PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO
EM CIÊNCIAS FISIOLÓGICAS - UFSCAR/UNESP
Rodovia Washington Luiz, Km 235 – Caixa Postal 676
Fone/Fax: (016) 3351-8328 – email: ppgcf@ufscar.br
13565-905 – São Carlos, SP - Brasil

Biomarcadores sanguíneos para a doença de Alzheimer: avaliação da expressão gênica da ADAM10 e de micro-RNAs

São Carlos - SP
Março 2016

PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO
EM CIÊNCIAS FISIOLÓGICAS - UFSCAR/UNESP
Rodovia Washington Luiz, Km 235 – Caixa Postal 676
Fone/Fax: (016) 3351-8328 – email: ppgcf@ufscar.br
13565-905 – São Carlos, SP - Brasil

Biomarcadores sanguíneos para a doença de Alzheimer: avaliação da expressão gênica da ADAM10 e de micro-RNAs

PATRICIA REGINA MANZINE MORALLES

Tese apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas, Associação Ampla UFSCar/UNESP, do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos como parte dos requisitos para a obtenção do título de Doutora em Ciências Fisiológicas, área de concentração: Ciências Fisiológicas.

Orientadora: Prof.ª Dr.ª MÁRCIA REGINA COMINETTI
Coorientadora: Prof.ª Dr.ª SOFIA CRISTINA IOST PAVARINI

São Carlos - SP
Março 2016

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária UFSCar
Processamento Técnico
com os dados fornecidos pelo(a) autor(a)

M828b

Moralles, Patricia Regina Manzine
Biomarcadores sanguíneos para a doença de Alzheimer : avaliação da expressão gênica da ADAM10 e de micro-RNAs / Patricia Regina Manzine Moralles. -- São Carlos : UFSCar, 2016.
117 p.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2016.

1. Idoso. 2. Doença de Alzheimer. 3. Biomarcadores. 4. ADAM10. 5. RT-qPCR. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado da candidata Patricia Regina Manzine Moralles, realizada em 11/03/2016:

Profa. Dra. Márcia Regina Cominetti
UFSCar

Profa. Dra. Márcia Radanovic
USP

Prof. Dr. Sebastião Gobbi
UNESP

Prof. Dr. Marcos Hortes Nisihara Chagas
UFSCar

Prof. Dr. Anderson Ferreira da Cunha
UFSCar

Dedico este trabalho:

A Deus e N.Sª de Fátima,
Sempre presentes em minha vida,
Minha luz e sabedoria em todos os momentos.
Ao meu esposo Herick,
Meu exemplo de grande professor e pesquisador,
Companheiro de sempre e para sempre,
A metade que traz o melhor de mim mesma,
O grande e único amor da minha vida.
À minha família,
Minha base e apoio,
Meu exemplo de união e respeito.
Aos meus pais, Ermelindo e Dionéia,
Meus exemplos de vida e de amor,
Que me direcionam no caminho do bem e da fé,
Que valorizam desde meus pequenos feitos,
Até minhas grandes conquistas,
Pais maravilhosos.
Aos idosos e seus familiares,
Pela amizade e acolhida.
Por tornarem possível a realização deste trabalho.

AGRADECIMENTOS

A Deus, pelo dom da vida e por me iluminar e abençoar em todos os momentos. Por ser meu guia e me conceder saúde, sabedoria e confiança em minhas decisões.

Ao meu querido esposo Herick, pelo amor que me completa e me faz feliz. Por me ajudar em todos os momentos desta pesquisa, sempre preocupado e dedicado em cada etapa. Por me encorajar a trilhar o mundo acadêmico e sempre me fortalecer nos momentos de fraqueza e indecisões.

Aos meus queridos pais, Ermelindo e Dionéia, pelo amor incondicional e pelo esforço na busca de um melhor amanhã para os filhos. Pelo apoio e incentivo aos estudos desde muito cedo, que certamente foram decisivos para a realização deste sonho.

À minha querida orientadora Prof.ª Dr.ª Márcia Regina Cominetti pela amizade, ensinamentos e dedicação durante todos os momentos. Por acreditar na minha capacidade e me incentivar na busca de novos desafios e conquistas.

À minha querida coorientadora Prof.ª Dr.ª Sofia Cristina Iost Pavarini, pelo carinho e preocupação de sempre com minha pesquisa e principalmente comigo! Pelos momentos inesquecíveis de conversa e conselhos para minha vida.

À querida Prof.ª Dr.ª Maria Aderuza Horst, pela imensa colaboração e parceria durante todo o processo metodológico desta pesquisa, sempre muito paciente, amiga e disposta a me ensinar.

Aos membros da banca de qualificação, Prof. Dr. Sebastião Gobbi, Prof. Dr. Iran Malavazi, Prof. Dr. Daniel Shikania Kerr e membros da banca de defesa, Prof.ª Dr.ª Márcia Radanovic, Prof. Dr. Sebastião Gobbi, Prof. Dr. Anderson Ferreira Cunha e Prof. Dr. Marcos Hortes Nisihara Chagas, pelas relevantes contribuições que foram essenciais para o aprimoramento deste trabalho.

Aos idosos participantes e seus familiares, pela compreensão e apoio para a realização desta pesquisa. Pelos momentos únicos em cada visita, regados com muita alegria e esperança.

Ao Ambulatório de Neurologia Cognitiva e Comportamental da USE e em especial ao Prof.º Dr.º Francisco de Assis Carvalho do Vale e aos seus discentes Nádia, Bruno e Natália pelos diagnósticos clínicos e pelo empenho nesta pesquisa.

À enfermeira e técnica de enfermagem da USE, Rosa e Neli, pela paciência e dedicação semanal diante das coletas.

À Universidade Federal de São Carlos (UFSCar) pela formação profissional e pela oportunidade de realizar o curso de Doutorado.

Aos professores e funcionários do Programa de Pós-Graduação em Ciências Fisiológicas pela competência e seriedade diante da consolidação deste curso.

Aos funcionários das instituições de saúde USE, CEME, USFs, Instituto de Biociências da UNESP – Rio Claro, pelo acolhimento aos discentes e pesquisadores e por facilitarem a obtenção dos dados.

Ao grupo de pesquisa italiano, em especial a Prof.ª Dr.ª Monica Di Luca e Prof.ª Dr.ª Elena Marcello, por compartilhar experiências laboratoriais relevantes para o desenvolvimento desta pesquisa e me proporcionar novas capacidades profissionais e pessoais.

À Dr.ª Cláudia Malheiros Coutinho Camillo pela recepção e auxílio durante os ensaios no Centro Internacional de Pesquisa - AC Camargo Cancer Center – SP.

Ao Instituto de Biociências da UNESP – Campus Rio Claro e seus integrantes, Carla, Marol e Gilson, pela amizade e colaboração durante as coletas dos idosos.

Ao Laboratório de Biologia do Envelhecimento (LABEN) e todas as amigas e amigos (Angelina, Amanda, Cecília, Marcela, Francine, Angélica, Carla, Matheus, Lucas, Júlio, Marina, Liany, Ana Luiza, Lia e Mariana) pelos momentos de trabalho e companheirismo durante toda minha trajetória. Pelos conselhos, conversas, comilanças e apoio de sempre!

Ao Laboratório de Bioquímica e Biologia Molecular pela acolhida, paciência, aprendizado e novas amizades, em especial a Prof.ª Dr.ª Heloisa Sobreiro Selistre de Araújo e a grande turma Livia, Kelly, Dani, Patty, Antônio, Patricia Bueno, Carol, Araceli, Rafa, Cyntia, Charles, Beth, Guilherme, Fabi, Anderson, Fernanda, Uliana, Grazi, Natália, Camila, Anabelle, Rita e João, muito obrigada!

À FAPESP e à CAPES pelo apoio financeiro para que este trabalho pudesse ser desenvolvido.

A todas as pessoas aqui não mencionadas, mas que participaram direta ou indiretamente do sucesso desta pesquisa,

Muito obrigada!

“Se tiver o hábito de fazer as coisas com alegria, raramente encontrará situações difíceis.”

Robert Baden-Powell

RESUMO

ADAM10 é uma α-secretase que cliva a APP através do caminho não amiloidogênico, inibindo desta forma a produção do peptídeo β-amiloide (Aβ) na doença de Alzheimer (DA). Estudos apresentam a diminuição plaquetária da proteína ADAM10 em idosos com DA, assim como a desregulação de microRNAs (miRNAs) relacionados com moléculas envolvidas com a fisiopatologia desta doença. O objetivo geral foi verificar e comparar a expressão gênica da ADAM10 e de miRNAs entre idosos com DA e controles sem alterações cognitivas. Trata-se de um estudo de comparação, baseado nos pressupostos da pesquisa quantitativa. Amostras biológicas foram coletadas, analisadas e armazenadas em um biorrepositório. A expressão gênica da ADAM10 em sangue total foi estudada em 47 sujeitos com DA, 32 controles saudáveis e 21 sujeitos com transtorno neurocognitivo leve (TNCL), através de técnicas de RT-qPCR e analisada pela expressão relativa por $2^{-\Delta\Delta Ct}$. Para análises dos miRNAs, utilizando Megaplex e a base de dados MiRWalk 2.0, foram analisados por RT-qPCR ~700 miRNAs no sangue total e 21 deles foram validados em uma amostra de 21 sujeitos com DA e 17 controles. Testes estatísticos de associação, regressão e acurácia diagnóstica foram realizados. Não foi observada diferença significante na expressão gênica da ADAM10 entre sujeitos com DA e controles. Assim, a diminuição dos níveis proteicos da ADAM10 plaquetária em pacientes com DA não foi devido a redução do mRNA codificante para ADAM10. Mir-144-5p, miR-374 e miR-221 estavam menos expressos em indivíduos com DA, com moderada acurácia diagnóstica. Entretanto, a associação da expressão dos miRNAs selecionados com o Mini Exame do Estado Mental (MEEM) foi significativamente melhor como uma ferramenta de diagnóstico em comparação com as análises individuais. Os miRNAs validados estão envolvidos na regulação de vias relacionadas a doenças neurodegenerativas (cascata beta-amiloide, ubiquitinação, reguladores de transcrição, transmissão sináptica, tráfego de vesículas). Especificamente, o miR-144-5p, miR-374 e miR-221 são relevantes para a DA, como reguladores da tradução da APP, BACE1 e da ADAM10. Segundo nosso conhecimento, este é o primeiro estudo a demonstrar a expressão reduzida desses miRNAs no sangue total de pacientes com DA, em comparação com controles cognitivamente saudáveis. Estes resultados estão de acordo com os resultados proteicos da DA e destacam os miRNAs avaliados como potenciais biomarcadores que podem ser utilizados para o aperfeiçoamento do diagnóstico da DA.

Palavras-chave: idoso, doença de Alzheimer, biomarcadores, ADAM10, RT-qPCR, miRNA, sangue total.

ABSTRACT

ADAM10 is an α-secretase that cleaves APP in the non-amyloidogenic pathway, thereby inhibiting β-amyloid peptide (Aβ) production in Alzheimer´s disease (AD). Studies have shown decreased ADAM10 platelet levels in AD patients as well as the deregulation of microRNAs (miRNAs) related to molecules involved in the pathophysiology of this disease. The objective was to verify and compare ADAM10 gene expression and micro-RNAs (miRNAs) between AD patients and controls without cognitive impairment. It is a comparative study, based on the assumptions of quantitative research. Biological samples were collected, analyzed and stored in a biorepository. The ADAM10 gene expression in whole blood was studied in 47 AD, 32 healthy controls and 21 mild cognitive impairment (MCI) subjects by RT-qPCR techniques and analyzed by relative expression by $2^{-\Delta\Delta Ct}$. For miRNAs analyses, using Megaplex™ and MirWalk 2.0 database, were analyzed by RT-qPCR ~700 miRNAs in total blood and 21 miRNAs of them were validated in a sample of 21 AD subjects and 17 healthy controls. Statistical association tests, regression and diagnostic accuracy were performed. No significant differences in ADAM10 gene expression were observed between AD and control groups. Therefore, the decrease of ADAM10 protein in platelets of AD patients was not caused by a reduction in mRNA encoding for ADAM10. Mir-144-5p, miR-374 and miR-221 were downregulated in AD subjects, with moderate accuracy diagnosis. However, the association of selected miRNAs expression and Mini Mental State Examination (MMSE) was significantly better as a diagnostic tool compared to their expression separately. The validated miRNAs are involved in the regulation of pathways related to neurodegenerative diseases (beta-amyloid cascade, ubiquitination, transcriptional regulator, synaptic transmission, vesicle trafficking). Specifically, miR-144-5p, miR-374 and miR-221 are relevant for AD, as regulators of APP, BACE1 and ADAM10 translation. To the best of our knowledge, this is the first study to demonstrate a downregulation of these specific miRNAs in total blood of Alzheimer's disease patients, compared to healthy cognitive controls. These findings are in agreement with AD protein outcomes and place the miRNAs evaluated as potential biomarkers that can be used to improve AD diagnosis.

Keywords: elderly, Alzheimer´s disease, biomarkers, ADAM10, RT-qPCR, miRNAs, total blood.

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

APRESENTAÇÃO

APRESENTAÇÃO

Este trabalho será apresentado inicialmente com breve conteúdo teórico sobre a temática da fisiopatologia da doença de Alzheimer e os biomarcadores sanguíneos relacionados com esta doença, incluindo essencialmente a ADAM10 e os microRNAs. Em seguida os objetivos do estudo serão apresentados e respondidos sobre a forma de dois artigos intitulados "*ADAM10 gene expression in the blood cells of Alzheimer´s disease patients and mild cognitive impairment subjects*" e "*Predicted blood-based microRNAs for ADAM10 are downregulated in Alzheimer´s disease compared to healthy controls*". Ao final do trabalho, o item Anexo apresentará mais dois artigos intitulados "*BACE1 levels are increased in plasma os Alzheimer´s disease patients compared to matched cognitively healthy controls*" e "*Serotoninergic antidepressants positively affect platelet ADAM10 expression in patients with Alzheimer's disease*", de autoria colaborativa da aluna com outros estudos do Laboratório de Biologia do Envelhecimento – LABEN, desenvolvidos durante o período do doutorado.

INTRODUÇÃO

1. INTRODUÇÃO

1.1 Envelhecimento populacional e alterações cognitivas

Alterações cognitivas podem ocorrer como parte do processo fisiológico do envelhecimento, ou como sintoma de doenças neurológicas e sistêmicas, ou ainda, como sintoma predominante nas síndromes demenciais (ALBERT *et al.*, 2011). Em decorrência do envelhecimento populacional nos países desenvolvidos e nos em desenvolvimento, é esperado um aumento progressivo da prevalência desses transtornos. Uma preocupação nestes países, como deveria ser também no nosso, é o grande impacto do envelhecimento e das demências sobre a economia (CHAIMOWICZ, 1997).

No Brasil segundo o IBGE, 7,5% da população tem mais de 60 anos e este número deverá dobrar até 2050 (IBGE, 2010). Associado ao envelhecimento populacional destaca-se o quadro das morbidades, entre as quais as doenças crônicas degenerativas e os transtornos mentais representam um importante problema de saúde pública, com a utilização maciça dos serviços de saúde, causando grande impacto aos mesmos (CHAIMOWICZ, 1997).

As queixas de dificuldade de memória podem ocorrer em associação com déficits no desempenho de testes cognitivos, muitas vezes com intensidade não suficiente para caracterizar uma síndrome demencial. Deste modo, diversos construtos são descritos na literatura com o intuito de definir esses transtornos cognitivos não demenciais (TCND). A prevalência estimada de TCND em idosos nos Estados Unidos foi de 22%, com progressão anual para demência de 12% (PLASSMAN *et al.*, 2008). No Brasil as estimativas de prevalência de demência em idosos de 65 anos ou mais variam de 5,1% a 8,8%, 20% nas pessoas de mais de 80 anos, podendo chegar a 47% naqueles acima de 85 anos, dados similares aos verificados em países desenvolvidos (NITRINI *et al.*, 2009).

O conceito de Comprometimento Cognitivo Leve (CCL) elaborado por (PETERSEN *et al.*, 1999) e recentemente atualizado pelo DSM-V como Transtorno Neurocognitivo Leve (TNCL) é o mais utilizado para o estudo dos TCND (DSM-V, 2013). Os critérios clínicos para TNCL são: evidência de moderado declínio cognitivo em relação ao nível de desempenho prévio em um ou mais domínio cognitivo, de acordo com informações do paciente, do informante que tem conhecimento ou do médico, e diminuição no desempenho neurocognitivo em testes formais ou avaliação clínica equivalente de um a dois desvios padrão abaixo do esperado. Além disso, as alterações cognitivas são insuficientes para interferir na independência, mesmo que esforço e estratégias compensatórias sejam

necessárias. Estudos apontam que o subtipo amnéstico do TNCL pode corresponder a uma fase sintomática pré-clínica da doença de Alzheimer (DA) (ALBERT *et al.*, 2011).

Segundo o DSM-V o termo "doença de Alzheimer" não é utilizado isoladamente como diagnóstico. A DA se encontra na categoria "Transtorno Neurocognitivo Maior ou Leve devido à DA". Para o transtorno neurocognitivo maior dois ou mais desvios padrão abaixo do esperado nos testes cognitivos devem ser considerados e as alterações cognitivas são suficientes para interferir na independência. A DA é diagnosticada na constatação dos três itens: 1. Mutação genética como causa da DA, por meio de história familiar ou teste genético; 2. Evidência de declínio na memória e na aprendizagem e em pelo menos outro domínio cognitivo; 3. Declínio constante e gradual da cognição; 4. Falta de evidências de etiologia mista (DSM-V).

A DA é a causa de mais da metade de todos os quadros demenciais, destacando-se como a principal causa de demência em idosos (JALBERT *et al.*, 2008; NITRINI *et al.*, 2009). Trata-se de uma doença crônica não transmissível, neurodegenerativa e progressiva caracterizada por deterioração das funções cognitivas, incluindo a memória, prejuízo das atividades de vida diária e sintomas comportamentais e psicológicos (BALLARD *et al.*, 2011).

Nas últimas décadas, a DA tem se configurado como um dos principais problemas de saúde pública entre idosos, mundialmente. É uma doença de grande impacto socioeconômico que acomete aproximadamente 2% da população em países industrializados (BALLARD *et al.*, 2011). As estimativas epidemiológicas atuais da DA apontam que mais de 24 milhões de pessoas no mundo são acometidas por esta doença, com 4,6 milhões de casos novos ao ano devido ao desenvolvimento demográfico mundial, sendo previsto para 2030 um aumento no número de casos para 63 milhões de pessoas com DA (BALLARD *et al.*, 2011). Estudos brasileiros mostram que entre os diagnósticos mais frequentes, a DA representa a maior proporção, em torno de 54% dos casos e DA associada à demência vascular, 15% (HERRERA, 1998; MEGURO *et al.,* 2001; MEGURO *et al.,* 2011).

1.2 Fisiopatologia da DA

As características patológicas do cérebro na DA incluem atrofia cortical predominantemente no lobo temporal medial, e microscopicamente, perdas neuronais extensas e depósitos fibrilares anormais intra e extracelulares, denominados emaranhados neurofibrilares e placas senis, respectivamente (JORISSEN *et al.*, 2010). Os emaranhados

neurofibrilares são formados a partir da proteína Tau em dissociação aos microtúbulos. A Tau é de grande importância para a manutenção estrutural e funcional do citoesqueleto dos axônios neuronais (LICHTENTHALER, 2011). Na DA, a Tau sofre o processo da hiperfosforilação, de modo que ocorre sua dissociação dos microtúbulos e em sua agregação nos emaranhados intraneuronais compostos de filamentos helicoidais pareados, induzindo o colapso da estrutura neuronal (KUHN *et al.*, 2010).

As placas senis surgem a partir da deposição excessiva e subsequente agregação do peptídeo β-amiloide (Aβ) no cérebro. A hipótese da cascata Aβ postula que as placas amiloides extracelulares consistem da agregação de peptídeos Aβ insolúveis gerados a partir de clivagens proteolíticas da proteína precursora do β-amiloide (APP) causando assim danos em regiões cerebrais e precipitando os sintomas da DA (LICHTENTHALER, 2011).

A APP tem sido o centro de intensas pesquisas nos últimos anos devido sua associação com a patogênese da DA. Do ponto de vista estrutural, a APP se assemelha a um receptor de membrana celular que compreende um peptídeo sinalizador, uma extensa região extracelular N-terminal, um único domínio transmembrana e uma pequena extremidade C-terminal, cada qual de grande relevância para a patogênese da doença (DI LUCA *et al.*, 2000). A função biológica da APP ainda não é clara, mas pode envolver um papel na adesão celular ou como um receptor de sinal (LICHTENTHALER, 2011). O ectodomínio da APP pode ser desprendido da membrana por duas alternativas proteolíticas que envolvem tanto α ou β-secretases (EVIN *et al.*, 2003). A β-secretase é a enzima que cliva a APP no sítio β (BACE1 - *Beta-site APP cleaving enzime*) (COLE e VASSAR, 2007). A clivagem da APP pela BACE1 na região N-terminal do Aβ produz uma forma solúvel da APP (sAPPβ) que é liberada para o meio extracelular e parece ter uma função pró-apoptótica (NIKOLAEV *et al.*, 2009) e um fragmento C-terminal ligado a membrana (C99 – fragmento C-terminal com 99 aminoácidos da APP). A subsequente clivagem do C99 por uma γ-secretase no domínio C-terminal do Aβ libera no meio extracelular o Aβ1-40 e Aβ1-42 e o domínio intracelular da APP (AICD) – *APP intracellular domain* (EVIN *et al.*, 2003). A γ-secretase é um complexo de proteases hetero-tetraméricas constituído por quadro subunidades: presenilina, nicastrina, Aph-1 (anterior pharynx-defective1) e Pen-2 (presenilina enhancer 2) (LICHTENTHALER, 2011). O Aβ1-40 termina na Valina40 (Val40) e o Aβ1-42 apresenta dois aminoácidos adicionais hidrofóbicos, Isoleucina (Ile41) e Alanina (Ala42). Como resultado, o Aβ42 é mais hidrofóbico e apresenta maior potencial de agregação, portanto, mais amiloidogênico (ZHANG *et al.*, 2011). As β e γ-secretases foram identificadas há mais de 10 anos e ambas são proteínas de membrana (LICHTENTHALER, 2011). Alguns estudos têm mostrado que

apesar do nível plasmático de Aβ42 isolado não ser suficiente para atuar como um biomarcador, ele está aumentado no início da DA e mudanças em seu nível podem indicar uma transição do estado não demencial para DA (GRAFF-RADFORD, 2007). Outro estudo de coorte mostrou que a relação plasmática do Aβ42/40 se apresentou como um útil biomarcador para identificar sujeitos cognitivamente normais em risco para desenvolver DA (BLASKO, 2008).

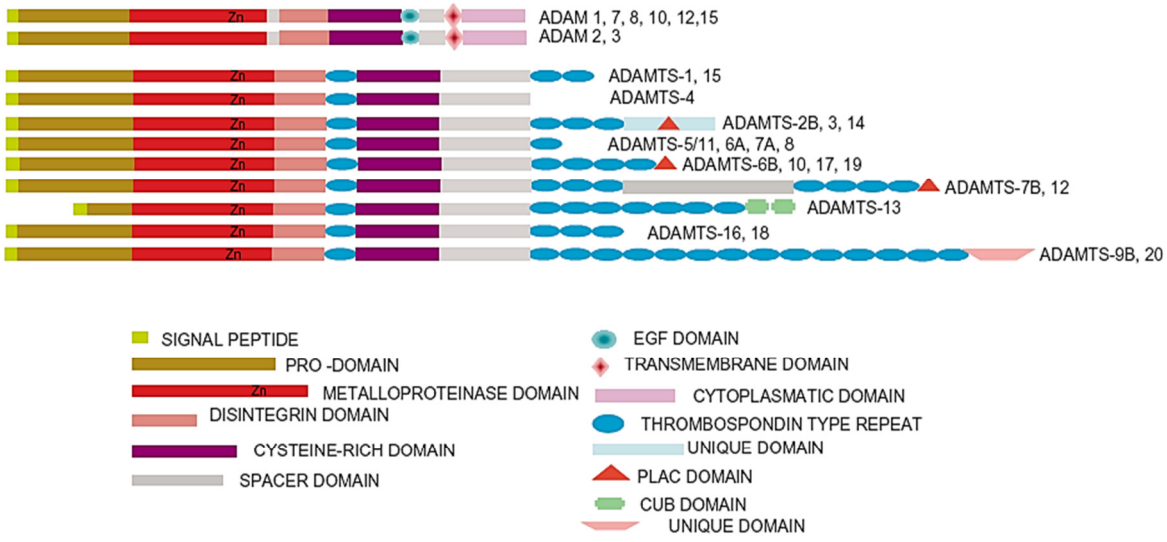
O peptídeo Aβ é composto de 39-43 aminoácidos, com massa molecular de aproximadamente 4kDa e compreende um fragmento proteolítico da APP liberado pelas clivagens sequenciais via β e γ-secretases aqui descritas (EVIN *et al.*, 2003). Contudo, a rota predominante de processamento da APP consiste de clivagens sucessivas por α e γ-secretases. Devido à formação do Aβ ocorrer no início da cascata de geração das placas amiloides, os mecanismos de formação, prevenção e remoção do Aβ são alvos de intensa pesquisa, tanto pela academia quanto pelas companhias farmacêuticas (LICHTENTHALER, 2011). A agregação do Aβ é neurotóxica, através de mecanismos ainda não bem esclarecidos. Contudo, os resultados desta agregação são acúmulo da proteína Tau dissociada, perda sináptica, neuroinflamação, neurodegeneração e morte neuronal, seguido do início dos sintomas da DA que posteriormente levam a morte (SAFTIG e LICHTENTHALER, 2015).

Apesar das evidências genéticas e neuroquímicas sustentarem a hipótese da cascata Aβ, outros mecanismos também estão presentes na patogênese da DA, o que a torna uma doença muito mais complexa. Estudos de neuroimagem mostram a presença de depósitos amiloides em sujeitos cognitivamente saudáveis, enquanto alguns pacientes com DA podem não apresentar tais formações (EDISON *et al.*, 2007; LI *et al.*, 2008). Além disso, perda sináptica e hiperfosforilação da Tau nos emaranhados parecem ter melhor correlação com a severidade da DA em comparação com o acúmulo do Aβ (SCHEFF e PRICE, 2003). Ensaios clínicos de fase III com drogas anti-amiloide também apresentaram resultados negativos na melhora da cognição, de modo a fortalecer a ideia que a DA pode ser causada por fatores independentes do Aβ (PIMPLIKAR *et al.*, 2010). Desta forma, outras propostas apontam que outros fragmentos da APP e não apenas o Aβ podem estar envolvidos no desenvolvimento da DA, de modo a ressaltar a importância das diferentes secretases atuantes neste processo proteolítico (GHOSAL *et al.*, 2009; NIKOLAEV *et al.*, 2009).

