.e., 2000; 1000; 500; 250; 125; 62.5; 0.0 pg/mL for pGSK3B, and 5000; 2500; 1250; 625; 312.5; 156.3; 78.1; 0.0 pg/mL for total GSK3B; 100 uL of platelet homogenates corresponding to samples of patients and controls were dispensed in distinct wells of 96-well EIA plates, and the reactivity to imbedded antibodies generates color arrays that were read at 450 nm (Zenyth 200 Spectrophotometer, Austria), yielding an estimate of the concentration of GSK3B present in distinct aliquots. GSK3B activity was indirectly inferred for each

subject by means of the calculation of the proportion of phosphorylated (inactive) forms of GSK3B in relation to the total content of the enzyme in platelets (GSK3B ratio = pGSK3B/total GSK3B), as previously described by our group (Forlenza et al. 2011a, Diniz et al., 2011, Joaquim et al. 2012). Therefore, the GSK3B ratio is an inverse estimation of enzymatic activity, higher values indicating lower GSK3B activity.

Statistical analysis

T- test were carried out to assess mean differences in socio-demographic variables of patients and controls, and Logistic Regression to compare iPLA2, cPLA2, sPLA2 total PLA2, total GSK3B, pGSK3B and GSK3B ratio between these groups at baseline. Kolmogorov-Smirnov Test was used to verify de normal distribution. To address the longitudinal effect of donepezil treatment on PLA2 and GSK3B, we used Wilcoxon Test and Repeated measures ANOVA.

All statistical analyses were done with the SPSS (Statistical Package for Social Sciences, for Windows, v. 14, Chicago, IL) and significance level was $p < 0,05$.

Results

Table 1 displays the socio-demographic characteristics of patients (n= 48) and controls (n = 42) CAMCOG and MMSE scores. Since the education grade was statistically different between groups the results were controlled by this variable.

At baseline, patients with AD had significantly lower iPLA2 activity as compared to controls ($p < 0,001$) (figure 1a); no such differences were found to c- and sPLA2. Regarding to GSK3B no differences were observed between AD patients and controls (totalGSK3B, pGSK3B or GSK3B ratio).

Among patients that complete the study ($n=30$), after three months of treatment we observed an increased in iPLA2 activity ($p=0.015$) (figure 1b). No differences were observed on others variables.

However, after 6 months of donepezil treatment we observed a significant increase in iPLA2 activity ($p=0.001$) (figure 1b), restoring enzymatic activity similar to that observed among controls (figure 1c). Regarding pGSK3B we found an increase in patients ($p=0.009$) as compared to controls subjects and ($p=0.02$) as compared to their baseline measurements (figure 2).

No differences were found in cognitive measurements, nevertheless 64% of patients showed an increase of at least one point in MMSE ($CI(95\%) = [0.438; 0.842]$) after 3 months of treatment with donepezil. After 6 months of treatment 46% of patients exhibited an increase of at least one point in MMSE score ($CI(95\%) = [0.256; 0.666]$) and 50% of them presented an improvement of at least one point in CAMCOG score ($CI(95\%) = [0.294; 0.706]$). When we sort out the patients with AD into two groups: those who showed improvement and those who showed no improvement in CAMCOG score with 6 months of treatment; we observed that patients who showed improvement were those who had a greater increase in iPLA2 activity in the first three months of treatment ($p=0.037$) (figure 3).

Discussion

The main purpose of this study was to investigate the effect of AChEIs on PLA2 activity and GSK3B expression in platelets of AD patients. We found an increase in Calcium-independent PLA2 group activity and pGSK3 expression after long term treatment with donepezil.

Numerous attempts at developing novel therapeutics targeting AD based on pathophysiological characteristic of the disease have failed and the AChEIs and N-methyl-D-aspartate antagonist are the only approved agents for AD treatment (Selkoe 2004).

Several studies have suggested the neuroprotective effects of AChEIs, especially donepezil, in addition to its acetylcholinesterase-inhibiting. These effects include up-regulation of anti-apoptotic protein Bcl-2, stimulation of nicotinic acetylcholine receptors and activation of PI3K/Akt pathway (Giacobini, 2001; Morris 2006).