1.3 ADAM10 e DA

Proteínas expressas na membrana celular são de grande importância para os processos que ocorrem nas células, como comunicação intercelular, sinalização intracelular assim como na transmissão de sinais. Deste modo, a modulação da superfície celular é um evento importante de regulação destas proteínas e de seus fragmentos. Dois caminhos celulares estão envolvidos nesta regulação, sendo pela endocitose, que remove as proteínas da membrana celular, ou através de proteases localizadas na membrana ou no espaço extracelular que são capazes de catalisar a clivagem de substratos proteicos ligados à membrana de modo a liberar seus domínios extracelulares e abolir ou alterar suas funções na superfície da célula (SAFTIG e LICHTENTHALER, 2015). Neste segundo contexto, insere-se a atividade das ADAMs (***A*** ***D***isintegrin ***A***nd ***M***etalloprotease), que são integrantes essenciais neste processo, sendo que 22 formas são conhecidas em humanos até o momento (ENDRES *et al.,* 2012). As ADAMs estão envolvidas em diversos processos proteolíticos, dentre eles da APP. São proteínas transmembrana tipo I com grande domínio extracelular e pequeno domínio citosólico (Figura 1), e membros da superfamília das proteases dependentes de zinco, que por sua vez, é dividida de acordo com a estrutura primária de seus sítios catalíticos e inclui os subgrupos das gluzincinas, metzincinas, inuzincinas, carboxipeptidades e DD carboxipeptidases (HOOPER e TURNER, 2002). As ADAMs também são chamadas de MDCs (***M***etalloprotease/***D***isintegrin/***C***ystein-rich) e recebem, seguido ao nome, um número que representa a ordem de sua descoberta.

Figura 1. Diagrama esquemático da estrutura das classes das ADAMs (*A Di*sintegrin *A*nd *M*etalloprotease). Extraído de Fox e Serrano, 2005.



A região N-terminal das ADAMs possui uma sequência sinal que direciona para a via secretória e um prodomínio que possui função na maturação, pois sua presença mantém o domínio metaloprotease inativo. A ativação do domínio metaloprotease se dá pelo mecanismo de *cystein-switch* (VAN WART e BIRKEDAL-HANSEN, 1990), no qual um resíduo conservado de cisteína, presente no prodomínio, coordena o íon zinco (Zn^{2+}) do sítio ativo e o mantém inativo. Após a remoção do prodomínio e a consequente liberação do sítio ativo, o domínio metaloprotease torna-se funcional e é capaz de realizar suas funções catalíticas. Outra suposta função do prodomínio seria o de chaperona, ou seja, o prodomínio poderia servir para fornecer estruturalmente a configuração apropriada para a proteína como um todo ou especificamente para o domínio metaloprotease (SEALS e COURTNEIDGE, 2003). O domínio metaloprotease é responsável pelo processamento hidrolítico dos substratos nas ADAMs. Ele possui um sítio ativo que contém um íon Zn^{2+} e moléculas de água, os quais são necessários para o mecanismo catalítico. Três resíduos conservados de histidina e um de metionina coordenam o íon Zn^{2+} do sítio ativo. O resíduo de metionina faz parte de um motivo denominado *Met turn* que rodeia o motivo consenso HExGHxxGxxHD. A maioria das ADAMs possui domínios catalíticos funcionais, mas aquelas que não possuem a sequência HExGHxxGxxHD, consequentemente não possuem atividade de protease (SEALS e COURTNEIDGE, 2003).

Uma vez que as ADAMs são ubiquamente expressas e conservadas evolutivamente, elas são consideradas como atuantes no desenvolvimento e diferenciação, na regulação da comunicação célula-célula assim como em eventos de sinalização intra e intercelulares (WEBER e SAFTIG, 2012).

Nos últimos anos, vários trabalhos avançaram no entendimento da estrutura e função das proteínas da família das ADAMs. Entre elas, a ADAM10 destaca-se como a principal protease de membrana e está envolvida em diferentes processos fisiológicos e patológicos, como desenvolvimento embrionário, adesão celular, transdução de sinal, sistema imune, câncer e DA (LICHTENTHALER, 2011). Um exemplo deste processo é a dependência da sinalização da *Notch* na presença ou ausência da clivagem pela ADAM10, de modo a interferir na transcrição de genes dependentes desta sinalização e que atuam principalmente no desenvolvimento de células embrionárias, coordenando a diversificação celular (ANDERSSON *et al.*, 2011). Uma vez que os receptores de *Notch* alcançam a membrana celular e interagem com ligantes de células vizinhas a endocitose da *Notch* e de seus ligantes expõe o sítio de clivagem próximo à membrana que, então, pode ser processado pela ADAM10 (VAN TETERING *et al.*, 2009).

A ADAM10 emergiu como um dos membros mais importantes desta classe de proteases com atuação em vários órgãos, incluindo o cérebro. Adicionalmente, seu envolvimento na patogênese molecular da DA atraiu muito interesse e proveu novos conhecimentos sobre a biologia celular e a regulação de sua atividade. Após a identificação da *Notch* como seu principal substrato durante o desenvolvimento, e a identificação de outros substratos da ADAM10 fora do sistema nervoso central, se tornou evidente que as funções da ADAM10 não se limitam apenas a clivagem de poucas proteínas de membrana, mas são essenciais *sheddases* (proteínas liberadoras de ectodomínios) para muitas proteínas de membrana (REISS e SAFTIG, 2009). Além do papel central da ADAM10 no desenvolvimento e na homeostase cerebral, destaca-se sua contribuição em doenças neurodegenerativas incluindo a DA, doença de Huntington e desordens priônicas (SAFTIG e LICHTENTHALER, 2015).

Dada as inconsistências relativas à identidade da α-secretase envolvida na via não amiloidogênica da DA, dois estudos recentes utilizaram uma gama de reagentes, neurônios primários e camundongos condicionados para resolver a identidade desta α-secretase (JORISSEN *et al.*, 2010; KUHN *et al.*, 2010). Ambos os estudos chegaram à mesma conclusão de que a ADAM10, mas não a ADAM9 ou a ADAM17, é a α-secretase primordial em neurônios primários, ou seja, nas células mais afetadas na DA.

Na via não amiloidogênica, a APP é clivada pela ADAM10 entre Lys16 e Leu17 no meio da região do Aβ, deste modo, liberando a sAPPα - uma estrutura com funções neurotrópica e neuroprotetora - retendo o resíduo C83 (fragmento C-terminal com 83 aminoácidos da APP) na membrana. A clivagem seguinte do C83 pela γ-secretase libera o p3 (que não possui os 16 aminoácidos terminais do Aβ) e é supostamente benéfico e não encontrado nas placas amiloides, com início na posição Aβ17 (Aβ17-40 e Aβ17-42), inibindo assim a produção do Aβ amiloidogênico (MORISHIMA-KAWASHIMA e IHARA, 2002) (Figura 2). Desta forma, o aumento proteolítico da APP pela ADAM10 é suposto como uma tentativa de desacelerar ou prevenir o processo patológico da DA, particularmente porque a α e β-secretases parecem competir para o início desta clivagem (SAFTIG e LICHTENTHALER, 2015). Além disso, estratégias para aumentar a atividade das α-secretases são consideradas abordagens terapêuticas para pacientes com DA (FAHRENHOLZ, 2010).

Enquanto a super expressão ou estimulação da ADAM10 causa aumento da sAPPα e redução dos níveis de Aβ tanto em neurônios quanto em células de origem não neural (KIM *et al.*, 2009; LAMMICH *et al.*, 1999; TIPPMANN *et al.*, 2009), a redução de sua

atividade não promove aumento dos níveis de Aβ em várias linhagens de células (KUHN *et al.*, 2010). Estas diferenças de especificidade nas linhagens celulares ainda não são bem compreendidas, mas precisam ser levadas em consideração dentro dos estudos de proteólise da APP pela ADAM10 (SAFTIG e LICHTENTHALER, 2015).

O gene da ADAM10 humana é formado por 16 exons e localizado em uma região com cerca de 160kbp no cromossomo 15 (YAMAZAKI *et al.*, 1997) (Figura 3). Ensaios caracterizaram a região promotora da ADAM10 mostrando a ausência da região clássica TATA box.

Figura 2. Clivagem da APP por α e β-secretases. A APP é clivada sequencialmente por β-secretases e γ-secretases para gerar o peptídeo Aβ. A formação do peptídeo Aβ (via amiloidogênica) é prevenida pela α-secretase (via não amiloidogênica). Extraído de COLE e VASSAR, 2007.

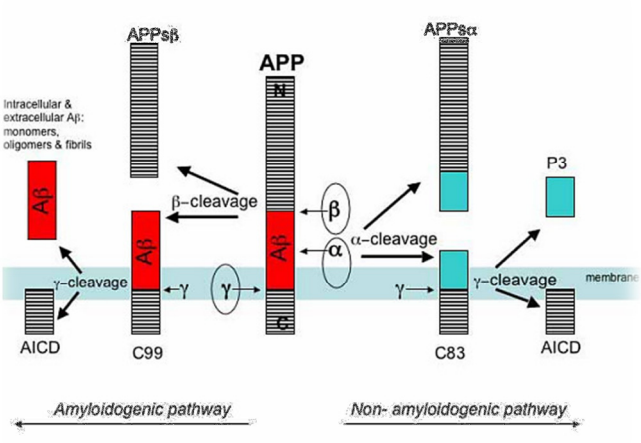
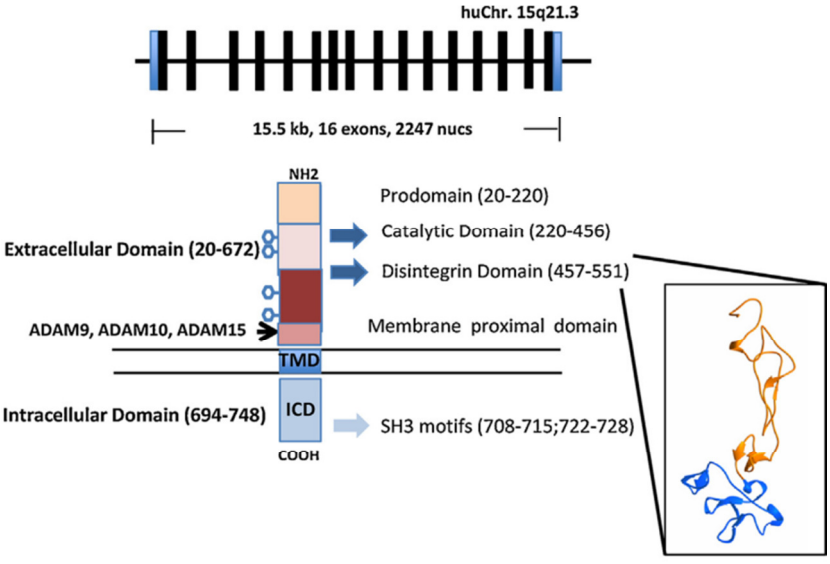


Figura 3. O gene da ADAM10 humana é formado pelas regiões 5´e 3´não-tradutoras (azul) e pelos 16 exons (preto) os quais geram o polipeptídio multimodular da ADAM10. A estrutura cristalográfica ilustra o domínio desintegrina, pertencente à porção extracelular da ADAM10. Extraído de SAFTIG e LICHTENTHALER, 2015.



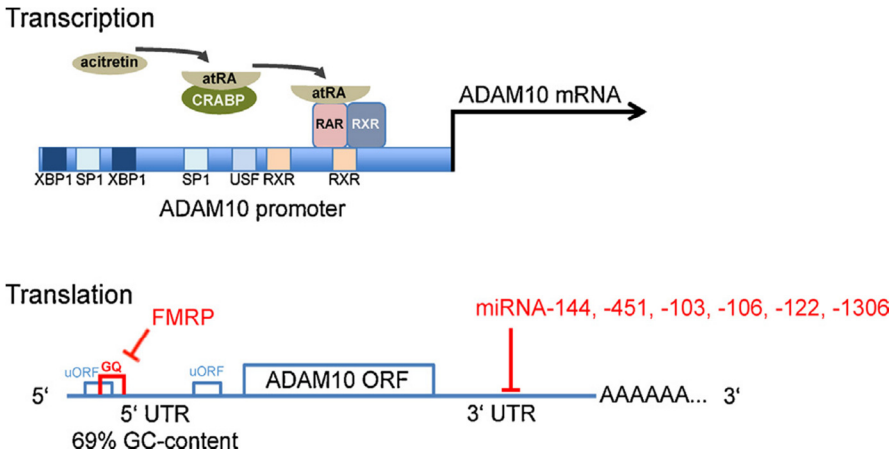
Vários fatores de transcrição se ligam a região promotora da ADAM10 e modulam sua transcrição (Figura 4). Em uma abordagem geral, a proteína de ligação X-box (XBP)-1 regula positivamente a expressão da ADAM10, de modo que a redução de sua atividade ou expressão foi observada em pacientes com DA e pode estar associada com aumento de placas amiloides e a progressão da doença (NIKOLAEV *et al.*, 2009).

Na região entre -508 e -300 são observados sítios de ligação para SP1, USF e também contém elementos responsivos ao ácido retinoico (Figura 4) localizados na região promotora -302 e -203 da ADAM10 (Figura 4). Neste contexto, foi observado que a deacetilase sirtuina-1 (SIRT1) coativa o receptor de ácido retinoico (RXR) levando a ativação da ADAM10 após sua ligação na região promotora (THEENDAKARA *et al.*, 2013). Estudos mostram que quando todos os sítios de ácido retinoico são ativos, os transcritos, níveis proteicos e atividade da ADAM10 são aumentados (ENDRES *et al.*, 2005). Em um recente ensaio clínico, 21 pacientes com DA leve à moderada foram tratados durante quatro semanas com a droga acitretina (derivada do ácido retinoico) ou placebo. Apesar do Aβ não ter apresentado redução significativa, os resultados deste estudo mostraram boa tolerância dos participantes à droga e aumento dos níveis de sAPPα no líquor do grupo tratado, de modo a tornar a APP o primeiro substrato da ADAM10 validado em humanos (ENDRES *et al.*, 2014).

A região promotora da ADAM10 também é responsível a melatonina, um hormônio envolvido no ritmo circadiano. A melatonina inibe a formação de placas amiloides e espécies tóxicas do Aβ, induzindo o processamento não amiloidogênico da APP pela ADAM10 (SHUKLA *et al.*, 2015).

O transcrito da ADAM10 (mRNA) apresenta tamanho de 4.4kb. A análise do mRNA da ADAM10 evidencia um segmento rico em GC localizado na região 5´UTR (*Untranslated Region*) formado por cerca de 450 nucleotídeos. Curiosamente, a presença desta região inibe a tradução da ADAM10 e sua deleção causa grande aumento da expressão em células hepáticas humanas (LAMMICH *et al.*, 2010). Este efeito de inibição da tradução da ADAM10 pela região 5´UTR deve-se a presença de uma estrutura estável secundária de RNA G-quadruplex (GQ), e pode envolver também a proteína do atraso mental frágil X (FMRP) (Figura 4) (PASCIUTO *et al.*, 2015).

Figura 4. Regulação da ADAM10 em nível transcricional e traducional. A transcrição da ADAM10 é regulada por vários fatores de transcrição. Seus sítios de ligação na região promotora da ADAM10 estão indicados pelos quadrados coloridos. Um deles é o heterômero RAR/RXR que pode se ligar aos dois sítios RXR localizados na região promotora da ADAM10. Em consequência da ligação do ácido retinoico *all-trans* (atRA) no RAR, o fator RAR/RXR estimula a transcrição da ADAM10. A droga acitretina, um derivado do ácido retinoico é capaz de remover o atRA da proteína celular ligada ao ácido retinoico (CRABP), levando a ligação do atRA no RAR e estimulando a expressão gênica da ADAM10. O mRNA da ADAM10 é formado por uma região 5´UTR rica em GC, pela estrutura de codificação aberta (ORF) e pela região 3´UTR. Duas regiões *upstream* de codificação aberta (uORF) são encontradas na região 5´UTR, mas não controlam a tradução da ADAM10. Por outro lado, uma estrutura secundária G-quadruplex (GQ) inibe a tradução da ADAM10 através da FMRP. Do mesmo modo, diferentes miRNAs inibem a tradução da ADAM10 pela ligação em diferentes sítios na região 3´UTR. Extraído de SAFTIG e LICHTENTHALER, 2015.



A expressão proteica da ADAM10 no cérebro é muito variada, de modo que apresenta-se em diferentes regiões, como mesencéfalo, telencéfalo e cerebelo (KARKKAINEN *et al.*, 2000). Entretanto, estudos mostram a co-expressão da APP, BACE1 e ADAM10 apenas em neurônios corticais (MARCINKIEWICZ e SEIDAH, 2000). Além da APP, a ADAM10 pode clivar outros substratos, como a *Notch*, Klotho, N-caderina, L1, Neuregulina-1, Nectina-1 e Eferin, o que implica em sua grande variedade de funções tanto no cérebro quanto em outros tecidos (SAFTIG e LICHTENTHALER, 2015). Recentemente o estudo de KUHN *et al.* (2016) identificou 91 novos possíveis substratos da ADAM10 em neurônios corticais. Dentre eles, NrCAM, LDLR, MT4MMP e CDH6 foram validados e apontam função central da ADAM10 na formação de sinapses e direcionamento dos axônios, de modo a concluir que a ADAM10 é a principal protease de membrana do sistema nervoso.

A ADAM10 é expressa precocemente no desenvolvimento cerebral, com maior prevalência durante a formação dos vasos sanguíneos, oligodendrócitos e subgrupos de neurônios (LIN *et al.*, 2008). Foi purificada pela primeira vez em preparações de membrana de mielina em 1989 (CHANTRY *et al.*, 1989) e clonada em 1996 (HOWARD *et al.*, 1996). Os primeiros estudos proteicos da ADAM10 evidenciaram sua atividade no processamento do

TNFα (fator de necrose tumoral tipo α) (LUNN *et al.*, 1997) e da APP (LAMMICH *et al.*, 1999). Após a remoção do peptídeo sinal, a proforma da ADAM10 humana é formada por 729 aminoácidos com um grande e complexo domínio extracelular, um domínio transmembrana e 55 aminoácidos na cauda citoplasmática (Figura 3). A ativação da ADAM10 é influenciada por vários fatores, dentre eles a remoção do prodomínio durante o tráfego pela rede trans-Golgi. A prohormônio convertase 7 (PC7) possivelmente atua neste processo de maturação (LOPEZ-PEREZ *et al.*, 2001) de modo que sua super expressão promove aumento da atividade da α-secretase na clivagem da APP (ANDERS *et al.*, 2001).

Evidências ainda suportam que as furinas (protease dependente de cálcio) contribuem para a ativação da ADAM10 (Figura 5A) (HWANG *et al.*, 2006). Dentro do retículo endoplasmático (RE) a ADAM10 ainda possui uma sequência para reconhecimento da PC entre o prodomínio e o domínio catalítico e somente após sua remoção no sistema trans-Golgi a porção extracelular é ativada, ficando constituída pelos domínios metaloprotease, desintegrina e proximal à membrana. Destaca-se a presença de um segundo sítio de processamento da PC na porção aminoterminal do prodomínio das ADAMs proteases (9, 10 e 17) que parece ser igualmente importante ao clássico sítio de processamento da PC no controle da função das ADAMs (WONG *et al.*, 2015). A função do prodomínio da ADAM10 foi recentemente esclarecida através de mutações (Q170H e R181G) observadas em dois pacientes com DA. A expressão destas mutações aumenta cerca de 2-3 vezes os níveis de Aβ e reduz a atividade de α-secretase sobre a APP (KIM *et al.*, 2009).

A importância do domínio transmembrana da ADAM10 em sua atividade proteolítica também é alvo de pesquisas, visto que este domínio sustenta uma estrutura pertencente ao domínio citoplasmático capaz de formar um homodímero na membrana, o que pode influenciar sua interação com outras estruturas e consequentemente o processo proteolítico (DENG *et al.*, 2014). A porção citosólica apresenta uma sequência rica em arginina. Mutações neste domínio mostram aumento da expressão da ADAM10 na superfície celular (MARCELLO *et al.*, 2010). Estudos recentes investigam a modulação da expressão da ADAM10 sináptica em neurônios a partir de proteínas carreadoras (Figura 6). Duas linhas de pensamento norteiam estes estudos e ambas relacionam-se com o tráfego intraneuronal da ADAM10. A primeira direciona-se ao estudo da proteína SAP97 (*synapse-associated protein* 97), a qual em associação com a ADAM10 favorece seu transporte citoplasmático em direção à membrana, de modo que aumenta sua capacidade de clivar a APP, como α-secretase (MARCELLO *et al.*, 2012). A segunda enfoca aspectos da endocitose da ADAM10 de membrana, realizada pela proteína adaptadora de clatrina (AP2), de modo que esta associação

favorece a internalização desta enzima e, portanto, influencia negativamente sua expressão e atividade como α-secretase (ZHONG *et al.*, 2007).

Figura 5. (A) A clivagem por furinas dentro do sistema trans-Golgi remove o prodomínio da ADAM10 e libera sua forma madura e ativa **(B)** A regulação da ADAM10 é mediada em diferentes níveis, por exemplo, na modulação do promotor, o grau de liberação do prodomínio, tráfego celular, sinalização celular e interações entre lipídeos e proteínas. Extraído de SAFTIG e LICHTENTHALER, 2015.

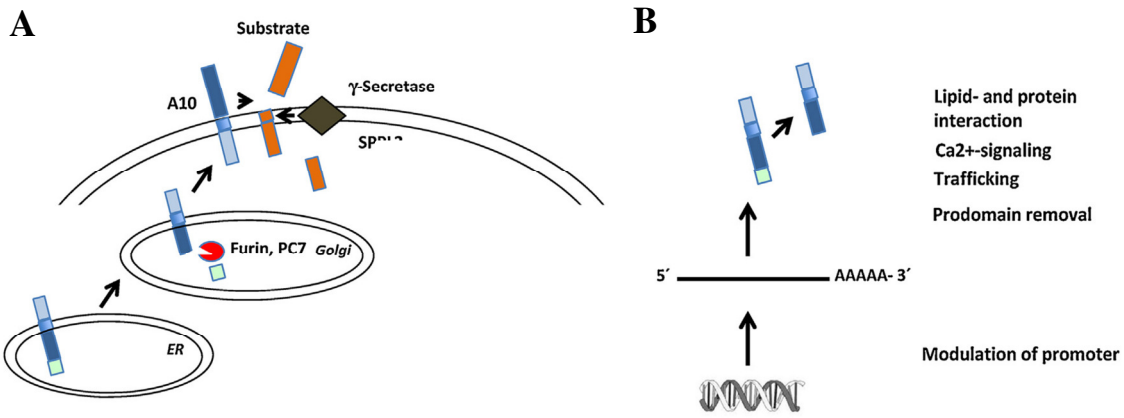
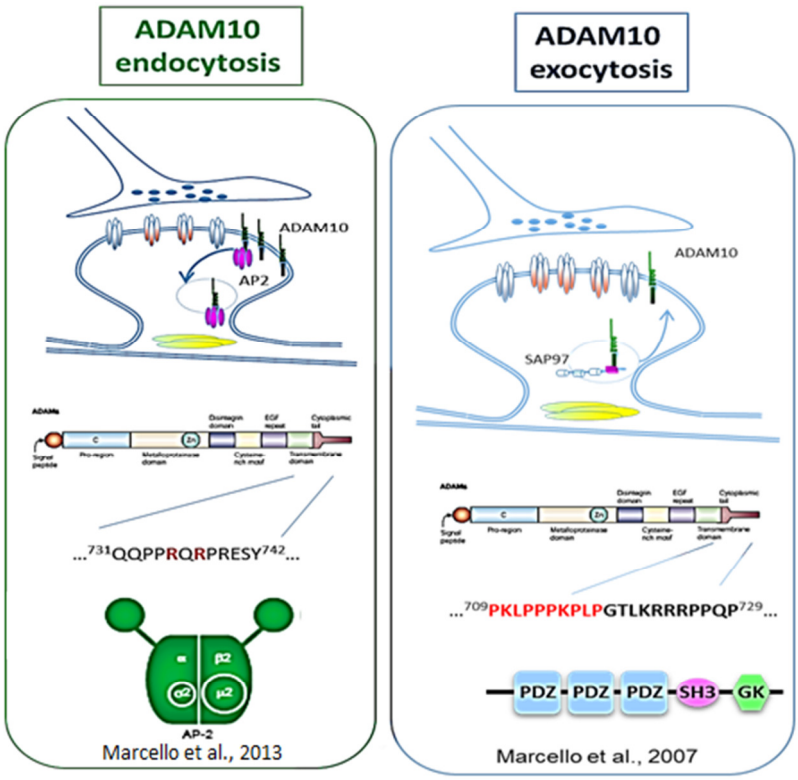


Figura 6. Mecanismos de tráfego da ADAM10 sináptica. Endocitose da ADAM10 de membrana realizada pela proteína adaptadora de clatrina (AP2), de modo que esta associação favorece a internalização desta enzima, diminuindo sua atividade. Exocitose da ADAM10 de membrana pela proteína SAP97, a qual favorece seu transporte citoplasmático em direção à membrana (MARCELLO *et al.,* 2012).



Entretanto, evidências sugerem a presença da ADAM10 ativa em diferentes componentes do caminho secretório além da membrana plasmática, como por exemplo, em microvesículas (exossomos) (GUTWEIN *et al.*, 2003). A maior parte de sua forma estável é encontrada na rede trans-Golgi e neste local a clivagem de substratos já pode ser iniciada, entretanto, na rota secretória para a membrana plasmática a ADAM10 também pode exercer considerável atividade proteolítica. Sua atividade também pode ser verificada no início da endocitose antes de sua degradação, de modo que ainda não é claro endereçar onde a ADAM10 está realmente ativa (SAFTIG e LICHTENTHALER, 2015).

Além da interação proteína-proteína, a composição da membrana celular também pode exercer importante papel no controle da atividade da ADAM10, visto que a depleção de moléculas de colesterol promove aumento da atividade da ADAM10 na APP (KOJRO *et al.*, 2001). Além disso, a presença de estruturas lipídicas também prejudica a mobilidade da ADAM10 e consequentemente sua atuação nos processos proteolíticos (REISS *et al.*, 2011). A ação de outras α-secretases também pode alterar a atividade da ADAM10. Um estudo com neuroblastos SH-SY5Y (célula que possui grande expressão de APP) observou que a utilização do inibidor específico para ADAM9 (proA9) acarreta aumento da atividade da ADAM10 ligada a membrana, com aumento do sAPPα e diminuição do sAPPβ no meio extracelular (MOSS *et al.*, 2011). Como vimos, a regulação da ADAM10 é mediada em diferentes níveis, como na modulação do promotor, no grau de liberação do prodomínio, no tráfego celular, na sinalização celular e nas interações entre lipídeos e proteínas (Figura 5B).

1.4 micro-RNAs e DA

Os micro-RNAs (miRNAs) são moléculas de aproximadamente 21 nucleotídeos, não codificantes, capazes de regular a expressão de genes em nível pós-transcricional (AMBROS, 2004). Estas moléculas têm provocado uma revolução na compreensão dos mecanismos de regulação gênica (AMBROS, 2010). Os miRNAs são processados a partir de moléculas precursoras (pri-miRNAs) as quais são transcritas pela RNA polimerase II a partir de genes independentes ou representam íntrons em genes codificantes para proteínas (KROL *et al.*, 2010). Os miRNAs se ligam à região 3'UTR de mRNAs alvos por meio de pareamento de bases resultando na degradação do mRNA ou em inibição traducional (Figura 4) (LEIDINGER *et al.*, 2013; SATOH, 2012).

Estima-se que 1 a 4% dos genes do genoma humano sejam codificantes para miRNAs e que um único miRNA pode regular até 200 moléculas de mRNA (ESQUELA-

KERSCHER e SLACK, 2006). Até 2009, mais de 400 espécies de miRNAs tinham sido identificadas no cérebro humano e estima-se que este órgão possa conter mais de 1.000 miRNAs (BEREZIKOV *et al.*, 2006). Entretanto, os miRNAs também podem ser detectados no sangue circulante, células sanguíneas, líquor, além de vários outros tecidos (DE SMAELE *et al.*, 2010). Por sua estabilidade (comparado ao mRNA) são moléculas menos susceptíveis à modificação química e degradação por RNAses. Assim sendo, os miRNAs podem ser detectados a partir de amostras frescas mas também de tecidos congelados e até mesmo fixados em parafina (DE SMAELE *et al.*, 2010). Sua detecção sanguínea os torna candidatos atraentes como biomarcadores, especialmente no caso da DA, aonde procedimentos invasivos para coleta de líquor requerem locais equipados com profissionais treinados.

Os miRNAs de tecidos cerebrais estão sendo utilizados como biomarcadores para doenças como câncer e doenças do sistema nervoso central, como DA, doença de Parkinson, esquizofrenia, doença de Huntington e autismo (DE SMAELE *et al.*, 2010). Evidências de pesquisa sobre a DA sugerem que alterações na rede de miRNAs podem contribuir para aumento do risco de desenvolver a doença (DELAY *et al.*, 2012; SCHONROCK *et al.*, 2012). Ainda não foi estabelecido como a desregulação dos miRNAs pode contribuir para a DA, mas este fator pode ser tanto causa, quanto consequência da doença (SCHONROCK *et al.*, 2011). Vários trabalhos recentes demonstraram a expressão alterada de alguns miRNAs no líquor e neurônios de pacientes com DA (COGSWELL *et al.*, 2008; LUKIW, 2007). SCHONROCK *et al.* (2010) mostraram que o acúmulo do peptídeo Aβ causa uma considerável desregulação dos miRNAs neuronais. Quando se compara cérebros e líquor humanos com neurônios hipocampais de ratos transgênicos tratados com o peptídeo Aβ, observa-se uma diminuição da expressão do miR-9 , mir-29 e miR-181c (COGSWELL *et al.*, 2008). LUKIW *et al.* (2007) demonstraram que seis miRNAs são diferencialmente expressos em cérebros de fetos e adultos. Em cérebros de pacientes com DA, o miR-9 e o miR-146a tiveram suas expressões aumentadas comparado com cérebros de sujeitos saudáveis e o miR-128 teve expressão ainda maior, comparado com cérebros de fetos e adultos. O gene que codifica para a APP é alvo do miR-20 e miR-101 (VILARDO *et al.*, 2010) e o miR-9 e miR-107 regulam o gene que codifica para a BACE1 e suas expressões estão diminuídas em cérebros de pacientes com DA (BOISSONNEAULT *et al.*, 2009; DELAY *et al.*, 2012; WANG *et al.*, 2008).

Entretanto, até o momento, poucos estudos avaliaram a presença de miRNAs no sangue circulante de pacientes com DA. SCHIPPER *et al.* (2007), verificaram que a expressão dos miR-34 e miR-181b está reduzida na DA em células periféricas sanguíneas.

SERPENTE *et al.* (2011) ao avaliarem nestas células o miRNA-369-3p, envolvido na expressão gênica de OLR1, um gene relacionado com a oxidação lipídica, verificaram sua redução em pacientes com DA em relação a controles. A desregulação de 140 miRNAs no sangue total de pacientes com DA foi observada no estudo de LEIDINGER *et al.* (2013). Destes, um painel de 12 miRNAs foram validados, sendo sete miRNAs super expressos (miR-112, miR-161, let-7d-3p, miR-5010-3p, miR-26a-5p, miR-1285-5p, miR-151a-3p) e cinco miRNAs sub expressos (miR-103a-3p, miR-107, miR-532-5p, miR-26b-5p e let-7f-5p) na DA. Neste estudo, o miR-144-5p apresentou o menor nível de expressão (não validado) na DA, entretanto, a literatura aborda a não especificidade deste miRNA para DA (SMITH-VIKOS e SLACK, 2013), visto que outros trabalhos também descrevem sua desregulação em outras patologias humanas, incluindo diversas neoplasias (KELLER *et al.*, 2011). Entretanto, em CHENG *et al.* (2013) a função do miR-144 na regulação da ADAM10 foi demonstrada, dado que a super expressão deste miRNA ocasionou a diminuição dos níveis desta proteína. Outro miRNA possivelmente envolvido na regulação da ADAM10 é o miR-451, o qual em conjunto com o miR-144 (miR144/451) podem atuar na inibição da expressão da ADAM10 (BARAO *et al.*, 2013).

O estudo investigativo de AUGUSTIN *et al.* (2012) utilizou abordagem computacional e validação experimental para sugerir possíveis miRNAs que atuem na regulação da expressão da ADAM10 na DA. Verificou-se que três miRNAs (miR-103, miR-107 e miR-1306) estão relacionados com a DA e possuem sítios de ligação conservados para ADAM10 entre as espécies, sendo que o miR-103 e miR-107 apresentaram sobreposição significante com a base de dados AlzGene[1]. Em células SH-SY5Y, estes três miRNAs mostraram importante atividade inibitória nos níveis de expressão da ADAM10.

Outra fonte periférica para análise de miRNAs é o plasma. No estudo de KIKO *et al.* (2014), a mensuração de seis miRNAs (miR-9, miR-29a, miR-29b, miR-34a, miR-125b e miR-146a) foi realizada no plasma e no líquor de sujeitos com DA com o intuito de avaliar o potencial destes miRNAs como biomarcadores para DA. Os resultados mostraram que os níveis dos miR-34a e miR-146a no plasma e miR-34a, miR-125b e miR-146 no líquor estão significantemente reduzidos na DA em comparação aos controles. Por outro lado, os miR-29a e miR-29b no líquor estão aumentados em sujeitos com DA (KIKO *et al.*, 2014).

Os estudos de SHEINERMAN *et al.* (2012) e KUMAR *et al.* (2013) também abordam o plasma como relevante fonte de miRNAs com potencial ação biomarcadora para

[1] http://www.alzgene.org/

DA. KUMAR *et al.*, (2013) sugerem um painel formado por 7 miRNAs com expressão reduzida na DA (let-7d-5p, let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p e miR-545-3p) que discrimina a doença com acurácia superior a 95%. A identificação em conjuntos de miRNAs plasmáticos permite a diferenciação mais eficiente da doença (SHEINERMAN *et al.*, 2012). Deste modo, os conjuntos (família miR-132 e família miR-134) foram capaz de discriminar sujeitos com TNCL de controles no estudo de SHEINERMAN *et al.* (2012). Nele, os pares super expressos na DA, miR-128/miR-491-5p, miR-132/miR491-5p, miR-874/miR-491-5p (família miR-132) e miR-134/miR-370, miR323-3p/miR-370, miR-382/miR-370 (família miR-134) alcançaram valores expressivos de sensibilidade e especificidade para distinção entre sujeitos com DA e controles. A tabela 1 resume os estudos sobre a desregulação de miRNAs na DA descritos acima.

Tabela 1. Estudos sobre a expressão de miRNAs na DA.

Referência	Fonte	miRNA	Expressão na DA
Lukiw *et al.* (2007)	Cérebro	9, 128, 146a	↑
		146b	↓
Cogswell *et al.* (2008)	Cérebro	9, 29, 181c	↓
Wang *et al.* (2008)	Cérebro	9, 20, 101, 107	↓
Boissonnealt *et al.* (2009)			
Delay *et al.* (2012)			
Kiko *et al.* (2014)	Líquor	29a, 29b	↑
		34a, 125b, 146	↓
Cogswell *et al.* (2008)	Líquor	15b, 181	↓
Schipper *et al.* (2007)	Células mononucleares	34, 181b	↓
Serpente *et al.* (2011)	Células mononucleares	369-3p	↓
Leidinger *et al.* (2013)	Sangue total	7d-3p, 26a-5p, 112, 151a-3p, 161, 1285-5p, 5010-3p	↑
		7f-5p, 26b-5p, 103a-3p, 107, 144-5p, 532-5p	↓
Sheinerman *et al.* (2012)	Plasma	132, 134	↑
Kumar *et al.* (2013)	Plasma	7d-5p, 7g-5p, 15b-5p, 142-3p, 191-5p, 301a-3p, 545-3p	↓
Kiko *et al.* (2014)	Plasma	34a, 146a	↓

Fonte: Elaborada pela autora.

1.5 Biomarcadores sanguíneos para DA

Biomarcadores ou marcadores biológicos podem ser utilizados como indicativos de processos biológicos como, por exemplo, monitoramento de respostas farmacológicas, detecção de patologias, entre outras. Através dos biomarcadores, busca-se uma resposta precoce ou um indicativo do estágio de algum processo biológico, normal ou patológico. Além disso, através dos biomarcadores espera-se obter algumas informações, tais como: (1) se um processo patogênico se iniciou; (2) qual o seu estágio e (3) se o organismo está respondendo de forma efetiva aos tratamentos empregados. Desta forma, os biomarcadores são de grande importância, não apenas para a detecção precoce de patologias, mas também para a monitoração de seu estágio e a eficiência de tratamentos (LESKO e ATKINSON, 2001).

Mesmo com o progresso da doença, o diagnóstico correto da DA ocorre somente com 65 a 90% de precisão, sendo que o diagnóstico definitivo somente pode ser realizado por autopsia (QIN, 2009). Neste sentido, há uma demanda urgente no desenvolvimento de biomarcadores para o diagnóstico da DA (TAKEUCHI, 2007).

Um biomarcador para DA deve idealmente ter algumas características. Ele deve detectar um marco fundamental da neuropatologia da doença, com resultados que podem ser validados em casos neurologicamente confirmados. Ainda, deve ter sensibilidade e especificidade maiores que 85% e 75% respectivamente, deve ser preciso, confiável, de baixo custo, conveniente e com baixo risco para os pacientes. Entretanto, até mesmo aqueles que preenchem parcialmente esses critérios ajudariam tanto para predizer diagnósticos de DA a partir de apresentações de transtorno neurocognitivo leve, como para monitorar a eficácia de terapias na modificação da doença (TANG e KUMAR, 2008).

A extensa literatura sobre candidatos a biomarcadores para a DA ilustra a constante busca de diferentes grupos de pesquisa ao redor do mundo, por moléculas que possam auxiliar no diagnóstico precoce e preciso da doença (DE BARRY *et al.*, 2010; TAKEDA *et al.*, 2010; TROJANOWSKI *et al.*, 2010). O desenvolvimento do estudo de biomarcadores para a DA pode ser obtido principalmente através do conhecimento sobre a fisiopatologia da doença (método dedutivo). Nos últimos anos, a abordagem dedutiva tem apresentado com sucesso vários marcadores moleculares para a DA, com destaque para os achados em tecidos periféricos como o sangue, dado seu reduzido custo e facilidade de acesso (EVIN *et al.*, 2003).

O dano à barreira hematoencefálica, um evento característico da DA, pode aumentar o movimento bidirecional de estruturas moleculares entre o cérebro e o sangue (HOLSINGER *et al.*, 2002). Uma vez que cerca de 500mL de líquor entram em contato com o plasma sanguíneo por dia, o sangue representa uma fonte rica para a prospecção de biomarcadores para a DA (HOLSINGER *et al.*, 2002).

Estudos anteriores mostram a redução dos níveis da ADAM10 em plaquetas na DA segundo subgrupos de CDR (*Clinical Dementia Rating*) em comparação com pacientes controles (COLCIAGHI *et al.*, 2002; MANZINE *et al.*, 2013c) e que esta redução se correlaciona com testes neuropsicológicos, como o MEEM (MANZINE *et al.*, 2013b) e Teste do Desenho do Relógio (TDR) (MANZINE *et al.*, 2013a). Além disso, alterações da APP, BACE1 e ADAM10 em plaquetas são observadas em estágios muito leves da doença (COLCIAGHI *et al.*, 2004). Recentemente o estudo de SCHUCK *et al.*, (2016) avaliou a expressão e atividade da ADAM10 plaquetária durante o envelhecimento normal, considerando faixas etárias de 22-85 anos em indivíduos cognitivamente saudáveis. Os achados mostraram que a expressão e atividade da ADAM10 aumentam com o avanço da idade, fortalecendo assim o papel da ADAM10 como um importante biomarcador sanguíneo para o diagnóstico da DA e sua importância no envelhecimento saudável.

Atualmente, avaliações da expressão gênica de moléculas relacionadas à fisiopatologia da DA em amostras de sangue periférico estão em destaque. A expressão anormal da APP, níveis alterados de enzimas antioxidantes, dano oxidativo ao DNA, RNA, e proteínas, secreção desregulada de citocinas e altas taxas de apoptose são características compartilhadas entre o cérebro e os linfócitos do sangue em pacientes com DA e dão ideia da complexidade da doença (SAYKIN *et al.*, 2010). As células sanguíneas periféricas na DA apresentam alterações em mais de 80% dos seus transcritos, sugerindo que estas células, portanto, podem ser utilizadas para análise do perfil molecular humano para auxílio no diagnóstico da doença (MAES *et al.*, 2007). Estudos da expressão gênica da ADAM10 são escassos na literatura e apresentam resultados apenas observáveis em amostras cerebrais (BANDYOPADHYAY *et al.*, 2006; DONMEZ *et al.*, 2010; MAO *et al.*; PRINZEN *et al.*, 2009). De posse destas evidências, e tendo em conta que a análise de materiais periféricos como o sangue é de fácil execução, o estudo da expressão gênica da ADAM10 em sangue circulante representa uma ferramenta clínica muito importante tanto para avaliar o início e a progressão de quadros demenciais, quanto para monitorar os efeitos de novas terapias baseadas na inibição da β e γ-secretases e/ou ativação das α-secretases (EVIN *et al.*, 2003).

Neste mesmo contexto, miRNAs que possam atuar como biomarcadores periféricos são uma alternativa viável, menos invasiva e confiável para auxiliar o diagnóstico da DA. A abordagem de miRNAs na DA encontra-se discrepante e controversa, de modo que o estudo de diferentes fontes periféricas menos invasivas (plasma, soro, células mononucleares, sangue total) ainda carece de metodologias e critérios de seleção amostral padronizados, de modo a reduzir a extensa variabilidade de resultados. O conhecimento do papel de marcadores específicos e as múltiplas vias que modulam a sua expressão podem proporcionar estratégias terapêuticas para alívio dos sintomas da DA. Além disso, o conhecimento sobre os mecanismos moleculares da DA, principalmente os relacionados com a cascata amiloide patogênica, pode oferecer múltiplos alvos potenciais para intervenções clínicas e diagnóstico precoce.

OBJETIVOS

2. OBJETIVOS

Este estudo teve como hipótese que a expressão gênica para ADAM10 e miRNAs em amostras de sangue encontram-se alteradas em idosos com DA em comparação a idosos sem alteração cognitiva.

Sendo assim, os objetivos gerais deste projeto foram:

1) Verificar e comparar a expressão gênica da ADAM10 em amostras de sangue entre sujeitos com TNCL, DA e sem alteração cognitiva;

2) Verificar e comparar a expressão gênica de miRNAs em amostras de sangue entre sujeitos com DA e sem alteração cognitiva;

3) Verificar se existe relação entre os níveis de mRNA para a ADAM10 e a expressão de miRNAs, visando fortalecer o papel da ADAM10 como molécula biomarcadora para a DA.

REFERÊNCIAS BIBLIOGRÁFICAS

3. REFERÊNCIAS BIBLIOGRÁFICAS

ALBERT, M. S.*, et al.* (2011). The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*, **7**, 270-279.

AMBROS, V. (2004). The functions of animal microRNAs. *Nature*, **431**, 350-355.

AMBROS, V. (2010). MicroRNAs: genetically sensitized worms reveal new secrets. *Curr Biol*, **20**, R598-600.

ANDERS, A., GILBERT, S., GARTEN, W., POSTINA, R. AND FAHRENHOLZ, F. (2001). Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. *Faseb J*, **15**, 1837-1839.

ANDERSSON, E. R., SANDBERG, R. AND LENDAHL, U. (2011). Notch signaling: simplicity in design, versatility in function. *Development*, **138**, 3593-3612.

AUGUSTIN, R.*, et al.* (2012). Computational identification and experimental validation of microRNAs binding to the Alzheimer-related gene ADAM10. *BMC Med Genet*, **13**, 35.

BALLARD, C., GAUTHIER, S., CORBETT, A., BRAYNE, C., AARSLAND, D. AND JONES, E. (2011). Alzheimer's disease. *Lancet*, **377**, 1019-1031.

BANDYOPADHYAY, S., HARTLEY, D. M., CAHILL, C. M., LAHIRI, D. K., CHATTOPADHYAY, N. AND ROGERS, J. T. (2006). Interleukin-1α stimulates non-amyloidogenic pathway by α-secretase (ADAM-10 and ADAM-17) cleavage of APP in human astrocytic cells involving p38 MAP kinase. *Journal of Neuroscience Research*, **84**, 106-118.

BARAO, S.*, et al.* (2013). BACE1 levels correlate with phospho-tau levels in human cerebrospinal fluid. *Current Alzheimer Research*, **10**, 671-678.

BEREZIKOV, E.*, et al.* (2006). Diversity of microRNAs in human and chimpanzee brain. *Nat Genet*, **38**, 1375-1377.

BLASKO, I. J., K.; KEMMLER, G. (2008). Conversion from cognitive health to mild cognitive impairment and Alzheimer's disease: Prediction by plasma amyloid beta 42, medial temporal lobe atrophy and homocysteine. *Neurobiology of aging*, **29**, 1-11.

BOISSONNEAULT, V., PLANTE, I., RIVEST, S. AND PROVOST, P. (2009). MicroRNA-298 and microRNA-328 regulate expression of mouse beta-amyloid precursor protein-converting enzyme 1. *J Biol Chem*, **284**, 1971-1981.

CHAIMOWICZ, F. (1997). [Health of Brazilian elderly just before of the 21st century: current problems, forecasts and alternatives]. *Rev Saude Publica*, **31**, 184-200.

CHANTRY, A., GREGSON, N. A. AND GLYNN, P. (1989). A novel metalloproteinase associated with brain myelin membranes. Isolation and characterization. *J Biol Chem*, **264**, 21603-21607.

COGSWELL, J. P.*, et al.* (2008). Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *J Alzheimers Dis*, **14**, 27-41.

COLCIAGHI, F.*, et al.* (2002). [alpha]-Secretase ADAM10 as well as [alpha]APPs is reduced in platelets and CSF of Alzheimer disease patients. *Mol Med*, **8**, 67-74.

COLCIAGHI, F.*, et al.* (2004). Platelet APP, ADAM 10 and BACE alterations in the early stages of Alzheimer disease. *Neurology*, **62**, 498-501.

COLE, S. L. AND VASSAR, R. (2007). The Alzheimer's disease beta-secretase enzyme, BACE1. *Mol Neurodegener*, **2**, 22.

DE BARRY, J., LIEGEOIS, C. M. AND JANOSHAZI, A. (2010). Protein kinase C as a peripheral biomarker for Alzheimer's disease. *Exp Gerontol*, **45**, 64-69.

DE SMAELE, E., FERRETTI, E. AND GULINO, A. (2010). MicroRNAs as biomarkers for CNS cancer and other disorders. *Brain Research*, **1338**, 100-111.

DELAY, C., MANDEMAKERS, W. AND HEBERT, S. S. (2012). MicroRNAs in Alzheimer's disease. *Neurobiology of Disease*, **46**, 285-290.

DENG, W., CHO, S., SU, P. C., BERGER, B. W. AND LI, R. (2014). Membrane-enabled dimerization of the intrinsically disordered cytoplasmic domain of ADAM10. *Proc Natl Acad Sci U S A*, **111**, 15987-15992.

DI LUCA, M., COLCIAGHI, F., PASTORINO, L., BORRONI, B., PADOVANI, A. AND CATTABENI, F. (2000). Platelets as a peripheral district where to study pathogenetic mechanisms of Alzheimer disease: the case of amyloid precursor protein. *European Journal of Pharmacology*, **405**, 277-283.

DONMEZ, G., WANG, D., COHEN, D. E. AND GUARENTE, L. (2010). SIRT1 Suppresses ²-Amyloid Production by Activating the ±-Secretase Gene ADAM10. *Cell*, **142**, 320-332.

DSM-V (2013). Diagnostic and statistical manual of mental disorders. 5th ed. *American Psychiatric Association*

EDISON, P.*, et al.* (2007). Amyloid, hypometabolism, and cognition in Alzheimer disease: an [11C]PIB and [18F]FDG PET study. *Neurology*, **68**, 501-508.

ENDRES, K.*, et al.* (2014). Increased CSF APPs-alpha levels in patients with Alzheimer disease treated with acitretin. *Neurology*, **83**, 1930-1935.

ENDRES, K., POSTINA, R., SCHROEDER, A., MUELLER, U. AND FAHRENHOLZ, F. (2005). Shedding of the amyloid precursor protein-like protein APLP2 by disintegrin-metalloproteinases. *Febs J*, **272**, 5808-5820.

ESQUELA-KERSCHER, A. AND SLACK, F. J. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, **6**, 259-269.

EVIN, G., ZHU, A., HOLSINGER, R. M. D., MASTERS, C. L. AND LI, Q.-X. (2003). Proteolytic processing of the Alzheimer's disease amyloid precursor protein in brain and platelets. *Journal of Neuroscience Research*, **74**, 386-392.

FAHRENHOLZ, F. (2010). The close link between retinoid signalling and the α-secretase ADAM10 and its potential for treating Alzheimer's disease (Commentary on Jarvis et al.). *European Journal of Neuroscience*, **32**, 1245-1245.

FOX, J. W. AND SERRANO, S. M. (2005). Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon*, **45**, 969-985.

GHOSAL, K., VOGT, D. L., LIANG, M., SHEN, Y., LAMB, B. T. AND PIMPLIKAR, S. W. (2009). Alzheimer's disease-like pathological features in transgenic mice expressing the APP intracellular domain. *Proc Natl Acad Sci U S A*, **106**, 18367-18372.

GRAFF-RADFORD, N. R. C., J.E.; LUCAS, et al. (2007). Association of low plasma Aβ42/Aβ40 ratios with increased imminent risk for mild cognitive impairment and Alzheimer disease. *Arch. Neurol*, **64**.

GUTWEIN, P.*, et al.* (2003). ADAM10-mediated cleavage of L1 adhesion molecule at the cell surface and in released membrane vesicles. *Faseb J*, **17**, 292-294.

HERRERA, E. C., P.; NITRINI, R. (1998). Estudo epidemiológico populacional de demência na cidade de Catanduva, Estado de São Paulo, Brasil. . *Rev. Psiquatr. Clin*, **25**, 70-73.

HOLSINGER, R. M., MCLEAN, C. A., BEYREUTHER, K., MASTERS, C. L. AND EVIN, G. (2002). Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann Neurol*, **51**, 783-786.

HOOPER, N. M. AND TURNER, A. J. (2002). The search for alpha-secretase and its potential as a therapeutic approach to Alzheimer s disease. *Curr Med Chem*, **9**, 1107-1119.

HOWARD, L., LU, X., MITCHELL, S., GRIFFITHS, S. AND GLYNN, P. (1996). Molecular cloning of MADM: a catalytically active mammalian disintegrin-metalloprotease expressed in various cell types. *Biochemical Journal*, **317**, 45-50.

HWANG, E. M.*, et al.* (2006). Furin is an endogenous regulator of alpha-secretase associated APP processing. *Biochem Biophys Res Commun*, **349**, 654-659.

IBGE (2010). Estimativa da população idosa brasileira.

JALBERT, J. J., DAIELLO, L. A. AND LAPANE, K. L. (2008). Dementia of the Alzheimer type. *Epidemiol rev*, **30**, 15-34.

JORISSEN, E.*, et al.* (2010). The Disintegrin/Metalloproteinase ADAM10 Is Essential for the Establishment of the Brain Cortex. *Journal of Neuroscience*, **30**, 4833-4844.

KARKKAINEN, I., RYBNIKOVA, E., PELTO-HUIKKO, M. AND HUOVILA, A. P. (2000). Metalloprotease-disintegrin (ADAM) genes are widely and differentially expressed in the adult CNS. *Mol Cell Neurosci*, **15**, 547-560.

KELLER, A.*, et al.* (2011). Toward the blood-borne miRNome of human diseases. *Nat Meth*, **8**, 841-843.

KIKO, T., NAKAGAWA, K., TSUDUKI, T., FURUKAWA, K., ARAI, H. AND MIYAZAWA, T. (2014). MicroRNAs in plasma and cerebrospinal fluid as potential markers for Alzheimer's disease. *J Alzheimers Dis*, **39**, 253-259.