Along these findings, alterations in PLA2 activity and GSK3B have been described as biomarker candidate to AD. A decrease in PLA2 activity, notably iPLA2 group, and an increase GSK3B has been described in brain and peripherally of AD patients (Gattaz et al, 1995; 1996; 2014, Smesny et al., 2008, Forlenza et al., 2011, Joaquim et al., 2012). In fact Noh and collaborators (2009 and 2013) described, in primary culture of rat cortical neurons the effects of donepezil on Akt and the GSK3B signaling pathway in the presence of AB42 toxicity. In particular, the neuroprotective effects of donepezil were related to the inhibition of GSK3B activity, and consequently reduced phosphorylation of tau (Noh et al., 2009 and 2013). The increase in pGSK3B as the result of long term donepezil treatment found in platelets of our patients is in line with these studies.

Besides the regulation mechanisms of GSK3B and nicotinic acetylcholine receptors, the neuroprotective effects of donepezil were mediated through the activation of protein phosphatase 2A (PP2A) that together PP1A are the dominant phosphatases in vivo (Moscardó et al., 2006). Studies from Moscardó et al (2006) provide evidences that active phosphatases inhibit cPLA2 activity, however there are no study about iPLA2 activity. Nevertheless our findings of increased iPLA2 in long term treatment corroborate with studies depicting iPLA2 activity is required for activation of PI3K, supposed as one of action mechanism ascribe to donepezil, and that both are redundant mediators of chemotactic proteins that are increased in patients treated with donepezil (Lee et al, 2011 and Reale et al 2006).

Furthermore, donepezil exerts protective effect against intracellular Ca^{2+} and glutamate toxicity in cultured cortical neurons (Akasofu et al., 2006). This effect corroborate with finding of displacement of inhibitory Calmodulin from iPLA2- Ca^{2+} -calmodulin complex that activate iPLA2 leading to intracellular Ca^{2+} homeostasis (Zhang et al., 2009)

Despite of lack of statistics on cognitive measurements our results are in line with clinical trials studies with donepezil standard doses (10 mg/d), which the majority of patients showed stabilized or improved less than 4 points in scores of cognitive tests (Yang et al., 2013). 64% of our patients showed after 3 months of treatment an improvement of 1 point or more in MMSE and after 6 months 46% of them improved 1 point or more in MEEM and 50% in CAMCOG tests.

The fact that the increase in iPLA2 precede cognitive improvement observed in CAMCOG is in line with our 4-year prospective study, which showed that patients with mild cognitive impairment (MCI) with decreased iPLA2 activity are at greater risk of conversion to AD. This decrease precedes global cognitive deterioration in these patients, since there is no difference in the ratings of the CAMCOG or MMSE of patients with MCI who converted to AD and those who did not convert (Gattaz et al 2013).

Our results confirms findings of our group of decreased iPLA2 activity and pGSK3B expression in Alzheimer disease and for the first time show the effect of donepezil on iPLA2 activity in platelets confirming its neuroprotective effects and hint iPLA2 as a therapeutic target.

References

1. Balaraman Y, Limaye ER, Levey AI, Srinivasan S. Glycogen synthase kinase 3B and Alzheimer's disease: pathophysiological and therapeutic significance. *Cellular and Molecular Life Science* 2006;63:1226e35.
2. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. *J Lipid Res.* 2009;50 Suppl:S237-42. Review.
3. Chin PC, Majdzadeh N, D'Mello SR. Inhibition of GSK3b is a common event in neuroprotection by different survival factors. *Molecular Brain Research* 2005; 137:193e201.
4. Dennis EA Diversity of group types, regulation, and function of phospholipase A2. *J Biol Chem.* 1994; 269(18):13057–13060
5. De-Paula VJ, Schaeffer EL, Talib LL, Gattaz WF, Forlenza OV Inhibition of phospholipase A2 increases tau phosphorylation at Ser214 in embryonic rat hippocampal neurons. *Prostaglandins Leukot Essent Fatty Acids.* 2010; 82:57–60
6. Diniz BS, Nunes PV, Yassuda MS, Pereira FS, Flaks MK, Viola LF, Radanovic M, Abreu ID, Borelli DT, Gattaz WF, Forlenza OV Mild cognitive impairment: cognitive screening or neuropsychological assessment? *Rev Bras Psiquiatr* 2008; 30:316–321
7. Diniz BS, Talib LL, Joaquim HPG, De-Paula VRJ, Gattaz WF, Forlenza OV. Platelet GSK3B activity in patients with late-life depression: marker of depressive episode severity and cognitive impairment? *The World Journal of Biological Psychiatry* 2011;12(3):216e22.
8. Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European Journal of Biochemistry* 1980;107:519e27
9. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res.* 1975; 12 (3): 189 – 198.
10. Forlenza OV, Diniz BS, Talib LL, Radanovic M, Yassuda MS, Ojopi EB, Gattaz WF Clinical and biological predictors of Alzheimer's disease in patients with amnesic mild cognitive impairment. *Rev Bras Psiquiatr* 2010; 32(3):216–222