KIM, M.*, et al.* (2009). Potential late-onset Alzheimer's disease-associated mutations in the ADAM10 gene attenuate {alpha}-secretase activity. *Hum Mol Genet*, **18**, 3987-3996.

KOJRO, E., GIMPL, G., LAMMICH, S., MÄRZ, W. AND FAHRENHOLZ, F. (2001). Low cholesterol stimulates the nonamyloidogenic pathway by its effect on the α-secretase ADAM 10. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 5815-5820.

KROL, J., LOEDIGE, I. AND FILIPOWICZ, W. (2010). The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*, **11**, 597-610.

KUHN, P. H.*, et al.* (2010). ADAM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons. *Embo Journal*, **29**, 3020-3032.

KUHN, P.H., COLOMBO, A.V., SCHUSSER, B. et al. (2016). Systematic substrate identification indicates a central role for the metalloprotease ADAM10 in axon targeting and synapse function. *eLife,* **23**, 10.7554/eLife.12748.

KUMAR, P.*, et al.* (2013). Circulating miRNA biomarkers for Alzheimer's disease. *PLoS One*, **8**.

LAMMICH, S.*, et al.* (2010). Expression of the anti-amyloidogenic secretase ADAM10 is suppressed by its 5'-untranslated region. *J Biol Chem*, **285**, 15753-15760.

LAMMICH, S.*, et al.* (1999). Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci U S A*, **96**, 3922-3927.

LEIDINGER, P.*, et al.* (2013). A blood based 12-miRNA signature of Alzheimer disease patients. *Genome Biol*, **14**, 2013-2014.

LESKO, L. J. AND ATKINSON, A. J., JR. (2001). Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu Rev Pharmacol Toxicol*, **41**, 347-366.

LI, Y.*, et al.* (2008). Regional analysis of FDG and PIB-PET images in normal aging, mild cognitive impairment, and Alzheimer's disease. *European Journal of Nuclear Medicine and Molecular Imaging*, **35**, 2169-2181.

LICHTENTHALER, S. F. (2011). Alpha-secretase in Alzheimer's disease: molecular identity, regulation and therapeutic potential. *Journal of Neurochemistry*, **116**, 10-21.

LIN, J., LUO, J. AND REDIES, C. (2008). Differential expression of five members of the ADAM family in the developing chicken brain. *Neuroscience*, **157**, 360-375.

LOPEZ-PEREZ, E., ZHANG, Y., FRANK, S. J., CREEMERS, J., SEIDAH, N. AND CHECLER, F. (2001). Constitutive α-secretase cleavage of the β-amyloid precursor protein in the furin-deficient LoVo cell line: involvement of the pro-hormone convertase 7 and the disintegrin metalloprotease ADAM10. *Journal of Neurochemistry*, **76**, 1532-1539.

LUKIW, W. J. (2007). Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport*, **18**, 297-300.

LUNN, C. A.*, et al.* (1997). Purification of ADAM 10 from bovine spleen as a TNFalpha convertase. *FEBS Lett*, **400**, 333-335.

MAES, O. C., XU, S., YU, B., CHERTKOW, H. M., WANG, E. AND SCHIPPER, H. M. (2007). Transcriptional profiling of Alzheimer blood mononuclear cells by microarray. *Neurobiology of aging*, **28**, 1795-1809.

MANZINE, P. R., BARHAM, E. J., VALE, F. A., SELISTRE-DE-ARAUJO, H. S., PAVARINI, S. C. AND COMINETTI, M. R. (2013a). Platelet a disintegrin and metallopeptidase 10 expression correlates with clock drawing test scores in Alzheimer's disease. *Int J Geriatr Psychiatry*, **22**, 414-420.

MANZINE, P. R., BARHAM, E. J., VALE, F. A. C., SELISTRE-DE-ARAUJO, H. S., IOST PAVARINI, S. C. AND COMINETTI, M. R. (2013b). Correlation Between Mini-Mental State Examination and Platelet ADAM10 Expression in Alzheimer's Disease. *J Alzheimers Dis*, **36**, 253-260.

MANZINE, P. R.*, et al.* (2013c). ADAM10 as a biomarker for Alzheimer's disease: a study with Brazilian elderly. *Dement Geriatr Cogn Disord*, **35**, 58-66.

MAO, X.*, et al.* The effects of chronic copper exposure on the amyloid protein metabolisim associated genes' expression in chronic cerebral hypoperfused rats. *Neuroscience Letters*.

MARCELLO, E., *et al.* (2013). Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. *The Journal of Clinical Investigation*, **123**, 2523-2538.

MARCELLO, E.*, et al.* (2012). SAP97-mediated local trafficking is altered in Alzheimer disease patients' hippocampus. *Neurobiol Aging*, **33**, 27.

MARCELLO, E., GARDONI, F., DI LUCA, M. AND PEREZ-OTANO, I. (2010). An arginine stretch limits ADAM10 exit from the endoplasmic reticulum. *J Biol Chem*, **285**, 10376-10384.

MARCELLO, E., *et al.* (2007). Synapse-associated protein-97 mediates alpha-secretase ADAM10 trafficking and promotes its activity. *J Neurosci*, **27**, 1682-1691.

MARCINKIEWICZ, M. AND SEIDAH, N. G. (2000). Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. *J Neurochem*, **75**, 2133-2143.

MEGURO, K., CHUBACI, R.Y.S., MEGURO, M., KAWAMORIDA, K., GOTO, N., CARAMELLI, P. (2011). Incidence of dementia and cause of death in elderly Japanese emigrants to Brazil before World War II. *Arch Gerontol Geriatr*, **52**, 75-78.

MEGURO, K., MEGURO, M., CARAMELLI, P., ISHIZAKI, J., AMBO, H., CHUBACI, R.Y., HAMADA, G.S., NITRINI, R., YAMADORI, A. (2001). Elderly Japanese emigrants to Brazil before World War II: II. Prevalence of senile dementia. *Int J Geriatr Psychiatry*, **16**, 775-779.

MORISHIMA-KAWASHIMA, M. AND IHARA, Y. (2002). Alzheimer's disease: β-Amyloid protein and tau. *Journal of Neuroscience Research*, **70**, 392-401.

MOSS, M. L.*, et al.* (2011). ADAM9 inhibition increases membrane activity of ADAM10 and controls alpha-secretase processing of amyloid precursor protein. *J Biol Chem*, **286**, 40443-40451.

NIKOLAEV, A., MCLAUGHLIN, T., O'LEARY, D. D. AND TESSIER-LAVIGNE, M. (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. *Nature*, **457**, 981-989.

NITRINI, R.*, et al.* (2009). Prevalence of dementia in Latin America: a collaborative study of population-based cohorts. *Int Psychogeriatr*, **21**, 622-630.

PAPADOPOULOS, G. L., ALEXIOU, P., MARAGKAKIS, M., RECZKO, M. AND HATZIGEORGIOU, A. G. (2009). DIANA-mirPath: Integrating human and mouse microRNAs in pathways. *Bioinformatics*, **25**, 1991-1993.

PASCIUTO, E.*, et al.* (2015). Dysregulated ADAM10-Mediated Processing of APP during a Critical Time Window Leads to Synaptic Deficits in Fragile X Syndrome. *Neuron*, **87**, 382-398.

PETERSEN, R. C., SMITH, G. E., WARING, S. C., et al. (1999). Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol*, **56**, 303-308.

PETERSEN, R. C., STEVENS, J. C., GANGULI, M., TANGALOS, E. G., CUMMINGS, J. L. AND DEKOSKY, S. T. (2001). 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*, **56**, 1133-1142.

PIMPLIKAR, S. W., NIXON, R. A., ROBAKIS, N. K., SHEN, J. AND TSAI, L. H. (2010). Amyloid-independent mechanisms in Alzheimer's disease pathogenesis. *J Neurosci*, **30**, 14946-14954.

PLASSMAN, B. L.*, et al.* (2008). Prevalence of cognitive impairment without dementia in the United States. *Ann Intern Med*, **148**, 427-434.

PRINZEN, C., TRUMBACH, D., WURST, W., et al. (2009). Differential gene expression in ADAM10 and mutant ADAM10 transgenic mice. *BMC Genomics*, **10**, 66.

QIN, W., et al. (2009). S100A7, a novel Alzheimer's disease biomarker with non-amyloidogenic alpha-secretase activity acts via selective promotion of ADAM-10. *PLoS One*, **4**.

REISS, K., CORNELSEN, I., HUSMANN, M., GIMPL, G. AND BHAKDI, S. (2011). Unsaturated fatty acids drive disintegrin and metalloproteinase (ADAM)-dependent cell adhesion, proliferation, and migration by modulating membrane fluidity. *J Biol Chem*, **286**, 26931-26942.

REISS, K. AND SAFTIG, P. (2009). The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. *Semin Cell Dev Biol*, **20**, 126-137.

SAFTIG, P. AND LICHTENTHALER, S. F. (2015). The alpha secretase ADAM10: A metalloprotease with multiple functions in the brain. *Prog Neurobiol*, **29**, 30046-30040.

SATOH, J. (2012). Molecular network of microRNA targets in Alzheimer's disease brains. *Exp Neurol*, **235**, 436-446.

SAYKIN, A. J.*, et al.* (2010). Alzheimer's Disease Neuroimaging Initiative biomarkers as quantitative phenotypes: Genetics core aims, progress, and plans. *Alzheimer's and Dementia*, **6**, 265-273.

SCHEFF, S. W. AND PRICE, D. A. (2003). Synaptic pathology in Alzheimer's disease: a review of ultrastructural studies. *Neurobiol Aging*, **24**, 1029-1046.

SCHIPPER, H. M., MAES, O. C., CHERTKOW, H. M. AND WANG, E. (2007). MicroRNA expression in Alzheimer blood mononuclear cells. *Gene Regul Syst Bio*, **1**, 263-274.

SCHONROCK, N., HUMPHREYS, D. T., PREISS, T. AND GOTZ, J. (2011). Target Gene Repression Mediated by miRNAs miR-181c and miR-9 Both of Which Are Down-regulated by Amyloid-beta. *J Mol Neurosci*.

SCHONROCK, N.*, et al.* (2010). Neuronal microRNA deregulation in response to Alzheimer's disease amyloid-beta. *PLoS ONE*, **5**, e11070.

SCHONROCK, N., MATAMALES, M., ITTNER, L. M. AND GÖTZ, J. (2012). MicroRNA networks surrounding APP and amyloid-β metabolism — Implications for Alzheimer's disease. *Experimental Neurology*, **235**, 447-454.

SCHUCK, F., WOLF, D., FELLGIEBEL, A. AND ENDRES, K. (2016). Increase of alpha-Secretase ADAM10 in Platelets Along Cognitively Healthy Aging. *J Alzheimers Dis*, **6**, 6.

SEALS, D. F. AND COURTNEIDGE, S. A. (2003). The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Genes Dev*, **17**, 7-30.

SERPENTE, M.*, et al.* (2011). Role of OLR1 and Its Regulating hsa-miR369-3p in Alzheimer's Disease: Genetics and Expression Analysis. *Journal of Alzheimer's Disease*, **26**, 787-793.

SHEINERMAN, K. S., TSIVINSKY, V. G., CRAWFORD, F., MULLAN, M. J., ABDULLAH, L. AND UMANSKY, S. R. (2012). Plasma microRNA biomarkers for detection of mild cognitive impairment. *Aging*, **4**, 590-605.

SHUKLA, M.*, et al.* (2015). Melatonin stimulates the nonamyloidogenic processing of betaAPP through the positive transcriptional regulation of ADAM10 and ADAM17. *J Pineal Res*, **58**, 151-165.

SMITH-VIKOS, T. AND SLACK, F. J. (2013). MicroRNAs circulate around Alzheimer's disease. *Genome Biol*, **14**.

TAKEDA, S., SATO, N., RAKUGI, H. AND MORISHITA, R. (2010). Plasma [small beta]-amyloid as potential biomarker of Alzheimer disease: possibility of diagnostic tool for Alzheimer disease. *Molecular BioSystems*, **6**.

TAKEUCHI, M., et al. (2007). Diagnostic utility of serum or cerebrospinal fluid levels of toxic advanced glycation end-products (TAGE) in early detection of Alzheimer's disease. *Med. Hypotheses*, **69**.

TANG, B. L. AND KUMAR, R. (2008). Biomarkers of mild cognitive impairment and Alzheimer's disease. *Ann Acad Med Singapore*, **37**, 406-410.

THEENDAKARA, V.*, et al.* (2013). Neuroprotective Sirtuin ratio reversed by ApoE4. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 18303-18308.

TIPPMANN, F., HUNDT, J., SCHNEIDER, A., ENDRES, K. AND FAHRENHOLZ, F. (2009). Up-regulation of the alpha-secretase ADAM10 by retinoic acid receptors and acitretin. *Faseb J*, **23**, 1643-1654.

TROJANOWSKI, J. Q.*, et al.* (2010). Update on the biomarker core of the Alzheimer's Disease Neuroimaging Initiative subjects. *Alzheimers Dement*, **6**, 230-238.

VAN TETERING, G., VAN DIEST, P., VERLAAN, I., VAN DER WALL, E., KOPAN, R. AND VOOIJS, M. (2009). Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. *J Biol Chem*, **284**, 31018-31027.

VAN WART, H. E. AND BIRKEDAL-HANSEN, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 5578-5582.

VILARDO, E., BARBATO, C., CIOTTI, M., COGONI, C. AND RUBERTI, F. (2010). MicroRNA-101 regulates amyloid precursor protein expression in hippocampal neurons. *J Biol Chem*, **285**, 18344-18351.

WANG, Y., LIANG, Y. AND LU, Q. (2008). MicroRNA epigenetic alterations: predicting biomarkers and therapeutic targets in human diseases. *Clin Genet*, 74, 307-315.

WEBER, S. AND SAFTIG, P. (2012). Ectodomain shedding and ADAMs in development. *Development*, **139**, 3693-3709.

WONG, E., MARETZKY, T., PELEG, Y., BLOBEL, C. P. AND SAGI, I. (2015). The Functional Maturation of A Disintegrin and Metalloproteinase (ADAM) 9, 10, and 17 Requires Processing at a Newly Identified Proprotein Convertase (PC) Cleavage Site. *J Biol Chem*, **290**, 12135-12146.

YAMAZAKI, K., MIZUI, Y. AND TANAKA, I. (1997). Radiation hybrid mapping of human ADAM10 gene to chromosome 15. *Genomics*, **45**, 457-459.

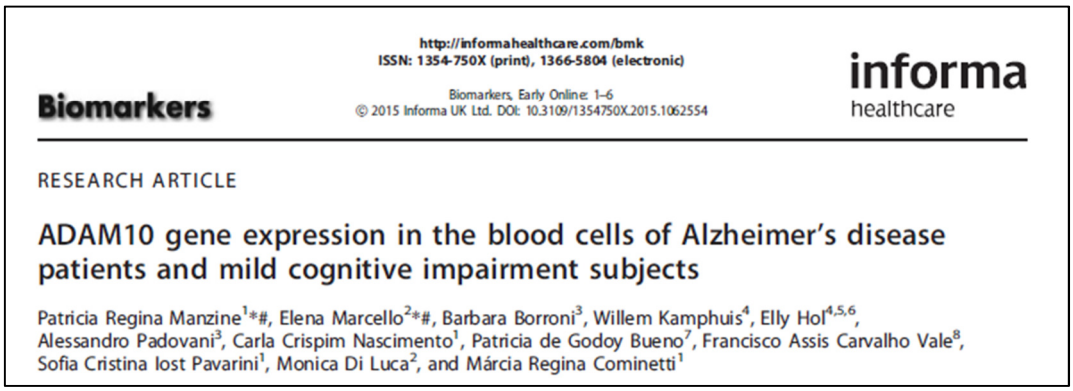
ZHANG, Y. W., THOMPSON, R., ZHANG, H. AND XU, H. (2011). APP processing in Alzheimer's disease. *Mol Brain*, **4**, 3.

ZHONG, Z*., et al.* (2007). Levels of beta-secretase (BACE1) in cerebrospinal fluid as a predictor of risk in mild cognitive impairment. *Arch Gen Psychiatry*, **64**, 718-726.

MANUSCRITOS

4. MANUSCRITOS

4.1 MANUSCRITO I – Publicado


http://informahealthcare.com/bmk
ISSN: 1354-750X (print), 1366-5804 (electronic)
Biomarkers
Biomarkers, Early Online: 1–6
© 2015 Informa UK Ltd. DOI: 10.3109/1354750X.2015.1062554
informa healthcare
RESEARCH ARTICLE

ADAM10 gene expression in the blood cells of Alzheimer's disease patients and mild cognitive impairment subjects

Patricia Regina Manzine[1]*#, Elena Marcello[2]*#, Barbara Borroni[3], Willem Kamphuis[4], Elly Hol[4,5,6], Alessandro Padovani[3], Carla Crispim Nascimento[1], Patricia de Godoy Bueno[7], Francisco Assis Carvalho Vale[8], Sofia Cristina Iost Pavarini[1], Monica Di Luca[2], and Márcia Regina Cominetti[1]

[a]Department of Gerontolgy, [g]Department of Physiological Sciences, [h]Department of Medicine, Federal University of São Carlos, São Carlos, SP, Brazil

[b]Department of Pharmacological and Biomolecular Sciences, University of Studies of Milan, Milan, Italy

[c]Department of Clinical and Experimental Science, Neurology Unit, University of Brescia, Brescia, Italy

[d]Netherlands Institute for Neuroscience - an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, The Netherlands

[e]Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, The Netherlands

[f]Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, The Netherlands

*** These authors equally contributed to this work**

Corresponding Author: Márcia Regina Cominetti, Departamento de Gerontologia, Universidade Federal de São Carlos, Rodovia Washington Luís, Km 235, São Carlos, SP, 13565-905, Brazil, phone: +55 16 3306 6663, fax +55 16 3351 9628. E-mail: mcominetti@ufscar.br

Keywords: aging; biomarkers; blood; platelets

Abstract

ADAM10 is a potential biomarker for Alzheimer´s disease (AD). ADAM10 protein levels are reduced in platelets of AD patients. The aim was to verify the total blood and platelet ADAM10 gene expression in AD patients and to compare with mild cognitive impartment (MCI) and healthy subjects. No significant differences in ADAM10 gene expression were observed. Therefore, the decrease of ADAM10 protein in platelets of AD patients is not caused by a reduction in ADAM10 mRNA. Further studies must be performed to investigate other pathways in the down regulation of ADAM10 protein.

1. Introduction

The pathological features in Alzheimer's disease (AD) brain include cortical atrophy, predominantly in the medial temporal lobe and, microscopically, extensive neuronal loss and abnormal extra and intracellular fibrillar deposits, termed senile plaques and neurofibrillary tangles, respectively (Hohsfield and Humpel, 2015). The senile plaques appear from excessive deposition and subsequent aggregation of β-amyloid peptide (Aβ) in the brain. AD pathogenesis is multifaceted and difficult to pinpoint, however genetic and cell biology studies led to the amyloid hypothesis, which posits that Aβ plays a pivotal role in AD pathogenesis (Hardy and Selkoe, 2002). Aβ derives from the concerted action of BACE1 (β-secretase) and the γ-secretase complex on the Amyloid Precursor Protein (APP) (Lichtenthaler, 2011). In healthy subjects the predominant route of APP processing consists of successive cleavages by α and γ-secretases. In the non-amyloidogenic pathway, APP is cleaved by α-secretases between lysine16 and leucine17 in the middle of Aβ region, thus releasing sAPPα - a structure with neurotrophic and neuroprotective functions (Meziane et al., 1998, Stein et al., 2004) retaining the C83 residue in the membrane. The following cleavage of C83 by γ-secretase releases the p3 - which is supposed to be beneficial, and is not found in amyloid plaques - and starts at position Aβ17 (Aβ17-40 and Aβ17-42), thereby inhibiting amyloidogenic Aβ production (Morishima-Kawashima and Ihara, 2002).

Several enzymes in the ''a disintegrin and metalloprotease'' (ADAM) family, including ADAM9, ADAM10, and ADAM17, have α-secretase activity in vitro, although recent studies have demonstrated that ADAM10 is the major α-secretase that catalyzes APP ectodomain shedding in the brain (Kuhn et al., 2010, Jorissen et al., 2010). Moreover, it has been demonstrated that ADAM10 is a susceptibility gene of late onset AD (LOAD), the most common form of the disease. Indeed, two rare highly penetrant nonsynonymous mutations associated with LOAD have been identified in ADAM10 prodomain (Kim et al., 2009).

In previous studies we have reported a marked reduction in ADAM10 platelet protein levels in CDR (Clinical Dementia Rating) subgroups compared to non-AD patients (Manzine et al., 2013, Colciaghi et al., 2002) and APP, BACE1 and ADAM10 alterations in platelets already in the very early stages of the disease in which dementia can be barely inferred by neuropsychological assessments (Colciaghi et al., 2004). Since ADAM10 is the most important α-secretase involved in cleavage of APP, in this work we raise the following question: is ADAM10 protein reduction in blood of AD patients a consequence of a decrease in ADAM10 transcription? In order to answer this question, we assessed the expression of

ADAM10 mRNA in total blood and in platelets of a selected population of AD, MCI and control subjects, using Reverse Transcription quantitative PCR (RT-qPCR).

2. Methods

2.1 Characteristics of the Subjects

Patients were recruited in reference (Public Center of Specialties) and counter-reference (Family Health Units) health services in Brazil and at the Department of Medical Sciences-Neurology (University of Brescia, School of Medicine - Italy). Subjects recruited for AD group were diagnosed with probable AD according to National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCS-ADRDA) criteria. For MCI group, subjects had CDR 0.5, MoCA (range 19-25), Pfeffer test without impairment and follow the Petersen criteria (Petersen, 2004). All participants underwent the exclusion criteria for head trauma, metabolic dysfunctions, haematological diseases, alcohol abuse, drug abuse, delirium, mood disorders, and treatment with medications affecting platelet functions, i.e., anticoagulants, antiplatelet drugs, serotoninergic agonists-antagonists, and corticosteroids. All subjects included had a standardised clinical workup based on neurological examinations, laboratory blood and urine analysis, a neuroimaging study (Head Computed Tomography and/or Magnetic Resonance Imaging), and a neuropsychological assessment, including a Mini Mental State Examination (MMSE) and a CDR. Before enrolment, subjects or their legal caregivers filled out an informed consent, after the nature and possible consequences of the study were explained. The research project was approved by Brazil Platform (CAAE: 02760312.0.0000.5504/ N°: 112.543).

2.2 Isolation of RNA from total blood

Personnel carrying out platelet and blood preparation, as well as subsequent analysis, were blind for diagnosis and treatment of subjects. Blood drawings were always taken in the morning, fasted and without tourniquet. For the isolation of total RNA, 2.5 ml of blood was collected in total RNA extraction tubes (PAXgene Blood RNA - Becton & Dickinson), according to the product manual. After collection, tubes were inverted 10 times and kept for two hours at room temperature in an upright position as manufacturer's instructions and were subsequently frozen at -80º C until use. Total RNA isolation from blood samples was performed using PAXgene Blood RNA isolation kit (Qiagen) according to the manufacture's manual. From the extracted total RNA, samples (1µl) were quantified with a

Nanodrop (Thermo Scientific) to obtain absorbance values and their ratios (A260/A280 and A260/A230).

2.3 Isolation of RNA from platelets

For the isolation of total RNA from platelets, 27 ml of blood was collected into 1 vol. 3.8% sodium citrate (in the presence of 136 mM glucose), mixed gently, and centrifuged at 200 × g for 10 min. The time interval between blood drawing and the first centrifugation was never longer than 20-25 min. Platelet rich plasma (PRP) was separated from blood cells using a plastic pipette, carefully avoiding the drawing in of the buffy coat. Subsequently, platelets were collected by centrifugation at 500 × g for 20 min. Platelet pellets were washed twice with Tris-HCl 10 mM pH 7.4 and total RNA was isolated using Trizol reagent, following the manufacturer's instructions (Invitrogen). Isolated RNA was dissolved in 12µl diethylpyrocarbonate (DEPC)-treated water. All RNA samples presented sharp ribosomal RNA bands with no sign of degradation (Agilent Technologies, 2100 Bioanalyser).

2.4 Reverse transcription

For the reverse transcription from total blood, cDNA was prepared using Enhanced Avian RT First Strand Synthesis (Sigma-Aldrich) kit according to the manufacturer's manual with the proportion of 700 ng of RNA/µL. cDNA dilutions series were performed in order to find the best reaction efficiency for each primer. RNA from platelets, 400 ng in 4 µl water, was DNase I treated (0.5 U DNAseI, Amplification Grade, Invitrogen), reverse transcribed into first strand cDNA with 100 U/µl of Superscript III (Invitrogen) and 50 ng random hexamer primers, during 50 min at 50 ºC. To the resulting cDNA sample, 15 µl of 10 mM Tris–1 mM EDTA was added, bringing the final volume to a total of 35 µl. From all samples a 1:20 dilution was made and used for RT-qPCR analysis.

2.5 Real-time quantitative PCR

RT-qPCR primer sequences were designed using PrimerExpress V 2.0 software (PE Applied Biosystems, Warrington, UK) and NetPrimer (Premier Biosoft). Specificity of the primers was confirmed by BLAST searching. The length of the resulting amplicons was verified by agarose gel electrophoresis.

For RT-qPCR from total blood, samples were amplified in a thermocycler (RotorGene RG6000 - Corbett Life Sciences) with SYBR Green Jump Start (Sigma-Aldrich), using specific primers for ADAM10 designed to amplify exons 9-10 in total blood

(ADAM109-10T) and the endogenous controls β-actin and GAPDH (Table 1), the two best reference genes tested. The conditions for the annealing temperature were optimized, and analyses of PCR products were performed on agarose (2%) ethidium bromide gel. PCR conditions were as follows: 10.0µl DEPC water, 12.5µl SYBR Green, 0.5µl pure cDNA, 1.0µl Primers Forward/Reverse [10nM], ADAM10 Tm 66° C; β-actin and GAPDH Tm 69° C. The RT-qPCR reactions used were standardized with a final volume of 25µl. Cycling conditions were: Hold 94ºC, 2min; Cycling (40 repeats) – Step 1:94ºC, 15 sec and Step 2: Tm (x)ºC, 1 min; Melt 72-95ºC, 45 sec on the 1st step.

The melting curves showed a single amplified product and the absence of primer–dimer formation. Non-template controls were included for each primer pair reactions. The amplification efficiency (E) was determined on a cDNA dilution series on threshold 0.05. ADAM10: E = 0.93, M = -3.49, R = 0.99; β-actin: E = 0.94, M = -3.47, R = 0.99 and GAPDH: E = 0.90, M = -3.55, R = 0.99. The internal calibrator used as a basis to standardize the results of expression was the control group ΔCts average. Calibration was determined by ΔΔCt = ΔCt (sample) - ΔCt (calibrator). Gene expression was assessed by relative quantification, using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

For RT-qPCR analysis from platelets RNA, transcript levels were derived from the accumulation of DNA concentration-dependent SYBR green fluorescence in an ABI Prism 5700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: SYBR Green PCR buffer, 3 mM MgCl2, 200 mM dATP, dGTP, dCTP, and 400 mM dUTP, 0.5 U AmpliTaq Gold, 2 pmol primers, and 2 µl of the 1:20 dilution of the cDNA in a total volume of 10 µl. Cycling conditions were: Hold 95ºC, 10min; Cycling (40 repeats) – Step 1:95ºC, 15 sec and Step 2: Tm (x)ºC, 1 min; Melt 72-95ºC, 45 sec on the 1st step.

ADAM10 platelet transcript levels were assessed by RT-qPCR with two different sets of primers: $ADAM10_{9\text{-}10P}$ and $ADAM10_{11\text{-}12P}$ recognizing sequences in exons 9-10 and 11-12, respectively. The melting curve analysis showed a single amplified product and the absence of primer–dimer formation. Non-template controls were included for each primer pair to check for any significant levels of contaminants. Primers designed for ADAM10 and β-actin intronic sequences ($ADAM10_{intron}$ and $\beta\text{-actin}_{intron}$ primers) were used for each sample to check genomic DNA contamination. These samples always resulted in at least in a difference of 8 cycles of the cycle threshold (Ct) values compared to template containing samples. All samples were analyzed together on a single 96-well plate.

All cDNA synthesis reactions were performed on 400 ng total RNA and it may be expected that the cDNA input in the qPCR is not different among the groups and that in

fact normalization for the amount of cDNA would not be required. This assumption was tested with a selection of reference genes. The remaining candidate reference genes were subjected to the geNorm-assisted analysis to select the most optimal set of reference genes (Vandesompele et al., 2002). For the normalization the formula fluorescence sample/fluorescence set of reference genes (geometric mean) was performed (Tricarico et al., 2002).

2.6 Data analysis and statistical evaluation

Statistical evaluations were performed according to tests of comparison (Mann-Whitney U-test and Kruskal-Wallis). The influence of gender, age and MMSE score on ADAM10 gene expression was analyzed through multiple regression, robust to heteroskedasticity, using Stata 12 software. The data were presented in figures and/or tables using GraphPad Prism 5 software.

3. Results

In order to investigate whether there is a variation in ADAM10 mRNA in the total blood, RNA was isolated from 32 healthy subjects (control), 21 MCI and 47 AD patients recruited in reference (Public Center of Specialties) and counter-reference (Family Health Units) health services in Brazil (Table 2), which were age, sex and education level-matched, according to the CDR. The influence of gender, age and MMSE score on ADAM10 gene expression was analyzed. Results demonstrated that gender, age and MMSE score were not statistically significant, and the adjustment coefficient R2 obtained was 0.0763 with $p>0.05$, demonstrating that the mentioned variables do not influence the dependent variable (ADAM10 mRNA). Figure 1A presents the findings of ADAM10 mRNA expression in control (1.06±0.36), MCI (1.18±0.42) and AD patients (0.97±0.37). No significant differences between groups were observed ($p>0.05$). Fluorescence, melting curves and agarose (2%) gels from total blood ADAM10, β-actin and GAPDH genes are presented in Supplementary Figure 1.

Analysis by CDR is presented in Figure 1B, which shows the results of ADAM10 mRNA expression between control (1.06±0.36), MCI (1.18±0.42) and AD subjects according to the CDR (CDR1, n=24, 1.00±0.29; CDR2, n=14, 0.98±0.44; CDR3, n=9, 0.85±0.445). There was no significant difference between control subjects and AD for CDRs 1, 2 and 3 ($p>0.05$).

Since we found no significant difference of ADAM10 mRNA levels in total blood of healthy controls compared to AD patients or MCI subjects, we decided to investigate whether ADAM10 expression would be altered specifically in platelets. For that, RNA was isolated from platelets obtained from 19 control subjects, 21 AD patients and 16 patients with MCI recruited at the Department of Medical Sciences-Neurology (University of Brescia, School of Medicine - Italy) (Table 2). Subjects included in these three groups were age and sex-matched. The internal gene stability measure M of analysed housekeeping genes and the pairwise variation analysis to determine the number of control genes required for accurate normalization is presented in Supplementary Figure 2. There was no significant statistical difference ($p>0.05$) in platelet gene expression between groups for both set of primers for all the analyses (Figure 2AB).

4. Discussion

Previous studies have demonstrated reduced ADAM10 protein levels in platelets from AD patients compared to healthy controls (Colciaghi et al., 2004), and this reduction was related to the advance of the dementia (Manzine et al., 2013). ADAM10 protein levels were found to be significantly reduced in platelets of sporadic AD patients (Manzine et al., 2013) and sAPPα levels in platelets and cerebrospinal fluid of AD patients were also found to be reduced (Colciaghi et al., 2002). Complementary to these findings is the observation that α-secretase activity was reduced in temporal cortex homogenates from AD patients (Tyler et al., 2002). By immunohistochemistry, ADAM10 was found associated with diffuse and neuritic plaques in AD brains and a reduction in the number of ADAM10 immunoreactive neurons was observed (Bernstein et al., 2003). In contrast, ADAM10 mRNA levels were found to be two-fold increased in hippocampal and cerebellar sections of AD patients (Gatta et al., 2002). These results were obtained analyzing brains of severe AD patients, and it is possible that in the later stages of the disease, ADAM10 expression is increased as a defense mechanism or as a secondary effect of inflammation and reactive gliosis.

Molecular biology and in vitro studies have a tremendous impact in our knowledge of AD, and clear-cut data obtained in accessible cells or biological fluids can provide crucial advancements in our understanding of the disease. Here we investigated whether the decrease in ADAM10 protein levels in platelets of AD patients could be ascribed to a reduction in ADAM10 blood mRNA. No significant differences in total blood ADAM10 or platelets gene expression were observed in AD or MCI patients compared to control

subjects, even with the advance of the disease, according to the CDR. These data reveal that the decrease of platelets ADAM10 protein levels in AD platelets is not caused by an alteration of ADAM10 mRNA. A different mechanism is probably involved and it would concern translation or proteolysis phenomena.

It has been shown that ADAM10 is regulated at different levels (transcription, translation and trafficking) and by multiple signaling pathways (Prinzen et al., 2005, Reinhardt et al., 2014, Endres and Fahrenholz, 2012). In Endres and Fahrenholz (2012) review, the regulation of ADAM10 via transcriptional mechanisms, translational events and topology on the membrane are presented, particularly the modulation of gene expression through derivatives of retinoic acid. Conflicting results are shown by these authors, suggesting that ADAM10 expression varies by tissue, disease stage and according to the study. In peripheral blood, although the protein levels of ADAM10 are reduced (Colciaghi et al., 2002, Tang et al., 2006) its activity is unaltered in both MCI and AD (Gorham et al., 2010). In CNS tissue, ADAM10 gene and protein expression appears to be reduced, as well as its activity (Marcinkiewicz and Seidah, 2000, Tyler et al., 2002, Bernstein et al., 2003). Moreover, Gatta et al. (2002) reported an increased ADAM10 gene expression, considering the same parameters. From a gene expression point of view, ADAM10 promoter activity could be induced by vitamin A, since Sp1, USF, and retinoic acid-responsive elements were identified in the core promoter (nucleotides -508 to -300) (Prinzen et al., 2005).

Possible impacts of genetics on ADAM10 expression are also reported in the literature. Bekris et al. (2011), analyzed genetic variations in 19 single nucleotide polymorphisms (SNP) related to APP and its cleavage in a sample of controls and AD, taking into account gender, age, race and APOEε4. The study concluded that genetic variations in the ADAM10 gene (SNP rs541049) correlate with CSF levels in sAPPα. Moreover, the ADAM10 expression, sAPPα levels and thus possibly Aβ accumulation, are modulated according to a promoter haplotype (Bekris et al., 2012).

The human ADAM10 gene contains an untranslated region that codes 444 nucleotides, which has a high content of GC base pairs and successive deletions within this gene region characterize a strong translational repressor to be located within the first 259 nucleotides of the UTR (Lammich et al., 2010). MicroRNAs (miRNAs) regulate protein expression by impeding translation of single genes or destabilizing their mRNAs. The miR-122 has been experimentally defined to regulate ADAM10 via its 3'UTR (Bai et al., 2009), while miR-144 is the sole miRNA that is consistently elevated in the brains of elderly humans and in AD patients (Persengiev et al., 2011).

On the other hand, a post-translational mechanism such as trafficking can affect ADAM10 protein levels. It has been recently shown that ADAM10 removal from the plasma membrane is mediated by the binding to the clathrin adaptor (Marcello et al., 2013). ADAM10/AP2 interaction is increased in AD patients hippocampus at the early stages of the disease (Marcello et al., 2013) and, therefore, could entail an increased delivery of the enzyme to the lysosomal system and thereby an enhanced degradation.

Although methodological differences used for ADAM10 gene expression analysis in whole blood and platelets (primers, RT kits, RT-qPCR machine) could represent a limitation of this study, the methods employed followed pre-established protocols from each laboratory and were based on parameters internationally standardized. Moreover, even with no differences on ADAM10 mRNA levels among groups, the number of medication taken by AD patients compared to healthy control subjects must be taken in account. In this study AD subjects used medications mainly for diabetes, hypertension and dyslipidemias, although control and MCI subjects also had taken these medications, however, in an inferior proportion. Finally, as far as we know, there are no studies reporting ADAM10 protein levels in total blood, and future studies should complete this gap to a better understanding of the ADAM10 synthesis.

5. Conclusions

Here we observed that the difference on platelets ADAM10 protein levels verified for AD patients is not result from differences in mRNA levels, suggesting that post-transcriptional or trafficking mechanisms could play a role. Therefore, in future studies of blood biomarkers, evaluation of both mRNA and protein expression in the same study sample is recommended.

Acknowledgements

The authors thank all the subjects and their families. The authors are grateful for the financial support of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, grant #2010/09497-7 and 2013/06879-4). P.R. Manzine has a scholarship sponsored by FAPESP (grant #2012/08654-7). E. Marcello had a fellowship sponsored by the Italian Society of Pharmacology.

Declaration of interest

All the authors declare no conflict of interest.

References

Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, Yadav A, Nuovo G, Kumar P & Ghoshal K. (2009). MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem, 284, 32015-27.

Bekris LM, Galloway NM, Millard S, Lockhart D, Li G, Galasko DR, Farlow MR, Clark CM, Quinn JF, Kaye JA, Schellenberg GD, Leverenz JB, Seubert P, Tsuang DW, Peskind ER & Yu CE. (2011). Amyloid precursor protein (APP) processing genes and cerebrospinal fluid APP cleavage product levels in Alzheimer's disease. Neurobiol Aging, 32, 31.

Bekris LM, Lutz F, Li G, Galasko DR, Farlow MR, Quinn JF, Kaye JA, Leverenz JB, Tsuang DW, Montine TJ, Peskind ER & Yu CE. (2012). ADAM10 Expression and Promoter Haplotype in Alzheimer's Disease. Neurobiology of aging, 33, 2229.e1-2229.e9

Bernstein HG, Bukowska A, Krell D, Bogerts B, Ansorge S & Lendeckel U. (2003). Comparative localization of ADAMs 10 and 15 in human cerebral cortex normal aging, Alzheimer disease and Down syndrome. J Neurocytol, 32, 153-60.

Colciaghi F, Borroni B, Pastorino L, Marcello E, Zimmermann M, Cattabeni F, Padovani A & Di Luca M. (2002). [alpha]-Secretase ADAM10 as well as [alpha]APPs is reduced in platelets and CSF of Alzheimer disease patients. Mol Med, 8, 67-74.

Colciaghi F, Marcello E, Borroni B, Zimmermann M, Caltagirone C, Cattabeni F, Padovani A & Di Luca M. (2004). Platelet APP, ADAM 10 and BACE alterations in the early stages of Alzheimer disease. Neurology, 62, 498-501.

Endres K & Fahrenholz F. (2012). Regulation of alpha-secretase ADAM10 expression and activity. Exp Brain Res, 217, 343-52.

Gatta LB, Albertini A, Ravid R & Finazzi D. (2002). Levels of beta-secretase BACE and alpha-secretase ADAM10 mRNAs in Alzheimer hippocampus. Neuroreport, 13, 2031-3.

Gorham P, Bark N, Bjorkhem I, Meaney S & Crisby M. (2010). Platelet alpha- and beta-secretase activities are not significantly affected by dementia or mild cognitive impairment in Swedish patients. Curr Alzheimer Res, 7, 134-9.

Hardy J & Selkoe DJ. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, 297, 353-6.

Hohsfield LA & Humpel C. (2015). Migration of blood cells to β-amyloid plaques in Alzheimer's disease. Experimental Gerontology, 65, 8-15.

Jorissen E, Prox J, Bernreuther C, Weber S, Schwanbeck R, Serneels L, Snellinx A, Craessaerts K, Thathiah A, Tesseur I, Bartsch U, Weskamp G, Blobel CP, Glatzel M, De Strooper B & Saftig P. (2010). The Disintegrin/Metalloproteinase ADAM10 Is Essential for the Establishment of the Brain Cortex. Journal of Neuroscience, 30, 4833-4844.

Kim M, Suh J, Romano D, Truong MH, Mullin K, Hooli B, Norton D, Tesco G, Elliott K, Wagner SL, Moir RD, Becker KD & Tanzi RE. (2009). Potential late-onset Alzheimer's disease-associated mutations in the ADAM10 gene attenuate α-secretase activity. Human Molecular Genetics, 18, 3987-3996.

Kuhn PH, Wang H, Dislich B, Colombo A, Zeitschel U, Ellwart JW, Kremmer E, Rossner S & Lichtenthaler SF. (2010). ADAM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons. Embo Journal, 29, 3020-3032.

Lammich S, Buell D, Zilow S, Ludwig AK, Nuscher B, Lichtenthaler SF, Prinzen C, Fahrenholz F & Haass C. (2010). Expression of the anti-amyloidogenic secretase ADAM10 is suppressed by its 5'-untranslated region. J Biol Chem, 285, 15753-60.

Lichtenthaler SF. (2011). Alpha-secretase in Alzheimer's disease: molecular identity, regulation and therapeutic potential. Journal of Neurochemistry, 116, 10-21.

Livak KJ & Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402-8.

Manzine PR, Bram JMF, Barham EJ, Vale FAC, Araujo HSS, Cominetti MR & Pavarini SCI. (2013). ADAM10 as a biomarker for Alzheimer's disease: a study with Brazilian elderly. Dement Geriatr Cogn Disord, 35, 58-66.

Marcello E, Saraceno C, Musardo S, Vara H, De La Fuente AG, Pelucchi S, Di Marino D, Borroni B, Tramontano A, Xe, Rez O, Xf OI, Padovani A, Giustetto M, Gardoni F & Di Luca M. (2013). Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. The Journal of Clinical Investigation, 123, 2523-2538.

Marcinkiewicz M & Seidah NG. (2000). Coordinated Expression of β-Amyloid Precursor Protein and the Putative β-Secretase BACE and α-Secretase ADAM10 in Mouse and Human Brain. Journal of Neurochemistry, 75, 2133-2143.

Meziane H, Dodart JC, Mathis C, Little S, Clemens J, Paul SM & Ungerer A. (1998). Memory-enhancing effects of secreted forms of the β-amyloid precursor protein in normal and amnestic mice. Proceedings of the National Academy of Sciences, 95, 12683-12688.

Morishima-Kawashima M & Ihara Y. (2002). Alzheimer's disease: β-Amyloid protein and tau. Journal of Neuroscience Research, 70, 392-401.

Persengiev S, Kondova I, Otting N, Koeppen AH & Bontrop RE. (2011). Genome-wide analysis of miRNA expression reveals a potential role for miR-144 in brain aging and spinocerebellar ataxia pathogenesis. Neurobiol Aging, 32, 2316 e17-27.

Petersen RC. (2004). Mild cognitive impairment as a diagnostic entity. J Intern Med, 256, 183-94.

Prinzen C, Muller U, Endres K, Fahrenholz F & Postina R. (2005). Genomic structure and functional characterization of the human ADAM10 promoter. FASEB J, 19, 1522-4.

Reinhardt S, Schuck F, Grosgen S, Riemenschneider M, Hartmann T, Postina R, Grimm M & Endres K. (2014). Unfolded protein response signaling by transcription factor XBP-1 regulates ADAM10 and is affected in Alzheimer's disease. FASEB J, 28, 978-97.

Stein TD, Anders NJ, Decarli C, Chan SL, Mattson MP & Johnson JA. (2004). Neutralization of Transthyretin Reverses the Neuroprotective Effects of Secreted Amyloid Precursor Protein (APP) in APPSw Mice Resulting in Tau Phosphorylation and Loss of Hippocampal Neurons: Support for the Amyloid Hypothesis. The Journal of Neuroscience, 24, 7707-7717.

Tang K, Hynan LS, Baskin F & Rosenberg RN. (2006). Platelet amyloid precursor protein processing: A bio-marker for Alzheimer's disease. Journal of the Neurological Sciences, 240, 53-58.

Tricarico C, Pinzan P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA & Orlando C. (2002). Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem, 309, 293-300.

Tyler SJ, Dawbarn D, Wilcock GK & Allen SJ. (2002). alpha- and beta-secretase: profound changes in Alzheimer's disease. Biochem Biophys Res Commun, 299, 373-6.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, 3, RESEARCH0034.

Table 1. Gene nomenclature, GenBank accession number and primer sequences.

Primer name	Gene	Gen Bank	Forward	Reverse
*$ADAM10_{9\text{-}10T}$	ADAM10	NM_001110.2	ACCTTCAGGAAG CTCTGGAGGAAT	CTGGTGTGCACTCTG TTCCAGAAT
†$ADAM10_{9\text{-}10P}$	ADAM10	NM_001110.2	CTTTTGCTCACGA AGTTGGACA	TGTCCCCAGATGTTG CTCTTG
†$ADAM10_{11\text{-}12P}$	ADAM10	NM_001110.2	AGATGAATGCTG CTTCGATGC	AAGGACCTTGACTTG GACTGCA
*β-actin	β-actin	NM_001101.3	GACGGCCAGGTC ATCACCATTG	AGCACTGTGTTGGCG TACAGG3
†β-actin 1	β-actin	NM_001101.2	CCTTCTACAATGA GCTGCGTGT	ACAGCCTGGATAGCA ACGTACA
†β-actin 2	β-actin	NM_001101.2	GCTCCTCCTGAGC GCAAG	CATCTGCTGGAAGGT GGACA
†HPRT	HPRT	NM_000194.1	ATGGACAGGACT GAACGTCTTG	TGATGTAATCCAGCA GGTCAGC
*GAPDH	GAPDH	NM_002046.4	GACTTCAACAGC GACACCCAC	CACCACCCTGTTGCT GTAG
†GAPDH	GAPDH	NM_017008	TGCACCACCAAC TGCTTAGC	GGCATGGACTGTGGT CATGA
†EF1α	EF1α	NM_001402	AAGCTGGAAGAT GGCCCTAAA	AAGCGACCCAAAGG TGGAT
†Cyclop	Cyclop	NM_021130	GCTCGCAGTATCC TAGAATCTTTGT	CTGCAATCCAGCTAG GCATG
†RPLPO	RPLPO	NM_053275	GTCGGAGGAGTC GGACGA	AGCCTTTATTTCCTT GTTTTGCA
†$ADAM10_{intron}$	ADAM10	NM_001110.2	GACTGAGGTTTG CCTTTCGGT	TTAGCCCCTGCATCC TTTCA
†$\beta\text{-actin}_{intron}$	β-actin	NM_001101.2	TGCTTTTTCCCAG ATGAGCTC	AATACACACTCCAAG GCCGCT

*$ADAM10_{9\text{-}10T}$ and endogenous controls = Primers sequences for total blood RT-qPCR. †$ADAM10_{9\text{-}10P}$ and $ADAM10_{11\text{-}12P}$ and endogenous controls = Primers sequences for platelet RT-qPCR.

Table 2. Subjects' demographic and clinical variables. Kruskal-Wallis test was used in all variables.

	Total Blood RNA			**Platelets RNA**			**p-value**
	Control	**MCI**	**AD**	**Control**	**MCI**	**AD**	
Cases (n)	32	21	47	19	16	21	
Mean age (range)	74 (64-86)	72 (60-84)	77 (60-90)	67 (63-71)	69 (61-74)	70 (65-75)	0.41
Female (%)	22 (68%)	14 (67%)	32 (68%)	13 (68.4%)	8 (50%)	15 (71%)	0.46
MMSE, mean ± SD	27.5 ± 1.5	24.5 ± 2.3*	13.4 ± 5.7*	29 ± 0.3	24.9 ± 0.5*	19.9 ± 3.6*	0.0001

*p = 0.0001 control *vs* MCI and control *vs* AD in total blood and platelet groups samples.

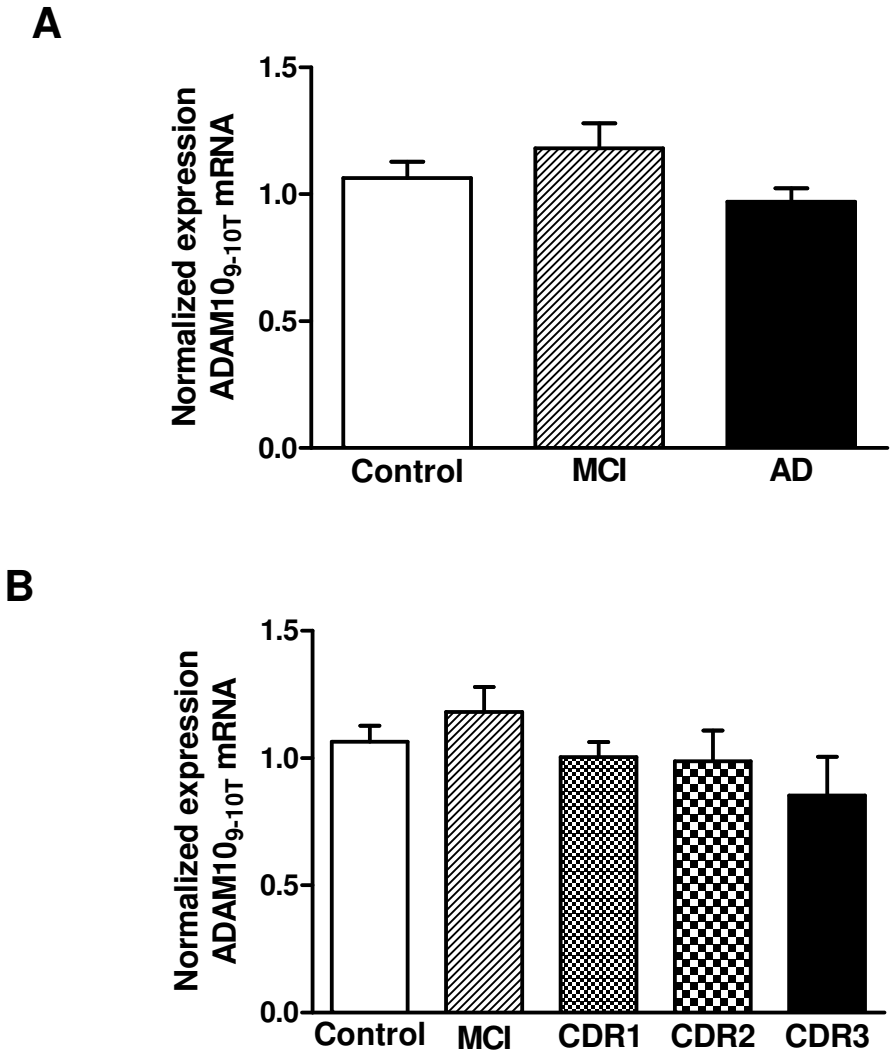


Figure 1. Normalized expression of total blood ADAM10. **(A)** Normalized expression of total blood ADAM10 in control *vs* MCI (p = 0.29), control *vs* AD (p = 0.38) and MCI *vs* AD (p = 0.08). **(B)** Normalized expression of total blood ADAM10 in control *vs* MCI and AD group according to CDR. Total blood was collected using PAXgene Blood RNA kit and RNA was reverse transcribed into cDNA using Enhanced Avian RT First Strand Synthesis. Samples were amplified in a thermocycler (Rotor Gene RG6000, Corbett Life Science) with SYBR Green Jump Start kit, using specific primers for ADAM10 designed to amplify exons 9-10 and the endogenous controls β-actin and GAPDH. Gene expression was assessed by relative quantification, using the $2^{-\Delta\Delta Ct}$ formula with the geometric mean of the endogenous controls. No significant statistical difference was observed using Kruskal-Wallis Test (p = 0.39). Graphs were prepared using GraphPad Prism 5.01.