11. Forlenza OV, Mendes CT, Marie SK, Gattaz WF. Inhibition of phospholipase A2 reduces neurite outgrowth and neuronal viability. *Prostaglandins Leukot Essent Fatty Acids* 2007; 76:47–55
12. Forlenza OV, Torres CA, Talib LL, de Paula VJ, Joaquim HP, Diniz BS, et al. Increased platelet GSK3B activity in patients with mild cognitive impairment and Alzheimer's disease. *Journal of Psychiatric Research* 2011;45(2):220e4
13. Forlenza OV, Wacker P, Nunes PV, Yacubian J, Castro CC, Otaduy MC, Gattaz WF. Reduced phospholipid breakdown in Alzheimer's brains: a ³¹P spectroscopy study. *Psychopharmacology* 2005; 180(2):359–365
14. Gattaz WF, Cairns NJ, Levy R, Foerstl H, Braus DF, Maras A. Decreased phospholipase A2 activity in the brain and in platelets of patients with Alzheimer's disease. *Eur Arch Psychiatry Clin Neurosci* 1996; 246(3):129–131,
15. Gattaz WF, Maras A, Cairns NJ, Levy R, Foerstl H. Decreased phospholipase A2 activity in Alzheimer brains. *Biol Psychiatry*. 1995; 37:13–17
16. Gattaz WF, Talib LL, Schaeffer EL, Diniz BS, Forlenza OV. Low platelet iPLA₂ activity predicts conversion from mild cognitive impairment to Alzheimer's disease: a 4-year follow-up study. *J Neural Transm*. 2014;121(2):193-200.
17. Giacobini E, Zhu XD, Williams E, Sherman KA. The effect of the selective reversible acetylcholinesterase inhibitor E2020 on extracellular acetylcholine and biogenic amine levels in rat cortex. *Neuropharmacology* 1996;35:205–211.
18. Gould TD, Manji HK. Glycogen synthase kinase-3: a putative molecular target for lithium mimetic drugs. *Neuropsychopharmacology* 2005;30:1223e37.
19. Grimes CA, Jope RS. The multifaceted roles of GSK3b in cellular signaling. *Progress in Neurobiology* 2001;65:391e426.
20. Hashimoto M, Kazui H, Matsumoto K, Nakano Y, Yasuda M, Mori E. Does donepezil treatment slow the progression of hippocampal atrophy in patients with Alzheimer's disease? *Am J Psychiatry*. 2005;162(4):676-82.
21. Joaquim HP, Talib LL, Forlenza OV, Diniz BS, Gattaz WF. Long-term sertraline treatment increases expression and decreases phosphorylation of glycogen synthase kinase-3B in platelets of patients with late-life major depression. *J Psychiatr Res*. 2012;46(8):1053-8.
22. Jope RS, Yuskaitis CJ, Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochemistry Research* 2007;32:577e95.