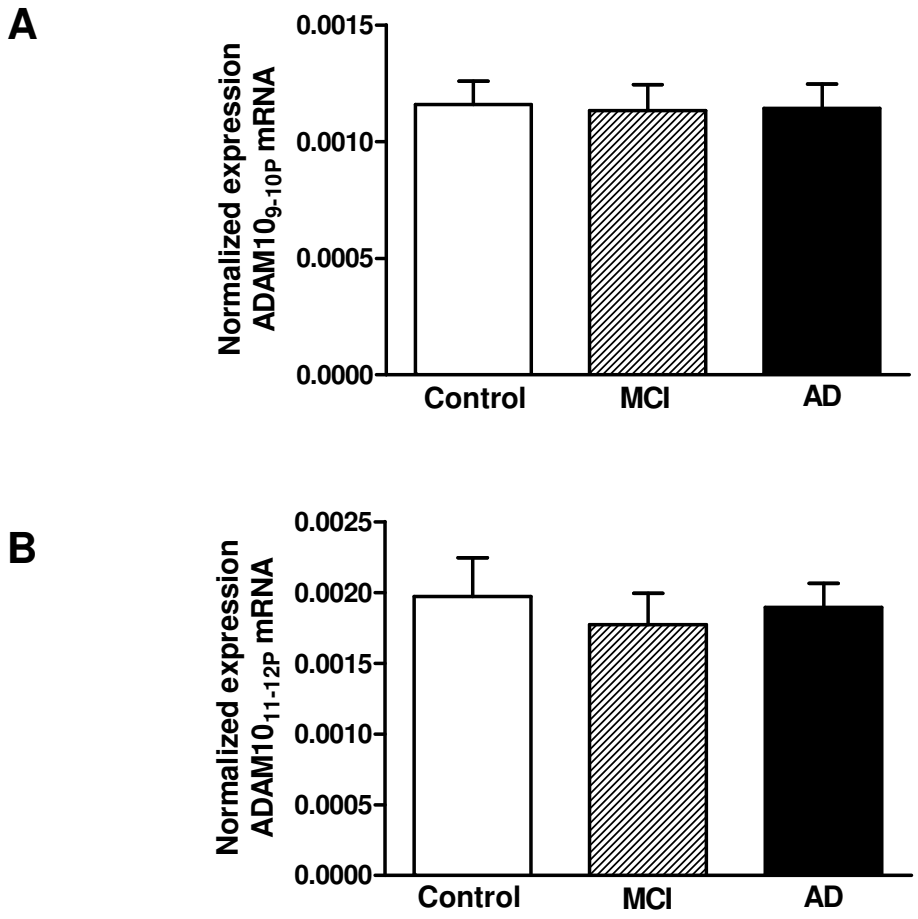
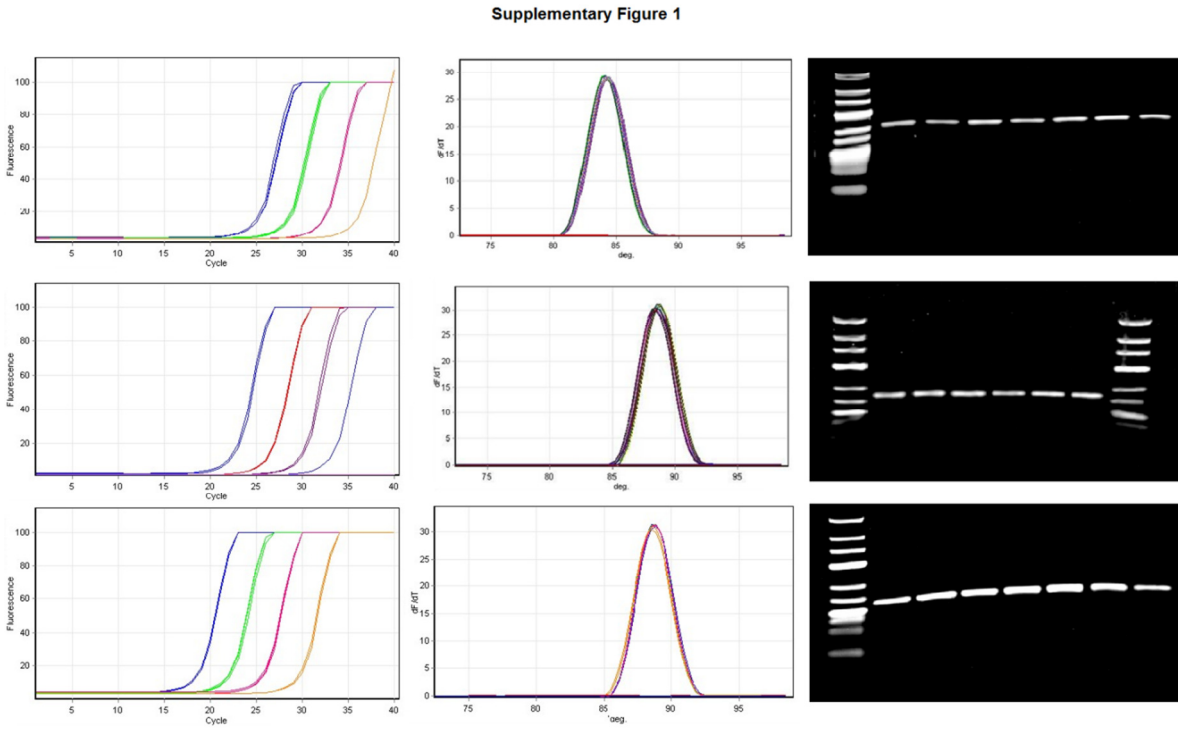
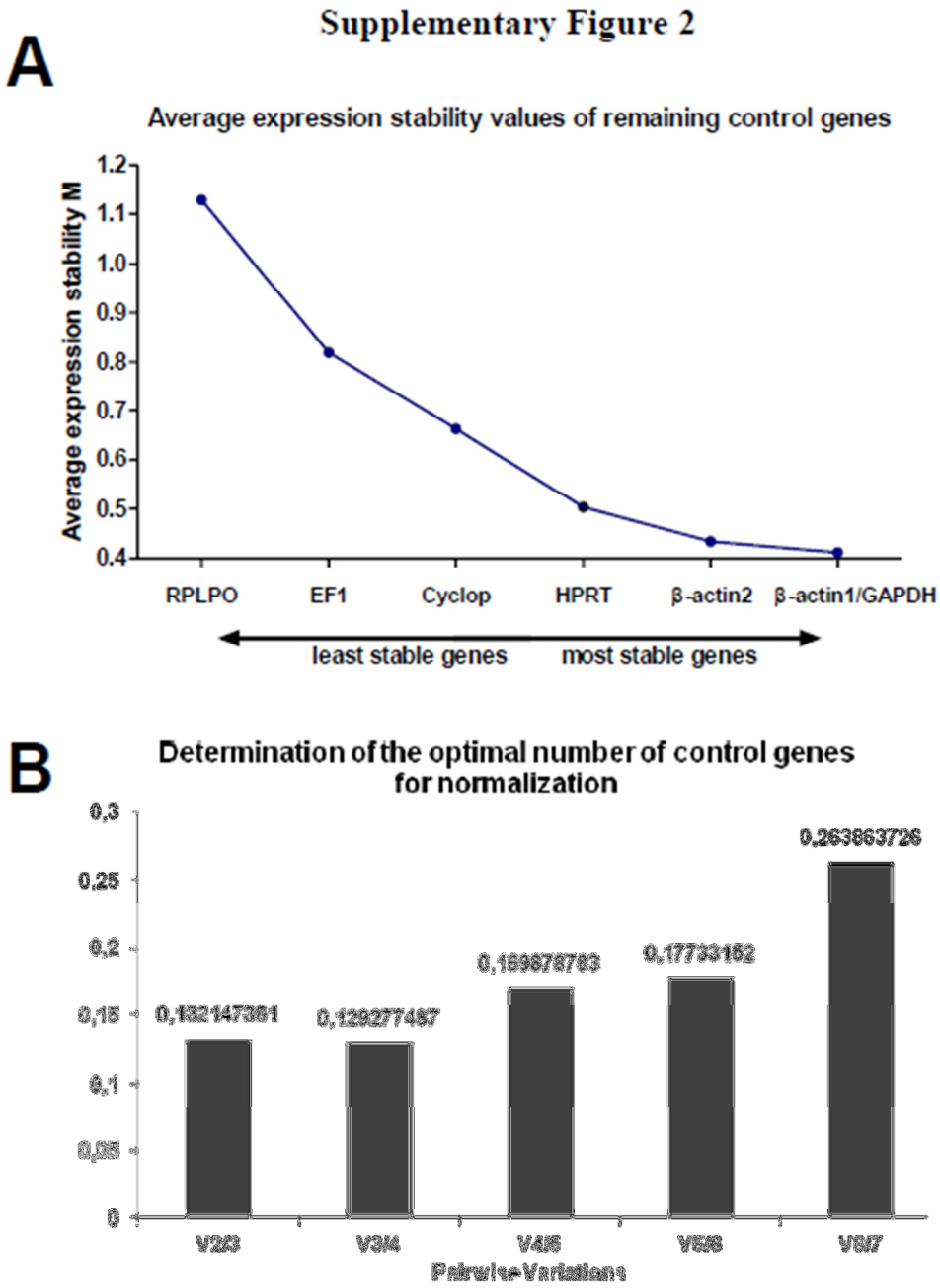


Figure 2. Normalized expression of platelet ADAM10. **(A)** Normalized expression of platelet ADAM10 exons 9-10 in the control, MCI and AD groups. Control = 0.00116±0.0004, MCI = 0.00113±0.0004, AD = 0.00114±0.0004. Control *vs* MCI (p = 0.81), control *vs* AD (p = 0.78) and MCI *vs* AD (p = 0.89). **(B)** Normalized expression of platelet ADAM10 exons 11-12 in the control, MCI and AD groups. Control = 0.00197±0.0013, MCI = 0.00177±0.0009, AD = 0.00189±0.0008. Control *vs* MCI (p = 0.34), control *vs* AD (p = 0.92) and MCI *vs* AD (p = 0.51). Blood was collected and centrifuged to obtain platelet rich plasma and a second centrifugation was used to obtain platelets. Platelets were washed and their total RNA was isolated using Trizol reagent. RNA was reverse transcribed into cDNA using Superscript III kit and random hexamer primers. Samples were amplified in a thermocycler (Applied Biosystems) with SYBR Green Jump Start, using specific primers for ADAM10 designed to amplify exons 9-10 and 11-12 and the reference genes. Transcript levels of several genes frequently selected for normalizing PCR were analyzed (Table 1) and according to Vandesompele et al. (2002), a normalization factor based on the expression levels of β-actin1, β-actin2, HPRT, and GAPDH was calculated by using the geometric mean of the Ct. Graphs were prepared using GraphPad Prism 5.01.



Supplementary Figure 1. Fluorescence, melting curves and agarose (2%) gel from total blood ADAM10, β-actin and GAPDH genes, respectively. No signal in NTCs was observed in fluorescence and melting curves. ADAM10: 200bp, β-actin: 160bp, GAPDH: 123bp.



Supplementary Figure 2. **(A)** The internal gene stability measure M of analyzed housekeeping genes. **(B)** Pairwise variation (Vn/n+1) analysis to determine the number of control genes required for accurate normalization. Note the increase between V3/4 and V4/5 caused by the addition of gene 5 cyclop. GraphPad Prism 5.01.

4.2 MANUSCRITO II – Em fase de redação

PREDICTED BLOOD-BASED MICRO-RNAS FOR ADAM10 ARE DOWNREGULATED IN ALZHEIMER´S DISEASE SUBJECTS COMPARED TO HEALTHY CONTROLS

Patricia Regina Manzine[a*], Maria Aderuza Horst[b], Francisco de Assis Carvalho do Vale[c], Sofia Cristina Iost Pavarini[a], Márcia Regina Cominetti[a]

[a]Department of Gerontology, [c]Department of Medicine, Federal University of São Carlos, São Carlos, SP, Brazil; [b]Department of Nutrition, Federal University of Goiás, Goiânia, MG, Brazil.

[*]**Corresponding Author:** Patricia Regina Manzine. Laboratório de Biologia do Envelhecimento – LABEN, Departamento de Gerontologia, Universidade Federal de São Carlos, Rodovia Washington Luís, Km 235, São Carlos, SP, 13565-905, Brazil, phone: +55 16 3306 6672. E-mail: patricia_manzine@yahoo.com.br

Abstract

ADAM10 is an α-secretase that cleaves APP in the non-amyloidogenic pathway, thereby inhibiting amyloidogenic Aβ production in Alzheimer´s disease (AD). Several studies address the deregulation of micro-RNAs (miRNAs) in a variety of human diseases, as well as in neurodegenerative processes. In this study we propose to explore and validate miRNAs that have directly or indirectly relations to the AD pathophysiology and ADAM10 gene. Using Megaplex^{TM} and MirWalk 2.0 database we analyzed by RT-qPCR ~700 miRNAs in total blood and validated 21 miRNAs in a sample of 21 AD subjects and 17 healthy controls. Mir-144-5p, miR-374 and miR-221 are downregulated in AD subjects, with moderate accuracy diagnosis. However, the association of selected miRNAs expression and MMSE was significantly better as a diagnostic tool compared to their expression separately. These miRNAs are involved in the regulation of pathways related to neurodegenerative diseases (beta-amyloid cascade, ubiquitination, transcriptional regulator, synaptic transmission, vesicle trafficking). Specifically, miR-144-5p, miR-374 and miR-221 are relevant for AD, as regulators of APP, BACE1 and ADAM10 translation. To the best of our knowledge, this is the first study to demonstrate a downregulation of these specific miRNAs in blood of Alzheimer's disease patients, compared to healthy cognitive controls. These findings are in agreement with AD protein outcomes and place the miRNAs evaluated as potential biomarkers that can be used to improve AD diagnosis.

Keywords: ADAM-10 protein; aging; Alzheimer disease; biomarkers; micrornas; Reverse Transcriptase PCR;

Introduction

Alzheimer's disease is a chronic, progressive and neurodegenerative disease that represents the main cause of dementia among people over 65 years (Colombo et al., 2013; Delay et al., 2012). Nowadays, more than 35 million people live with AD worldwide and this number is estimated to double in the next 20 years (Agostinho et al., 2015). Therefore, it is urgent for the scientific community and the entire population deeply understand the causes and molecular mechanisms of AD in order to find therapeutic solutions that reduce the rising incidence of the disease (Wimo et al., 2014).

The amyloid cascade hypothesis presumes that AD may be caused by the age-dependent and progressive accumulation and deposition of extracellular amyloid-β (Aβ) peptides in brain (Hardy and Selkoe, 2002). Aβ derives from the concerted action of BACE1 (β-secretase) and the γ-secretase complex on the Amyloid Precursor Protein (APP) (Lichtenthaler, 2011). In healthy subjects the predominant route of APP processing consists of successive cleavages by the α-secretase ADAM10 (A Disintegrin And Metalloprotease 10) and γ-secretases. In the non-amyloidogenic pathway, APP is cleaved by ADAM10 between lysine16 and leucine17 in the middle of Aβ region, thus releasing sAPPα (Colombo et al., 2013), a structure with neurotrophic and neuroprotective functions - retaining the C83 residue in the membrane. The following cleavage of C83 by γ-secretase releases the p3 - which is supposed to be beneficial, and is not found in amyloid plaques - and starts at position Aβ17 (Aβ17-40 and Aβ17-42), thereby inhibiting amyloidogenic Aβ production (Morishima-Kawashima and Ihara, 2002). Currently, the final diagnosis of AD can only be confirmed via autopsy, which makes early detection, reliable and non-invasive biomarkers a significant challenge (Leidinger et al., 2013). The development of easily accessible and high sensitivity and specificity molecular diagnostic markers from minimally-invasive sources, such as blood, plasma or serum has been the focus of studies over the last decade.

MiRNAs are small non-coding RNAs fragments (~ 23 nucleotides) that regulate gene expression by binding to complementary regions of specific transcripts in order to repress translation or destabilize their respective mRNAs (Satoh, 2012). Several studies address the deregulation of miRNAs in a variety of human diseases (Keller et al., 2011), as well as in neurodegenerative processes (Leidinger et al., 2013).

In previous studies we and others have described a marked reduction in platelet ADAM10 levels of AD subjects, compared to cognitive healthy controls (Colciaghi et al., 2002; Manzine et al., 2013c; Manzine et al., 2013d). These alterations were also observed for

APP, BACE1 and ADAM10 in platelets of AD patients already in the very early stages of the disease (Colciaghi et al., 2004). Additionally, no significant differences were observed for ADAM10 gene expression in total blood or platelets of AD or MCI patients compared to control subjects, even with the advance of the disease (Manzine et al., 2015). These data reveal that the decrease of platelets ADAM10 protein levels in the AD is not caused by reduced ADAM10 mRNA. In this sense, we hypothesized that a different mechanism is rather involved other than gene expression regulation. This mechanism would concern miRNAs specific regulation on ADAM10, translation mechanisms or proteolysis phenomena. In order to investigate this hypothesis, we aimed in this study to explore and validate miRNAs that are directly or indirectly related to the pathophysiology of AD and ADAM10 gene.

Materials and Methods

Characteristics of the Subjects

Patients were recruited in reference (Public Center of Specialties) and counter-reference (Family Health Units) health services in São Carlos city, São Paulo - Brazil. All subjects recruited were diagnosed with probable AD according to National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCS-ADRDA) criteria. All participants underwent the exclusion criteria for head trauma, metabolic dysfunctions, haematological diseases, alcohol abuse, drug abuse, delirium, mood disorders, and treatment with medications affecting platelet functions, i.e., anticoagulants, antiplatelet drugs, serotoninergic agonists-antagonists, and corticosteroids. All subjects included had a standardised clinical workup based on neurological examinations, laboratory blood and urine analysis, a neuroimaging study (Head Computed Tomography and/or Magnetic Resonance Imaging), and a neuropsychological assessment, including a Mini Mental State Examination (MMSE) and Clinical Dementia Rating (CDR). In this study, we used a Brazilian version of MMSE (Brucki et al., 2003). Before enrolment, subjects or their legal caregivers filled out an informed consent, after the nature and possible consequences of the study were explained. The research project was approved by Brazilian ethics committee (CAAE: 02760312.0.0000.5504/ N°: 112.543).

Isolation of RNA from total blood

Personnel carrying blood preparation, as well as subsequent analysis, were blind for diagnosis and statistics. For the isolation of total RNA, 2.5 ml of blood was collected in total RNA extraction tubes (PAXgene Blood RNA – Becton & Dickinson), according to the product manual. After collection, tubes were inverted 10x and kept at 4°C during storage and transportation with maximum time of 30-40 minutes until the first centrifugation step. PAXgene tubes were kept for two hours at room temperature in an upright position as manufacturer's instructions and were subsequently frozen at -80ºC until use. Total RNA isolation from blood samples was performed using PAXgene Blood RNA isolation kit (Qiagen) according to the manufacture's manual. From the extracted total RNA, samples (1µl) were quantified with a Nanodrop (Thermo Scientific) to obtain absorbance values and their ratios (A260/A280 and A260/A230).

Reverse transcription and RT-qPCR blood screening

cDNAs were prepared from 450 ng of total RNA by using the Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems) and specific stem-loop primers for microRNAs (Megaplex^{TM} - Applied Biosystems). Gene expression levels of different miRNAs were determined by reverse transcription quantitative real time PCR (RT-qPCR) analyzes by 7900HT Fast Real-Time PCR System equipment (Applied Biosystems) using the TaqMan® Human MicroRNA Array Cards - Pool A v2.1 and B v3.0 (Life Technologies). Pool A and pool B, containing 377 and 290 miRNAs respectively, are a pair of plates for RT-qPCR that provide primers and probes for assessing the expression levels of 667 different miRNAs. Both pools include five endogenous controls and a non-human expressed as a negative control (ath-miR-159a). In this first screening 16 plates were prepared (Pool A and Pool B for each patient and for four patients in each study group). This analysis allowed the selection of differentially expressed miRNAs between the two experimental groups. The results of RT-qPCR-array were analyzed using the DataAssist v3.01 software (Thermo Scientific) and the calculations were carried out using the average Cts of the five endogenous controls. The most stable endogenous control, determined by DataAssist v3.01 software, was U6. Data were analyzed using the following parameters: Ct 35 maximum; inclusion of maximum Cts; excluding outliers among replicates; p value adjustment using the Benjamini-Hochberg test (False Discovery Rate - FDR); standardization method from the endogenous U6 control and use of the control group as calibrator.

Validation of miRNAs screened by RT-qPCR

Cohort sample for validation was formed by n = 21 AD subjects and n = 17 healthy controls. cDNAs were prepared from 10 ng of total RNA using TaqMan® MicroRNA Reverse Transcription Kit. The miRNAs gene expression levels were determined through analysis by RT-qPCR in StepOne Plus (Applied Biosystems) using Taqman ® MicroRNA Assays (Life Technologies) and Taqman ® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems) for targets miRNAs and endogenous control (has-miR-U6). Reactions were performed in 96 well plates (MicroAmp Optical 96-well Fast and MicroAmp Optical Adhesive Film, Applied Biosystems) in duplicate. Non-template controls were included for each primer reactions. RT-qPCR conditions were as follows: 7.0 µl DEPC water, 10.0 µl MasterMix [2x], 1.0µl Primer Assay Mix [20x] and 2.0µl cDNA. The RT-qPCR reactions used were standardized with a final volume of 20µl. Cycling conditions were: Hold 50°C, 2 min; Hold 95°C, 10 min; Cycling (40 repeats) – Step 1: 95°C, 15 s and Step 2: Tm 60°C, 1 min. MiRNAs were analyzed considering thresholds 0.2 for all analyzes. The internal calibrator used as a basis to standardize the results of expression was the control group ΔCts average. Calibration was determined by ΔΔCt = ΔCt (sample) - ΔCt (calibrator). Gene expression was assessed by relative quantification, using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Data analysis and statistical evaluation

Statistical tests of comparison (Mann-Whitney U-test and Kruskal-Wallis - Dunn's Multiple Comparison Test) were performed. Spearman's correlation tests were subsequently performed between selected miRNAs and MMSE score. Sensitivity and specificity were calculated using the receiver operating characteristic (ROC) curves for miRNAs diagnostic analyses. To compare the ROC curves in isolated miRNAs and their combination with or without MMSE score, a method that evaluates the areas under the curves (AUCs) was employed. The cutoff with the highest Younden index (sensitivity plus specificity -1) was chosen (DeLong et al., 1988). Data were analyzed and the figures or tables were built using Graphpad Prism 5.01 (GraphPad Software Inc) and Medcalc 14.8.1 (MedCalc Software) softwares. Results with a probability of error below 5% were considered significant.

Results

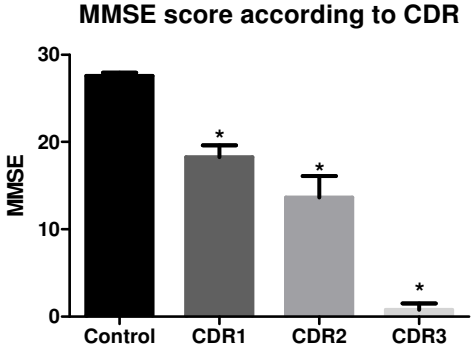
Cohort sample for validation was formed by n = 21 AD subjects and n = 17 healthy controls. The demographic and clinical data of the subjects are presented in table 1. Subjects with AD and their matched cognitively healthy controls were mostly female, 60 years or older. The most frequent disease observed among all subjects was hypertension (Table 1). Age and gender did not differ significantly between groups (Table 1). Regarding CDR levels, 52% of the elderly had CDR= 1; 29% presented CDR= 2; and 19%, CDR= 3 (Table 1). MMSE scores were significantly different between patients with AD and controls ($p \leq 0.001$), and also along the disease's progression ($p < 0.05$) (Table 1 and Fig. 1). Considering the MMSE score, the average (±SD) was 27±7 (control) and 14±8 (AD) (Table 1).

Table 1. Subjects' demographic and clinical variables according to CDR.

Variable	Control	AD	CDR1	CDR2	CDR3	*p*-value
Cases (n)	17	21	11	6	4	
Age, mean (range)	73 (65-86)	77 (60-89)	77 (67-85)	82 (78-89)	70 (60-79)	0.40
Gender, female (%)	72%	73%	92%	67%	25%	0.40
MMSE, mean ± SD	27 ± 7	14 ± 8	18 ± 5	14 ± 6	0.75 ± 1.5	*
Comorbidities						
Hypertension	4	11	6	5	0	
Diabetes Mellitus	3	8	4	3	1	
Hypothyroidism	0	2	2	0	0	

CDR, Clinical Dementia Rating; MMSE, Mini Mental State Examination; SD, Standard Deviation; Mann-Whitney U-test and Test ANOVA One Way (Kruskal-Wallis test). *< 0.001 Control≠AD; Control≠CDR1; Control≠CDR2; Control≠CDR3; < 0.05 CDR1≠CDR2; CDR1≠CDR3; CDR2≠CDR3. GraphPad Prism 5.01.

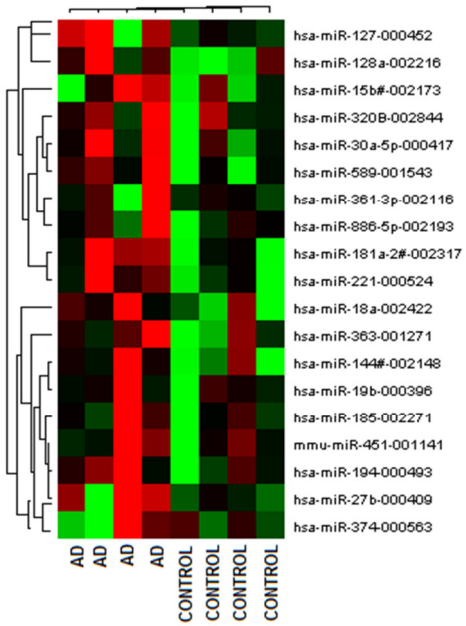
Fig. 1. MMSE score according to CDR.



Test ANOVA One Way (Kruskal-Wallis test). *p < 0.001 Control≠CDR1; Control≠CDR2; Control≠CDR3; p < 0.05 CDR1≠CDR2; CDR1≠CDR3; CDR2≠CDR3. GraphPad Prism 5.01.

Figure 2 shows the heat map reflecting miRNA expression in AD and control groups, which were subsequently selected for validation in larger scale sample (AD and controls).

Fig. 2. Heat Map of miRNAs with greater differentiation between AD and control groups. Red = upregulation, black = no change, green = downregulation. Data Assist v.3.01.



The intersection of the results obtained through the Megaplex™ Kits with MirWalk 2.0[2] database was performed. Twenty one miRNAs that have directly or indirectly relations to the AD pathophysiology (21 miRNAs) or to the ADAM10 and AD (13 miRNAs) were selected for validation in a larger sample, in addition miR-103 and miR-107, which were described in the literature as having important roles in ADAM10 protein expression (Augustin et al., 2012).

Among the nearly 700 miRNAs analyzed, 19 miRNAs that had higher fold changes (3.49 - 1.70) in the AD group compared to the control group were selected for validation, besides miR-103 and miR-107 (Fig. 2).