23. Lambeau G, Gelb MH. Biochemistry and physiology of mammalian secreted phospholipases A2. *Annu Rev Biochem* 2008; 77:495–520
24. Lee SH, Schneider C, Higdon AN, Darley-Usmar VM, Chung CY. Role of iPLA(2) in the regulation of Src trafficking and microglia chemotaxis. *Traffic*. 2011;12(7):878-89.
25. Leyhe T, Hoffmann N, Stransky E, Laske C. Increase of SCF plasma concentration during donepezil treatment of patients with early Alzheimer's disease. *Int J Neuropsychopharmacol*. 2009;12(10):1319-26.
26. Ly PT, Wu Y, Zou H, Wang R, Zhou W, Kinoshita A, Zhang M, Yang Y, Cai F, Woodgett J, Song W. Inhibition of GSK3 β -mediated BACE1 expression reduces Alzheimer-associated phenotypes. *J Clin Invest*. 2013 2;123(1):224-35.
27. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. 1984;34(7):939-44.
28. Morris JC. Mild cognitive impairment is early-stage Alzheimer Disease [editorial]. *Arch Neurol*. 2006; 63 (1): 15 – 16.
29. Moscardó A, Vallés J, Piñón M, Aznar J, Martínez-Sales V, Santos MT. Regulation of cytosolic PLA2 activity by PP1/PP2A serine/threonine phosphatases in human platelets. *Platelets*. 2006;17(6):405-15.
30. Muyliaert D, Kremer A, Jaworski T, Borghgraef P, Devijver H, Croes S, et al. Glycogen synthase kinase-3 β , or a link between amyloid and tau pathology? *Genes, Brain and Behavior*. 2008;7:57e66.
31. Noh MY, Koh SH, Kim SM, Maurice T, Ku SK, Kim SH. Neuroprotective effects of donepezil against A β 42-induced neuronal toxicity are mediated through not only enhancing PP2A activity but also regulating GSK-3 β and nAChRs activity. *J Neurochem*. 2013;127(4):562-74.
32. Noh MY, Koh SH, Kim Y, Kim HY, Cho GW, Kim SH. Neuroprotective effects of donepezil through inhibition of GSK-3 activity in amyloid-beta-induced neuronal cell death. *J Neurochem*. 2009;108(5):1116-25.
33. Nunes PV, Diniz BS, Radanovic M, Abreu ID, Borelli DT, Yassuda MS, Forlenza OV CAMCOG as a screening tool for diagnosis of mild cognitive impairment and dementia in a Brazilian clinical sample of moderate to high education. *Int J Geriatr Psychiatry* 2008; 23:1127–1133

34. Peineau S, Bradley C, Taghibiglou C, Doherty A, Bortolotto ZA, Wang YT, et al. The role of gsk-3 in synaptic plasticity. *British Journal Pharmacology* 2008; 153(Suppl. 1):S428e37.
35. Roth M, Tym E, Mountjoy CQ, Huppert FA, Hendrie H, Verma S, Goddard R CAMDEX: a standardized Instrument for the diagnosis of mental disorders in the elderly with special reference to early detection of dementia. *Br J Psychiatry* 1986; 149:698–709
36. Schaeffer EL, da Silva ER, Novaes Bde A, Skaf HD, Gattaz WF. Differential roles of phospholipases A2 in neuronal death and neurogenesis: implications for Alzheimer disease. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010; 34(8):1381–1389
37. Schaeffer EL, Forlenza OV, Gattaz WF. Phospholipase A2 activation as a therapeutic approach for cognitive enhancement in early-stage Alzheimer disease. *Psychopharmacology* 2009; 202(1–3):37–51
38. Schaeffer EL, Gattaz WF. Inhibition of calcium-independent phospholipase A2 activity in rat hippocampus impairs acquisition of short- and long-term memory. *Psychopharmacology* 2005; 181:392–400
39. Schaeffer EL, Gattaz WF. Requirement of hippocampal phospholipase A2 activity for long-term memory retrieval in rats. *J Neural Transm*. 2007;114:379–385
40. Schaeffer EL, Skaf HD, Novaes Bde A, da Silva ER, Martins BA, Joaquim HD, Gattaz WF. Inhibition of phospholipase A2 in rat brain modifies different membrane fluidity parameters in opposite ways. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011; 35(7):1612–1617
41. Schaeffer EL, Zorron PuL, Gagliotti DA, Gattaz WF. Conditioning training and retrieval increase phospholipase A(2) activity in the cerebral cortex of rats. *J Neural Transm* 2009; 116:41–50
42. Schaloske RH, Dennis EA. The phospholipase A2 superfamily and its group numbering system. *Biochim Biophys Acta*. 2006;1761(11):1246-59.. Review.
43. Selkoe DJ; American College of Physicians; American Physiological Society. Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann Intern Med*. 2004; 20:140(8):627-38. Review.
44. Shen H, Kihara T, Hongo H, Wu X, Kem WR, Shimohama S, Akaike A, Niidome T, Sugimoto H. Neuroprotection by donepezil against glutamate excitotoxicity involves