Descriptive data for each miRNA expression between the AD and control groups is shown in Supplementary Table 1. Fig. 3 shows validated miRNAs expression (miR-144-5p, miR-374, miR-221, miR-18a, miR-27b, miR-185, miR-107, miR-103; miR-19b, miR-320B, miR-363, miR-15b-3p, miR-127, miR-194, miR-128a, miR-30a-5p, mmu-miR-

[2] http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/

451, miR-589, miR-181a- 2-3p; miR-886-5p, miR-361-3p). From the 21 miRNAs analyzed, only miR-144-5p, miR-374 and miR-221 presented statistically significant differences ($p < 0.05$) between groups (Fig. 3 and Table S1), with mean expression of 1.67 ± 1.74, 1.87 ± 1.85 and 1.37 ± 1.20 and 0.69 ± 0.87, 0.78 ± 1.05 and 0.66 ± 0.63 in control and AD groups, respectively (Table S1). According to Spearman´s test, individual correlations of miR-144-5p, miR-374 and miR-221 with MMSE were $r = 0.3971$ ($p = 0.01$), $r = 0.4036$ ($p = 0.01$) and $r = 0.4066$ ($p = 0.01$), respectively. When in combination (miR-144-5p + miR-374 + miR-221), it was found a positive correlation $r = 0.4091$, 95% CI 0.1029-0.6445, $p = 0.01$ (Fig. 4).

Results showed a moderate and positive correlation between such miRNAs (individually or in combination) with MMSE, meaning that, when miRNAs expression values increase, there is also a rise in the MMSE score, as illustrated in the scatter diagram in Fig. 4. Supplementary Table 2 and Fig. S1 present AUC values in ROC curves considering Youden index with sensitivity and specificity for all validated miRNAs. Regarding AUC values, miR-144-5p, miR-374 and miR-221 presented moderate values of sensitivity and specificity with significant p-value. The best AUC value between miRNAs individually analyzed was obtained for miR-144-5p (0.70, 95% CI 0.5390 to 0.8755) at a cutoff ≤ 0.36, which presented sensitivity of 66.7 and specificity of 76.5 ($p = 0.017$) for AD detection. Likewise, given the significant correlation between these variables (miR-144-5p, miR-374, miR-221 and MMSE), AUC analysis in ROC curves were performed considering such miRNAs, the association between them and their association with MMSE (Table S2 and S3; Fig. 5).

Fig. 3. Differentially expressed miRNAs between control and DA groups. GraphPad Prism 5.01.

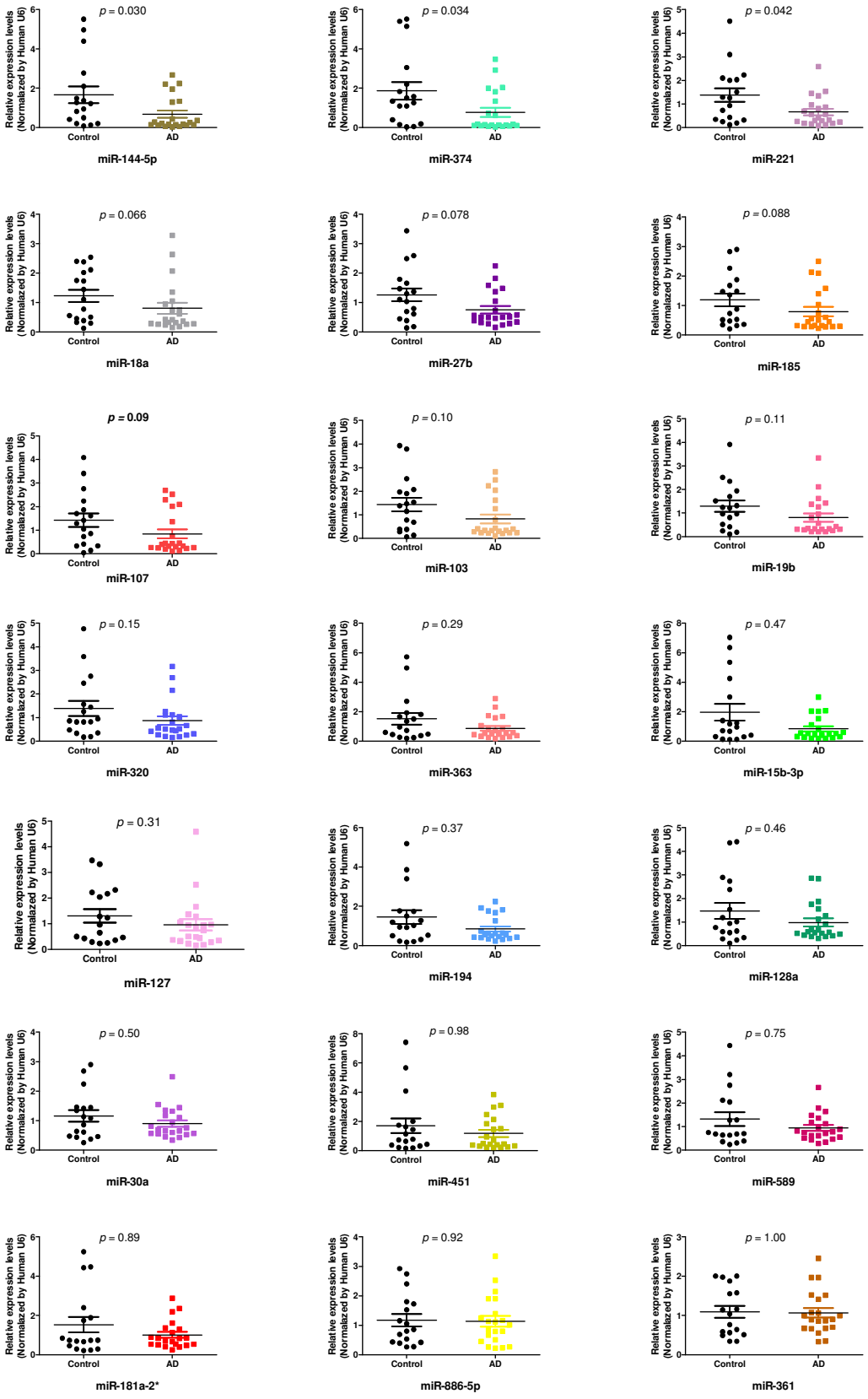


Fig. 4. Correlation in scatter diagram plotted with selected miRNAs as function of MMSE score. Medcalc 14.8.1

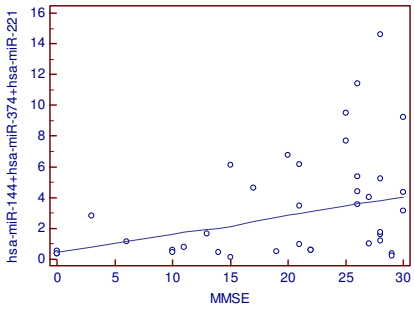
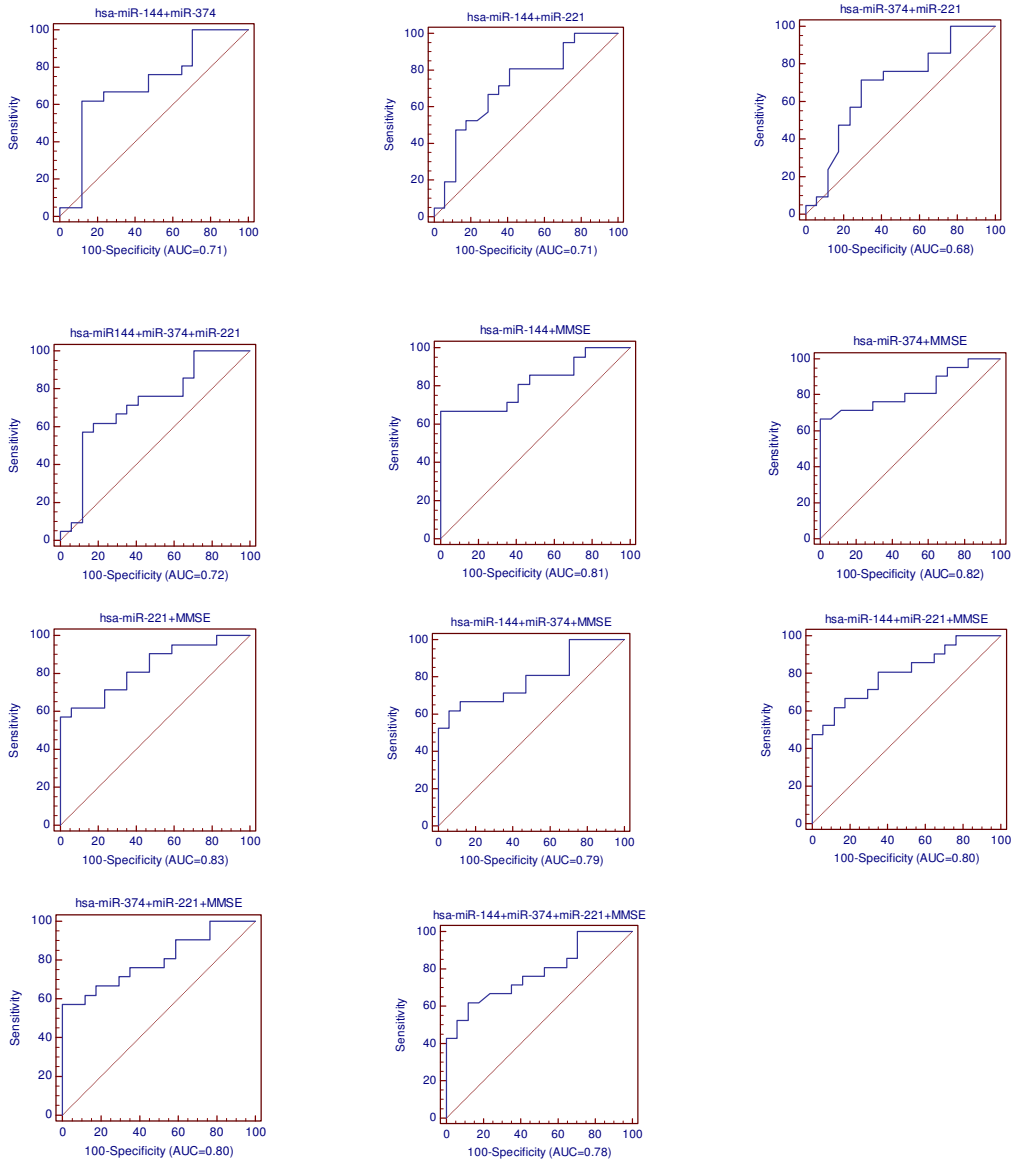


Fig. 5. AUC analysis in ROC curves performed considering selected miRNAs association and their association with MMSE. The sensitivity is plotted as a function of specificity. Medcalc 14.8.1



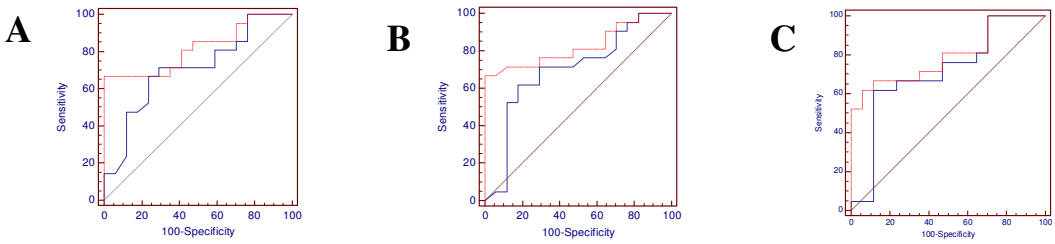
Data showed that the association between the selected miRNAs presented AUC variation from 0.68 to 0.72, sensitivity from 61.9 to 80.95 and specificity from 58.8 to 88.24, however the best values were observed for miR-144-5p+miR-374 associations (0.71, 95% CI 0.535-0.888, p = 0.01) at a cutoff ≤ 0.43, with 61.9 of sensitivity and 88.2 of specificity and miR-144-5p+miR-374+miR-221 (0.72, 95% CI 0.543-0.892, p = 0.01) at a cutoff ≤ 1.18 presented 61.9 of sensitivity and 82.35 of specificity. When MMSE score was added, AUC changed to 0.78 to 0.83, 57.14-66.67 of sensitivity and 88.24-100 of specificity, so that the best and most significant values were achieved with the association of miR-144-5p or miR-374 with MMSE (0.81, 95% CI 0.681 to 0.955, p ≤ 0.0001) and (0.82, 95% CI 0.689-0.961, p ≤ 0.0001), respectively (Table S3 and Fig. 5). ROC curves comparisons revealed that MMSE association with miR-144-5p and miR-374 significantly increases the difference between AUC values (p = 0.01). The same was observed when both are associated with MMSE (miR-144-5p+miR-374+MMSE), so that this combination reached a cutoff ≤ 1.31, sensitivity of 61.9 and specificity of 94.1 (Table 2 and S3).

Table 2. Data of pairwise comparison of ROC curves between the selected miRNAs and their association with MMSE. Medcalc 14.8.1

	AUC	**SE [a]**	**95% CI [b]**
miR-144-5p	0.71	0.087	0.537 to 0.878
miR-144-5p+MMSE	0.81	0.07	0.681 to 0.955
Difference between areas			0.111
Standard Error[c]			0.0445
95% Confidence Interval			0.0235 to 0.198
Significance level			*p* = 0.0129
	AUC	**SE [a]**	**95% CI [b]**
miR-374	0.7	0.091	0.521 to 0.877
miR-374+MMSE	0.82	0.07	0.689 to 0.961
Difference between areas			0.126
Standard Error[c]			0.0518
95% Confidence Interval			0.0245 to 0.228
Significance level			*p* = 0.0150
	AUC	**SE [a]**	**95% CI [b]**
miR-144-5p+miR-374	0.71	0.089	0.535 to 0.888
miR-144-5p+miR-374+MMSE	0.79	0.073	0.648 to 0.937
Difference between areas			0.0812
Standard Error[c]			0.0429
95% Confidence Interval			-0.00276 to 0.165
Significance level			*p* = 0.0580

[a] DeLong et al., 1988; [b] AUC ± 1.96 SE; [c] DeLong et al., 1988

Fig. 6. Pairwise comparison of ROC curves. **(A)** Pairwise comparison of ROC curves between miR-144-5p (blue line) and miR-144-5p+MMSE (red dotted line). **(B)** Pairwise comparison of ROC curves between miR-374 (blue line) and miR-374+MMSE (red dotted line). **(C)** Pairwise comparison of ROC curves between miR-144-5p+miR-374 (blue line) and miR-144-5p+miR-374+MMSE (red dotted line).



In this study, a cohort point was selected by Youden index J, so that the best relations considering the AUC values were chosen for further analysis. To better understand the data, interactive dot diagrams were prepared for miRNAs analysis and their association with MMSE (Fig. S2). As a result, the association of miRNAs with MMSE improved the AUC, as well as the sensitivity and specificity, in correctly classifying AD diagnosis with greater statistical significance ($p \leq 0.0001$) for miR-144-5p and miR-374.

The analysis of the pathways regulated by these miRNAs, according to the DIANA LAB site (multiple.php http://diana.cslab.ece.ntua.gr/pathways/index_) in DIANA micro v4.0 (Beta version) multiple microRNA analysis, demonstrated that miR-144-5p, miR-221 and miR-374 together are responsible for the regulation of 1.619 genes in 355 different pathways and their intersection for 22 genes and four pathways (Table 3).

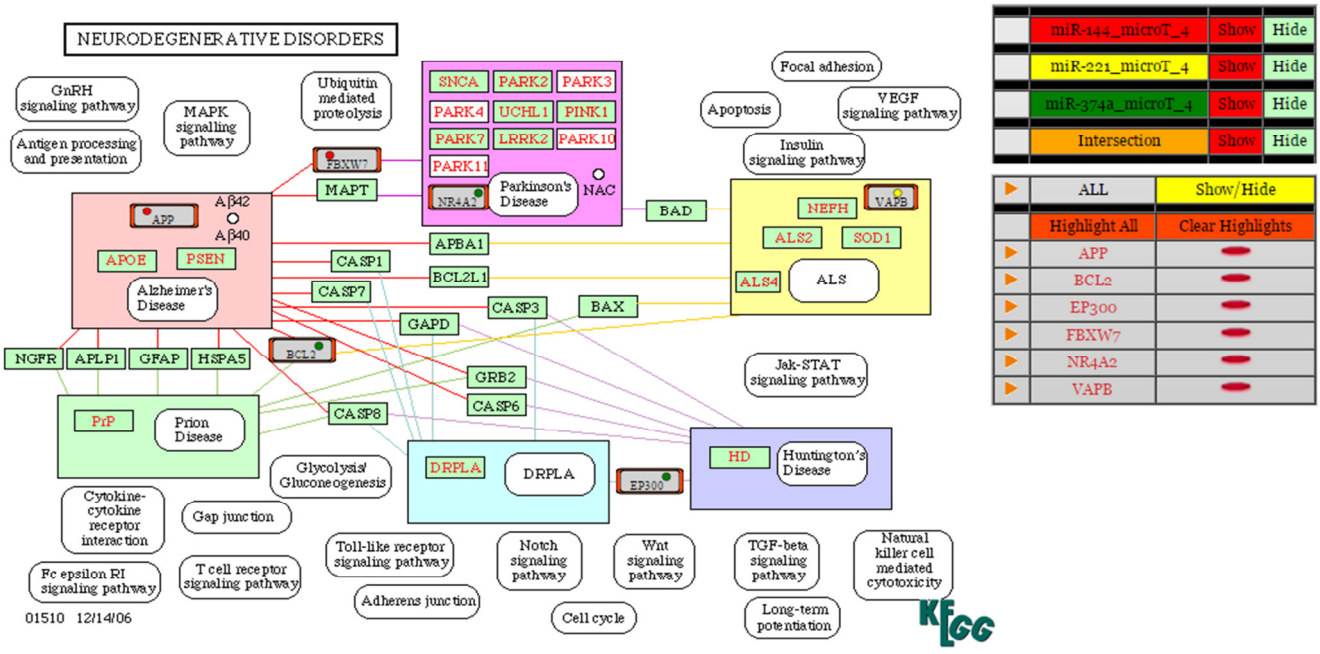
Table 3. MiRNAs target genes and pathways. DIANA LAB (Papadopoulos *et al.*, 2009)

Input List Name	Number of Genes	Number of Genes in Pathways
Union	1619	355
miR-144-5p_	708	160
miR-221_	366	85
miR-374_	814	166
Intersection	22	4

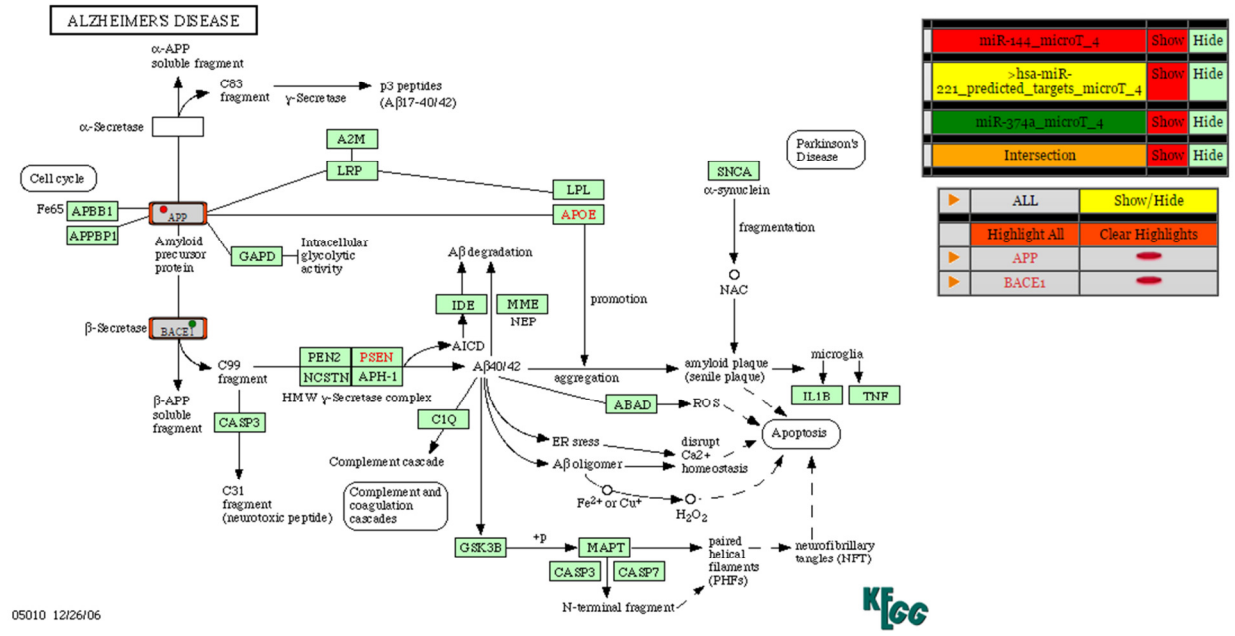
Among several active pathways of such miRNAs, stands out the *"Neurodegenerative disorders"* and *"Alzheimer's disease"*, which are shown in Fig. 7A,B.

Fig. 7A,B. **(A)** miRNAs target genes and pathways in neurodegenerative disorders or **(B)** Alzheimer´s disease. DIANA LAB[3] (Papadopoulos *et al.*, 2009)

A



B



ALS, amyotrophic lateral sclerosis**.**

[3] http://diana.cslab.ece.ntua.gr/pathways/view_results.php

In *"Neurodegenerative disorders"* pathway these miRNAs regulate different target genes, which act on different dementia types. However, in *"Alzheimer's disease"* pathway, miR-144-5p and miR-374 directly regulate relevant AD genes (APP and BACE1, respectively).

Discussion

Changes in miRNAs networks can result in brain neurodegenerative diseases (Kim et al., 2007). In addition, deregulation of specific miRNAs, such as miR-9 and miR-107, which are associated with AD due their impact on the insulin resistance control and innate immunological pathways, can be related to several types of neurological disorders (Ghelani et al., 2012). MiRNAs also demonstrate unique pattern of expression in accordance with their location, for example, in brain miR-221 and miR-222 are preferably found in the hippocampal region and miR-195, miR-497 and miR-30b in the cerebellum (Feng and Feng, 2011).

In the current literature, only three publications highlight miRNAs deregulation in peripheral blood mononuclear cells (PBMC) in AD patients. Villa et al. (2011) verified gene expression of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 engaged in the maturation of APP (mRNA) and demonstrated a negative correlation between this gene and miR-590-3p. According to these findings, hnRNP-A1 and its regulatory transcription factor (miR-590-3p) are deregulated in AD patients (Villa et al., 2011). Schipper et al. (2007) analyzed PBMC's expression profile of 462 miRNAs in 16 AD patients and 16 healthy controls in order to compare these findings with sub-regulated gene targets described previously in the AD literature. In this study, many miRNAs had moderate overexpression (range 1.1 to 1.4-fold) in AD subjects, compared to control group, especially miR-34a and miR-181b, which have high association with reduced AD gene expression and regulate transcription/translation factors, synaptic activity and cellular homeostasis (Schipper et al., 2007).

Leidinger et al. (2013) observed deregulation of 140 miRNAs in whole blood of AD patients. Of these, a panel of 12 miRNAs, 7 miRNAs upregulated (miR-112, miR-161, let-7d-3p, miR-5010-3p, miR-26a-5p, miR-1285-5p, miR-151a- 3p) and 5 downregulated (miR-103a-3p, miR-107, miR-532-5p, miR-26b-5p and let-7f-5p) were selected for validation by RT-qPCR in 202 subjects. The selected set of miRNAs showed 95% specificity and 92% sensitivity for the AD diagnosis. Moreover, these 12 miRNAs also achieved accuracy of 74%-78% in distinction AD from other neurological diseases (Leidinger et al., 2013).

Using RT-qPCR techniques we tested through Megaplex^{TM} 667 miRNAs extracted from whole blood of AD subjects and controls. The objective was search for miRNAs acting in AD and ADAM10 regulation. According to data analysis, all miRNAs tested in this study were downregulated, associated with AD or associated with AD and ADAM10 regulation, according to MirWalk 2.0.

Our study found a strong tendency to downregulation for 21 analyzed miRNAs normalized by miR-U6 endogenous control, however, only miR-144-5p, miR-374 and miR-221 showed significantly reduced levels in AD compared to the control group. These miRNAs were also the only ones to present significant AUC values for AD diagnosis. As also noted by (Leidinger et al., 2013), miR-144-5p had the lowest level of expression (not validated) in AD (AUC 0.9138; p = 8.35*10-6). However, the literature does not address the specificity of this miRNA to AD (Smith-Vikos and Slack, 2013), whereas other studies also describe its deregulation in other human diseases, including several cancers (Keller et al., 2011). Our study, however, did not observe any other similarity with miRNAs presented in this study. On the other hand, Cheng and co-workers (2013) was the first group to demonstrate the function of miR-144 in ADAM10 regulation, since its overexpression resulted in decreased levels of ADAM10 in human neuroblastoma SH-SY5Y cells, in vitro. Another important finding is the regulatory activity of AP-1 in miR-144 transcription, as the increase of its regulation by AP-1 promoted the suppression of ADAM10. Another miRNA possibly involved in ADAM10 regulation is miR-451, which together with miR-144 may act in the inhibition of ADAM10 expression (Cheng et al., 2013). However, our study found no significant change in the expression of miR-451 in the analyzed subjects.

Persengiev et al. (2011) also revealed that miR-144 activity is increased in AD cerebellum/cortex and present a central role in regulating Ataxin 1 (ATXN1) gene expression that is associated with development of spinocerebellar ataxia type 1 (SCA1). Pathogenic ATXN1 contains an elongated glutamine stretch, which triggers spontaneous misfolding and self-assembly of the protein into aggregates (Petrakis et al., 2012). Thus, activation of miR-144 can induce a reduction of the cytotoxic ATXN1 (Persengiev et al., 2011). The investigative study of Augustin et al. (2012) used computational approach and experimental validation to suggest possible miRNAs acting in the ADAM10 regulation in AD (Augustin et al., 2012). It was found that three miRNAs (miR-103, miR-107 and miR-1306) are related to AD and have conserved binding sites for mRNA coding for ADAM10 among species. From these, miR-103 (p = 0.0065) and miR-107 (p = 0.0009) showed a significant overlap with AlzGene database. In SH-SY5Y cells, these three miRNAs showed significant inhibitory

activity in ADAM10 expression. However, miR-103 and miR-107 analyzed in our study also presented downregulated in AD (not significant).

Hippocampal and prefrontal cortex analysis of subjects with LOAD (late onset disease Alzheimer's), evidenced miRNAs deregulation (Lau et al., 2013). This study confirmed a strong decrease of miR-132-3p and of three family-related miRNAs encoded by the same miRNA cluster on chromosome 17. Besides this miRNA family, miR-374 was also tested and called differentially expressed by next-generation sequencing provided a log2 fold change of 1.99 ($p = 0.0001$) in AD subjects, compared to healthy controls (Lau et al., 2013). This result is not in agreement with our data that showed a significant downregulation of this miRNA in total blood of AD group.