- stimulation of $\alpha 7$ nicotinic receptors and internalization of NMDA receptors. *Br J Pharmacol.* 2010;161(1):127-39.
45. Smesny S, Stein S, Willhardt I, Lasch J, Sauer H. Decreased phospholipase A2 activity in cerebrospinal fluid of patients with dementia. *J Neural Transm.* 2008;115(8):1173-9.
 46. Sun GY, Xu J, Jensen MD, Simonyi A. Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res.* 2004; 45(2):205–213
 47. Talbot K, Young RA, Jolly-Tornetta C, Lee VM, Trojanowski JQ, Wolf BA. A frontal variant of Alzheimer's disease exhibits decreased calcium-independent phospholipase A2 activity in the prefrontal cortex. *Neurochem Int.* 2000; 37(1):17–31
 48. Talib LL, Diniz BS, Zainaghi IA, Forlenza OV, Gattaz WF. A radioenzymatic assay to identify three groups of phospholipase A(2) in platelets. *Prostaglandins Leukot Essent Fatty Acids* 2012; 86(4–5):149–153
 49. Talib LL, Yassuda MS, Diniz BS, Forlenza OV, Gattaz WF. Cognitive training increases platelet PLA2 activity in healthy elderly subjects. *Prostaglandins Leukot Essent Fatty Acids.* 2008; 78:265–269
 50. Wang QM, Fiol CJ, DePaoli-Roach AA, Roach PJ. Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *Journal of Biological Chemistry* 1994;269:14566e74.
 51. Yang YH, Chen CH, Chou MC, Li CH, Liu CK, Chen SH. Concentration of donepezil to the cognitive response in Alzheimer disease. *J Clin Psychopharmacol.* 2013Jun;33(3):351-5.
 52. Zhang D, Shooshtarizadeh P, Laventie BJ, Colin DA, Chich JF, Vidic J, de BarryJ, Chasserot-Golaz S, Delalande F, Van Dorsselaer A, Schneider F, Helle K, Aunis D, Prévost G, Metz-Boutigue MH. Two chromogranin a-derived peptides induce calcium entry in human neutrophils by calmodulin-regulated calcium independent phospholipase A2. *PLoS One.* 2009;4(2):e4501

Table 1 : socio-demographic characteristics of patients and controls

		Diagnosis		p
		Controls (n=42)	AD (n=48)	
Gender	Male	12	13	0.653
	Female	30	35	
Age		71.92(6.6)	72.25(7.4)	0.510
Education (years)		13.3 (5.1)	6.06 (3.5)	0.001
MMSE		28.9 (1.6)	17.9 (4.3)	0.0001
CAMCOG		96.9 (6.2)	54.1 (15.6)	0.0001

(a)

(b)

(c)

Legend Figure 1 :

iPLA₂ activity in platelets (a) baseline assessment of patients and controls (b) longitudinal evaluation (T0- baseline, T1 and T2- 3 and 6 months treatment respectively) (c). Patients versus controls after 6 months of treatment. The bars represent the mean ($\pm$ standard error).

Legend figure 2:

Expression of phosphorylated GSK in controls and in patients (T0- baseline, T1 and T2- 3 and 6 months respectively). The bars represent the mean ($\pm$ standard error).

Legend figure 3:

Patients who improved in CAMCOG with six months of treatment were those who had increased iPLA₂ activity in the first three months treatment ($p = 0.037$).

APÊNDICE B

Modelo de termo de consentimento livre e esclarecido:

HOSPITAL DAS CLÍNICAS

DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

DADOS DE IDENTIFICAÇÃO DO SUJEITO DA PESQUISA OU RESPONSÁVEL LEGAL

1. NOME:
 DOCUMENTO DE IDENTIDADE Nº : SEXO : ☐ M ☐ F ☐
 DATA NASCIMENTO:/...../.....
 ENDEREÇO Nº APTO:
 BAIRRO: CIDADE
 CEP: TELEFONE: DDD (.....)

2. RESPONSÁVEL LEGAL
 NATUREZA (grau de parentesco, tutor, curador etc.)
 DOCUMENTO DE IDENTIDADE : SEXO: ☐ M ☐ F ☐
 DATA NASCIMENTO:/...../.....
 ENDEREÇO: Nº APTO:

 BAIRRO: CIDADE:

 CEP: TELEFONE: DDD
 (.....).....

DADOS SOBRE A PESQUISA

1. TÍTULO DO PROTOCOLO DE PESQUISA : **BIOMARCADORES NA DOENÇA DE ALZHEIMER: GSK3B E PLA₂ NA RESPOSTA AOS INIBIDORES DE COLINESTERASE**

2. PESQUISADOR : Wagner Farid Gattaz

CARGO/FUNÇÃO: Professor Titular/ Psiquiatra INSCRIÇÃO CONSELHO REGIONAL Nº 25.956

UNIDADE DO HCFMUSP: Instituto de Psiquiatria

3. AVALIAÇÃO DO RISCO DA PESQUISA:

RISCO MÍNIMO (X)	RISCO MÉDIO ()
RISCO BAIXO ()	RISCO MAIOR ()

4. DURAÇÃO DA PESQUISA : 24 meses

HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO-HCFMUSP

1 – Justificativa e objetivos da pesquisa – A busca de biomarcadores capazes de auxiliar no diagnóstico diferencial e na avaliação do prognóstico dos transtornos demenciais, sobretudo nas fases iniciais, é objetivo perseguido por inúmeros centros de pesquisa nos últimos anos. O presente estudo volta-se para o estudo destes biomarcadores e suas alterações em indivíduos portadores de doença de Alzheimer e Comprometimento Cognitivo. O objetivo principal do projeto é comprovar alterações destes biomarcadores, nestes pacientes antes e após o início do tratamento com inibidores da colinesterase.

2 – Procedimentos que serão utilizados e propósitos, incluindo a identificação dos procedimentos que são experimentais – Se você decidir participar, deverá ser preenchida uma ficha de histórico e avaliação clínica (psiquiátrica e cognitiva) e será feita uma coleta de 10-40 mL (equivalente a 3 colheres de sopa) de sangue por punção periférica da veia do antebraço. As amostras serão utilizadas para as determinações bioquímicas. Todo material será codificado de modo que a identidade dos participantes não seja revelada.

3 – Relação dos procedimentos rotineiros e como são realizados – coleta de sangue por punção periférica da veia do antebraço.

4 – Desconfortos e riscos esperados – Os desconfortos e riscos esperados são mínimos e são decorrentes apenas do processo da coleta de sangue. Você poderá apresentar dor, desconforto ou hematoma quando o sangue for retirado.

5 – Benefícios que poderão ser obtidos – Não há benefício direto para o participante; trata-se de estudo experimental testando a existência de variantes bioquímicas associadas à depressão. É possível, mas não garantido, que os pacientes com este transtorno possam ser beneficiados pelos resultados deste estudo no futuro, bem como seus familiares. O estudo visa maior esclarecimento dos mecanismos biológicos responsáveis pelo aparecimento da depressão. De posse destes conhecimentos, espera-se conseguir, no futuro, melhores estratégias para o tratamento do referido transtorno.

6 – Garantia de acesso - em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas. O principal investigador é o Dr Wagner Farid Gattz que pode ser encontrado no endereço Rua Ovídio Pires de Campos, 785, São Paulo – SP. Telefone 3069-8010. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) – Rua Ovídio Pires de Campos, 225 – 5º andar – tel: 3069-6442 ramais 16, 17, 18 ou 20, FAX: 3069-6442 ramal 26 – E-mail: cappesq@hcnet.usp.br

8 – É garantida a liberdade da retirada de consentimento a qualquer momento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na Instituição.

9 – Direito de confidencialidade – As informações obtidas serão analisadas em conjunto com outros pacientes, não sendo divulgado a identificação de nenhum paciente.

10 – Direito de ser mantido atualizado sobre os resultados parciais das pesquisas, quando em estudos abertos, ou de resultados que sejam do conhecimento dos pesquisadores.

11 – Despesas e compensações: não há despesas pessoais para o participante em qualquer fase do estudo, incluindo exames e consultas. Também não há compensação financeira relacionada à sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.

12 - Compromisso do pesquisador de utilizar os dados e o material coletado somente para esta pesquisa.

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo **BIOMARCADORES NA DOENÇA DE ALZHEIMER: GSK3B E PLA₂ NA RESPOSTA AOS INIBIDORES DE COLINESTERASEs**

Eu discuti com o Dr. Wagner Farid Gattaz sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Assinatura do paciente/representante legal

Data ____/____/____

Assinatura da testemunha

Data ____/____/____

para casos de pacientes menores de 18 anos, analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual.

(Somente para o responsável do projeto)

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

Assinatura do responsável pelo estudo

Data ____/____/____