Transcriptional activation of miR-221 regulate several genes involved in human cancers such as glioblastoma, melanoma, gastric, pancreatic, renal, ovarian, and prostate cancer, as well as in the metastatic process (Teixeira et al., 2012). Recently it was observed that miR-221 downregulation acts indirectly on the ADAM10 protein levels through suppressor gene metalloprotease inhibitor 3 (TIMP3) (Teixeira et al., 2012). TIMP3 acts in ADAM10 inhibition and also in APP (Amour et al., 2000; Hoe et al., 2007), in order to promote endocytosis of these structures and β-secretase cleavage, so that these findings corroborate with our results.

According to the DIANA LAB, genes related to miRNAs selected in our study are APP and BACE1 (for AD) and APP, BCL2, EP300, FBXW7, NR4A2 and VAPB (for neurodegenerative disorders) (Figure 7AB). The literature is clear and consistent about the activities of APP and BACE1 in AD, where through the amyloidogenic pathway generate Aβ formation (Lichtenthaler, 2011). Moreover, cleavage of APP by ADAM10 prevents this formation besides having neuroprotective characteristics (Morishima-Kawashima and Ihara, 2002). It also highlights the indirect action of miR-221 in regulating ADAM10 through TIMP3, which inhibits the α-secretase action on APP. Therefore, the downregulation of miR-144-5p, miR-374 and miR-221 corroborates with AD protein findings and places such miRNAs as potential biomarkers molecules to improve AD diagnosis. The other genes involved, although do not correlate directly with AD, have relevant roles in several indirect mechanisms and are new targets in AD researches.

In previous studies, we and others observed a significant difference in platelet ADAM10 expression between AD patients and cognitively healthy matched according to gender, age and education level (Colciaghi et al., 2002; Manzine et al., 2013c). This difference was also seen with the clinical progression of the disease and correlated with

patients cognitive performance, as measured by e.g. clock drawing test (Manzine et al., 2013a) or MMSE scores (Manzine et al., 2013b), revealing that ADAM10 expression can be used as a potential peripheral biomarker in AD. In addition, this reduction of platelet ADAM10 levels has been shown not be caused by reduced mRNA levels both in platelets or total blood (Manzine et al., 2015)

In order to investigate possible predictive miRNAs in ADAM10 regulation, this study tested 21 miRNAs of whole blood, not being observed upregulation of any miRNA with direct activity, that can answer and clarify our earlier ADAM10 findings, on the opposing, all miRNAs showed downregulated in AD. These findings may be explained by a compensatory strategy for increasing ADAM10 expression, which does not occur in AD due the presence of some other complicating mechanisms present in pathogenesis or even processes that involve indirect ADAM10 regulation may be present.

Future studies should attempt to inactivate or reduce the expression of these miRNAs (separately or together) checking for possible changes in ADAM10 expression and activity or even analyzing indirect action of these miRs on others genes in ADAM10 regulation. It should be noted however, that the small sample size, the influence of administered drugs on miRNAs profiles (Bocchio-Chiavetto et al., 2013) and the difference of biological material (platelets and total blood) may have influenced the outcomes of this study.

Therefore, the hypothesis that post-transcriptional or trafficking mechanisms can affect ADAM10 protein levels may explain its reduction in AD platelets. In this regard, it has been recently shown that ADAM10 removal from the plasma membrane is mediated by the binding to the clathrin adaptor (Marcello et al., 2013). ADAM10/AP2 interaction is increased in AD patients hippocampus at the early stages of the disease (Marcello et al., 2013) and, therefore, could entail an increased delivery of the enzyme to the lysosomal system, enhancing its degradation.

Peripheral biomarkers are usually a feasible, less invasive and reliable alternative to help AD diagnosis. The knowledge of the role of these markers and the multiple pathways that modulate their expression may provide therapeutic strategies to relief AD symptoms. Moreover, the increasing insights into the molecular mechanisms of AD, mainly those related to the amyloid pathogenic cascade can offer multiple potential targets for clinical interventions and for the early clinical diagnosis.

Conclusions

This study demonstrated that miR-144-5p, miR-374 and miR-221 are downregulated in AD subjects, with moderate accuracy diagnose. However, the association of selected miRNAs expression and MMSE was significantly better as an AD diagnostic tool, compared to its expression separately. Therefore, the use of these miRNAs, preferably in association with MMSE, appears to be a potential tool that can improve the AD clinical diagnosis.

Disclosure statement

The authors do not have any actual or potential conflicts of interests to disclose.

Acknowledgements

The authors thank all the subjects and their families. We would like to thank the sponsoring agency São Paulo Research Foundation (FAPESP – 2012/08654-7 and 2013/06879-4). We are also grateful to the nurse team for the biological material collection and to all family members and elderly who accepted to participate in this research.

References

Agostinho, P., et al., 2015. Localization and Trafficking of Amyloid-beta Protein Precursor and Secretases: Impact on Alzheimer's Disease. J Alzheimers Dis. 45, 329-47.

Amour, A., et al., 2000. The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett, 473.

Augustin, R., et al., 2012. Computational identification and experimental validation of microRNAs binding to the Alzheimer-related gene ADAM10. BMC Med Genet. 13, 35.

Bocchio-Chiavetto, L., et al., 2013. Blood microRNA changes in depressed patients during antidepressant treatment. Eur Neuropsychopharmacol. 23, 602-11.

Brucki, S. M., et al., 2003. [Suggestions for utilization of the mini-mental state examination in Brazil]. Arq Neuropsiquiatr. 61, 777-81.

Cheng, C., et al., 2013. MicroRNA-144 Is Regulated by Activator Protein-1 (AP-1) and Decreases Expression of Alzheimer Disease-related A Disintegrin and Metalloprotease 10 (ADAM10). Journal of Biological Chemistry. 288, 13748-13761.

Colciaghi, F., et al., 2002. alpha-secretase ADAM10 as well as alpha APPs is reduced in platelets and CSF of Alzheimer disease patients. Molecular Medicine. 8, 67-74.

Colciaghi, F., et al., 2004. Platelet APP, ADAM 10 and BACE alterations in the early stages of Alzheimer disease. Neurology. 62, 498-501.

Colombo, A., et al., 2013. Constitutive α- and β-secretase cleavages of the amyloid precursor protein are partially coupled in neurons, but not in frequently used cell lines. Neurobiology of Disease. 49, 137-147.

Delay, C., et al., 2012. MicroRNAs in Alzheimer's disease. Neurobiology of Disease. 46, 285-290.

DeLong, E. R., et al., 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics. 44, 837-45.

Feng, W., Feng, Y., 2011. MicroRNAs in neural cell development and brain diseases. Sci China Life Sci. 54, 1103-12.

Ghelani, H. S., et al., 2012. MicroRNAs as newer therapeutic targets: A big hope from a tiny player. J Pharmacol Pharmacother. 3, 217-27.

Hardy, J., Selkoe, D. J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 297, 353-6.

Hoe, H. S., et al., 2007. The metalloprotease inhibitor TIMP-3 regulates amyloid precursor protein and apolipoprotein E receptor proteolysis. J Neurosci, 27, 10895-10905.

Keller, A., et al., 2011. Toward the blood-borne miRNome of human diseases. Nat Meth. 8, 841-843.

Kim, J., et al., 2007. A MicroRNA feedback circuit in midbrain dopamine neurons. Science. 317, 1220-4.

Lau, P., et al., 2013. Alteration of the microRNA network during the progression of Alzheimer's disease. EMBO Mol Med. 5, 1613-34.

Leidinger, P., et al., 2013. A blood based 12-miRNA signature of Alzheimer disease patients. Genome Biol. 14, 2013-14.

Lichtenthaler, S. F., 2011. Alpha-secretase in Alzheimer’s disease: molecular identity, regulation and therapeutic potential. Journal of Neurochemistry. 116, 10-21.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25, 402-8.

Manzine, P. R., et al., 2013a. Platelet a disintegrin and metallopeptidase 10 expression correlates with clock drawing test scores in Alzheimer's disease. Int J Geriatr Psychiatry. 22, 414-20.

Manzine, P. R., et al., 2013b. Correlation Between Mini-Mental State Examination and Platelet ADAM10 Expression in Alzheimer's Disease. J Alzheimers Dis. 36, 253-60.

Manzine, P. R., et al., 2013c. ADAM10 as a Biomarker for Alzheimer s Disease: A Study with Brazilian Elderly. Dementia and Geriatric Cognitive Disorders. 35, 58-66.

Manzine, P. R., et al., 2013d. ADAM10 as a Biomarker for Alzheimer s Disease: A Study with Brazilian Elderly. Dementia and Geriatric Cognitive Disorders. 11.

Manzine, P. R., et al., 2015. ADAM10 gene expression in the blood cells of Alzheimer's disease patients and mild cognitive impairment subjects. Biomarkers. 20, 196-201.

Marcello, E., et al., 2013. Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. The Journal of Clinical Investigation. 123, 2523-2538.

Morishima-Kawashima, M., Ihara, Y., 2002. Alzheimer's disease: β-Amyloid protein and tau. Journal of Neuroscience Research. 70, 392-401.

Papadopoulos, G. L., et al., 2009. DIANA-mirPath: Integrating human and mouse microRNAs in pathways. Bioinformatics. 25, 1991-3.

Persengiev, S., et al., 2011. Genome-wide analysis of miRNA expression reveals a potential role for miR-144 in brain aging and spinocerebellar ataxia pathogenesis. Neurobiol Aging. 32, 6.

Petrakis, S., et al., 2012. Identification of Human Proteins That Modify Misfolding and Proteotoxicity of Pathogenic Ataxin-1. PLoS Genet. 8, e1002897.

Satoh, J., 2012. Molecular network of microRNA targets in Alzheimer's disease brains. Exp Neurol. 235, 436-46.

Schipper, H. M., et al., 2007. MicroRNA expression in Alzheimer blood mononuclear cells. Gene Regul Syst Bio. 1, 263-74.

Smith-Vikos, T., Slack, F. J., 2013. MicroRNAs circulate around Alzheimer's disease. Genome Biol. 14.

Teixeira, A. L., Gomes, M. and Medeiros, R., 2012. EGFR signaling pathway and related-miRNAs in age-related diseases: the example of miR-221 and miR-222. Front Genet, 3.

Villa, C., et al., 2011. Role of hnRNP-A1 and miR-590-3p in neuronal death: genetics and expression analysis in patients with Alzheimer disease and frontotemporal lobar degeneration. Rejuvenation Res. 14, 275-81.

Wimo, A., et al., 2014. Health economic evaluation of treatments for Alzheimer's disease: impact of new diagnostic criteria. J Intern Med. 275, 304-16.

Supplementary Table 1. Descriptive values for miRNAs individually in control and AD groups. GraphPad Prism 5.01.

	miR-144-5p		**miR-374**		**miR-221**		**miR-18a**		**miR-27b**		**miR-185**		**miR-107**	
	Control	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**
Minimum	0.08	0.03	0.03	0.03	0.12	0.08	0.13	0.15	0.14	0.16	0.21	0.23	0.05	0.12
25% Percentile	0.32	0.14	0.29	0.10	0.34	0.21	0.43	0.27	0.53	0.33	0.42	0.30	0.38	0.26
Median	1.22	0.21	1.34	0.14	1.26	0.40	1.10	0.42	1.10	0.51	0.88	0.45	1.29	0.40
75% Percentile	2.44	1.32	2.63	1.59	2.07	0.91	2.07	1.00	1.73	1.21	1.78	1.22	2.05	1.69
Maximum	5.51	2.67	5.52	3.47	4.51	2.58	2.54	3.28	3.43	2.24	2.90	2.50	4.08	2.69
Mean	1.67	0.69*	1.87	0.78*	1.37	0.66*	1.23	0.81	1.27	0.76	1.19	0.79	1.43	0.85
Std. Deviation	1.74	0.87	1.85	1.05	1.20	0.63	0.86	0.86	0.91	0.59	0.88	0.71	1.16	0.89
Std. Error	0.42	0.19	0.45	0.23	0.29	0.14	0.21	0.19	0.22	0.13	0.21	0.16	0.28	0.19
Lower 95% CI of mean	0.78	0.29	0.92	0.30	0.76	0.38	0.79	0.42	0.80	0.49	0.74	0.47	0.83	0.45
Upper 95% CI of mean	2.56	1.08	2.83	1.26	1.99	0.94	1.67	1.20	1.74	1.03	1.65	1.12	2.02	1.26

	miR-103		**miR-19b**		**miR-320B**		**miR-363**		**miR-15b-3p**		**miR-127**		**miR-194**	
	Control	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**
Minimum	0.07	0.11	0.10	0.20	0.17	0.15	0.19	0.17	0.11	0.20	0.24	0.16	0.17	0.23
25% Percentile	0.39	0.24	0.47	0.30	0.41	0.30	0.41	0.35	0.30	0.29	0.40	0.34	0.41	0.42
Median	1.38	0.35	1.22	0.35	0.86	0.52	0.96	0.53	0.96	0.52	0.96	0.74	1.03	0.55
75% Percentile	2.02	1.44	1.82	1.32	2.01	1.11	1.87	1.31	3.63	1.31	2.20	1.18	1.76	1.47
Maximum	3.93	2.82	3.91	3.33	4.76	3.17	5.72	2.88	7.03	2.99	3.47	4.58	5.19	2.24
Mean	1.44	0.83	1.29	0.81	1.39	0.87	1.52	0.87	1.97	0.85	1.31	0.97	1.46	0.85
Std. Deviation	1.18	0.87	0.99	0.80	1.29	0.84	1.61	0.74	2.33	0.80	1.08	1.01	1.42	0.63
Std. Error	0.28	0.19	0.24	0.18	0.31	0.18	0.39	0.16	0.56	0.17	0.26	0.22	0.34	0.14
Lower 95% CI of mean	0.83	0.43	0.78	0.44	0.72	0.49	0.69	0.53	0.77	0.48	0.75	0.51	0.73	0.56
Upper 95% CI of mean	2.04	1.22	1.80	1.17	2.05	1.25	2.35	1.21	3.17	1.21	1.86	1.43	2.19	1.14

Supplementary Table 1 Continuation

	miR-128a		miR-30a-5p		miR-451		miR-589		miR-181a-2-3p		miR-886-5p		miR-361-3p	
	Control	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**	**Control**	**AD**
Minimum	0.10	0.31	0.25	0.34	0.15	0.22	0.24	0.28	0.21	0.26	0.26	0.22	0.34	0.33
25% Percentile	0.45	0.47	0.47	0.56	0.35	0.32	0.51	0.53	0.37	0.52	0.43	0.48	0.54	0.67
Median	0.94	0.59	1.08	0.69	0.77	0.47	0.70	0.78	0.72	0.82	0.85	1.01	1.04	0.90
75% Percentile	2.56	1.42	1.45	1.25	1.87	1.99	2.08	1.27	2.13	1.33	1.76	1.65	1.72	1.46
Maximum	4.41	2.86	2.90	2.49	7.42	3.84	4.44	2.66	5.24	2.87	2.92	3.34	2.00	2.45
Mean	1.47	0.98	1.17	0.90	1.70	1.18	1.32	0.95	1.53	1.01	1.17	1.14	1.09	1.06
Std. Deviation	1.39	0.77	0.80	0.51	2.09	1.13	1.20	0.58	1.64	0.71	0.87	0.83	0.62	0.55
Std. Error	0.34	0.17	0.20	0.11	0.51	0.25	0.29	0.13	0.40	0.16	0.21	0.18	0.15	0.12
Lower 95% CI of mean	0.75	0.63	0.75	0.67	0.63	0.66	0.71	0.69	0.68	0.68	0.73	0.76	0.77	0.81
Upper 95% CI of mean	2.18	1.34	1.58	1.14	2.77	1.69	1.94	1.21	2.37	1.33	1.62	1.51	1.41	1.32

***** <0.05 (Mann-Whitney U-test). GraphPad Prism 5.01

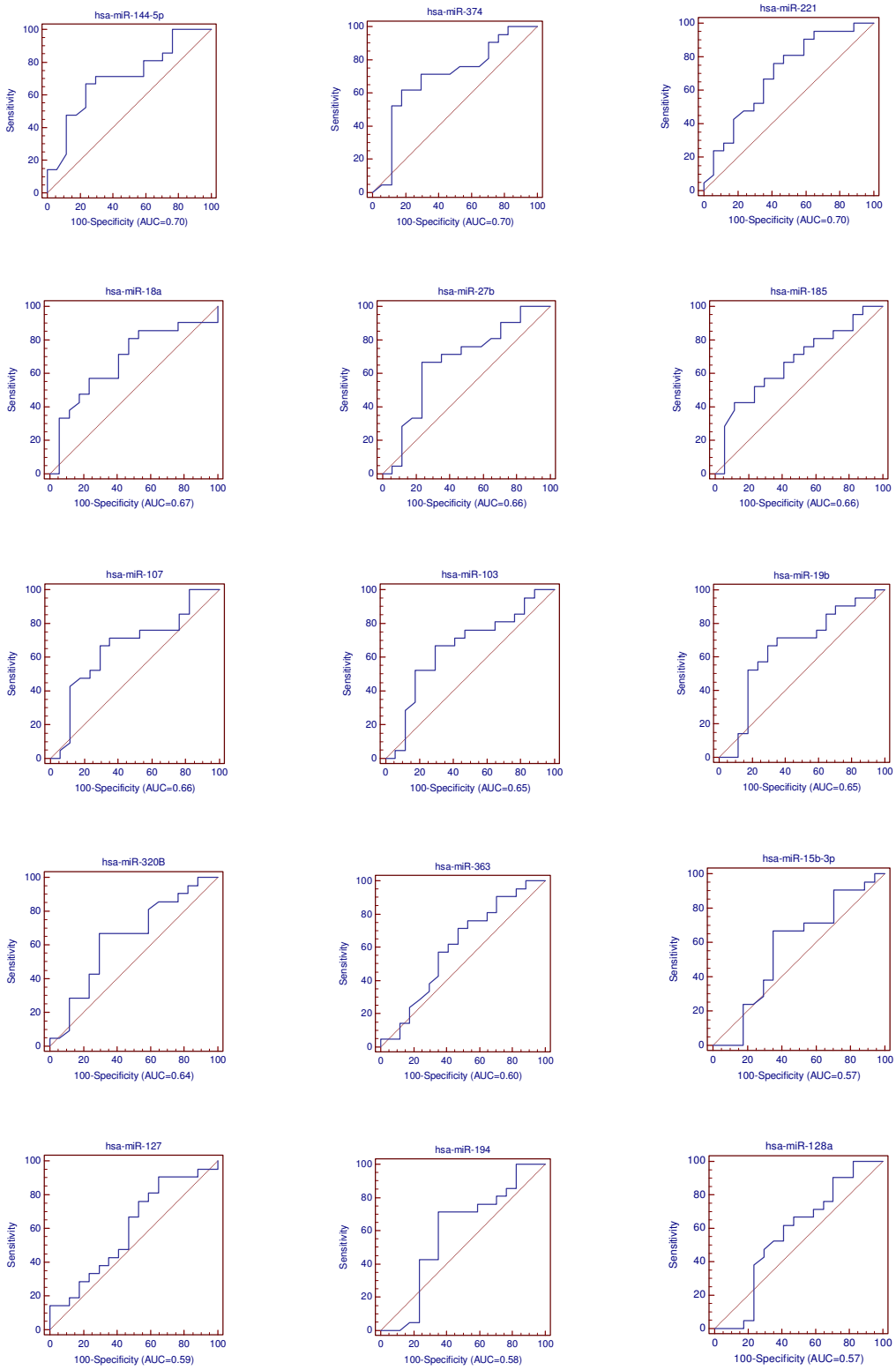
Supplementary Table 2. Descriptive values of area under the ROC curve (AUC) for miRNAs individually. with associated criterion (best Youden index J). sensitivity and specificity. GraphPad Prism 5.01.

	miR-144-5p	**miR-374**	**miR-221**	**miR-18a**	**miR-27b**	**miR-185**	**miR-107**
AUC	0.7073	0.6989	0.6947	0.6765	0.6695	0.6639	0.6611
Std. Error	0.08582	0.08950	0.08762	0.08978	0.09221	0.08938	0.09199
95% confidence	0.5390 to	0.5234 to	0.5229 to	0.5005 to	0.4887 to 0.8502	0.4886 to	0.4807 to
P value	*0.017*	*0.029*	*0.029*	0.063	0.076	0.070	0.080
Youden index J	≤ 0.36	≤ 0.17	≤ 0.86	≤ 1.05	≤ 0.58	≤ 0.33	≤ 0.46
Sensitivity	66.7	61.9	76.2	81	66.7	42.9	66.7
Specificity	76.5	82.4	58.8	52.9	76.5	88.2	70.6
	miR-103	**miR-19b**	**miR-320B**	**miR-363**	**miR-15b-3p**	**miR-127**	**miR-194**
AUC	0.6569	0.6527	0.6387	0.6008	0.57	0.5966	0.5868
Std. Error	0.09240	0.09437	0.09298	0.09554	0.09930	0.09469	0.09977
95% confidence	0.4757 to	0.4677 to	0.4564 to	0.4135 to	0.4062 to 0.7955	0.4110 to	0.3912 to
P value	0.09	0.112	0.1462	0.2906	0.48	0.3112	0.39
Youden index J	≤ 0.43	≤ 0.8	≤ 0.8	≤ 0.82	≤ 0.6	≤ 1.67	≤ 0.74
Sensitivity	66.7	66.7	66.7	71.4	66.7	90.5	71.4
Specificity	70.6	70.6	70.6	52.9	64.7	35.3	64.7
	miR-128a	**miR-30a-5p**	**miR-451**	**miR-589**	**miR-181a-2-3p**	**miR-886-5p**	**miR-361-3p**
AUC	0.5714	0.5644	0.5322	0.5308	0.5140	0.5098	0.5014
Std. Error	0.09936	0.09916	0.09789	0.1001	0.1007	0.09606	0.1015
95% confidence	0.3766 to	0.3700 to	0.3403 to	0.3346 to	0.3166 to 0.7114	0.3215 to	0.3023 to
P value	0.48	0.52	0.74	0.76	0.88	0.92	0.99
Youden index J	≤ 0.67	≤ 1.32	≤ 0.21	≤ 1.79	≤ 1.61	≤ 0.25	≤ 0.64
Sensitivity	61.9	81	0	95.2	85.7	14.3	14.3
Specificity	58.8	41.2	82.35	29.4	35.3	100	58.8

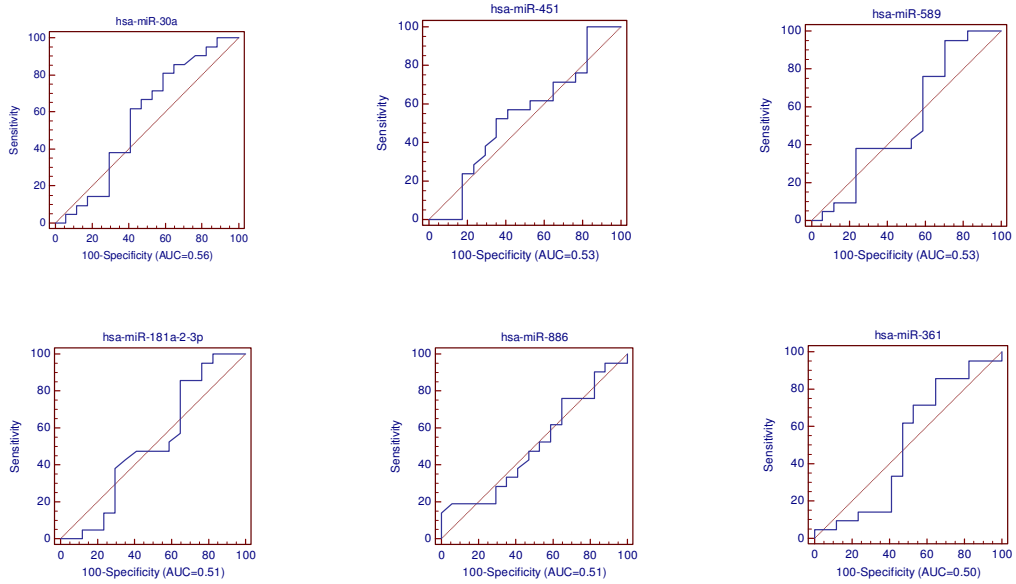
Supplementary Table 3. Descriptive values of area under the ROC curve (AUC) for combination of selected miRNAs and their association with MMSE with associated criterion (best Youden index J), sensitivity and specificity. GraphPad Prism 5.01.

	miR-144-5p+miR374	**miR-144-5p+miR-221**	**miR-374+miR-221**	**miR-144-5p+miR374+miR221**
AUC	0.71	0.71	0.68	0.72
Std. Error	0.0899	0.0869	0.0911	0.0880
95% confidence interval	0.535 to 0.888	0.545 to 0.886	0.508 to 0.865	0.547 to 0.892
P value	0.0187	0.0131	0.0409	0.0125
Youden index J	≤ 0.43	≤ 2.13	≤ 1.31	≤ 1.18
Sensitivity	61.9	80.95	71.43	61.9
Specificity	88.24	58.82	70.59	82.35
	miR-144-5p+MMSE	**miR-374+MMSE**	**miR-221+MMSE**	**miR-144-5p+ miR-374+MMSE**
AUC	0.81	0.82	0.83	0.79
Std. Error	0.0697	0.0695	0.0657	0.0738
95% confidence interval	0.681 to 0.955	0.689 to 0.961	0.700 to 0.958	0.648 to 0.937
P value	< 0.0001	< 0.0001	< 0.0001	0.0001
Youden index J	≤ 1.18	≤ 1.12	≤ 1.11	≤ 1.31
Sensitivity	66.67	66.7	57.14	61.9
Specificity	100	100	100	94.1
	miR-144-5p+miR-221 +MMSE	**miR-374+miR-221 +MMSE**	**miR-144-5p+miR374 +miR221+MMSE**	
AUC	0.80	0.80	0.78	
Std. Error	0.0704	0.0716	0.0747	
95% confidence interval	0.666 to 0.942	0.661 to 0.941	0.634 to 0.927	
P value	< 0.0001	< 0.0001	0.0002	
Youden index J	≤ 1.68	≤ 1.25	≤ 1.87	
Sensitivity	61.9	57.1	61.9	
Specificity	88.24	100	88.24	

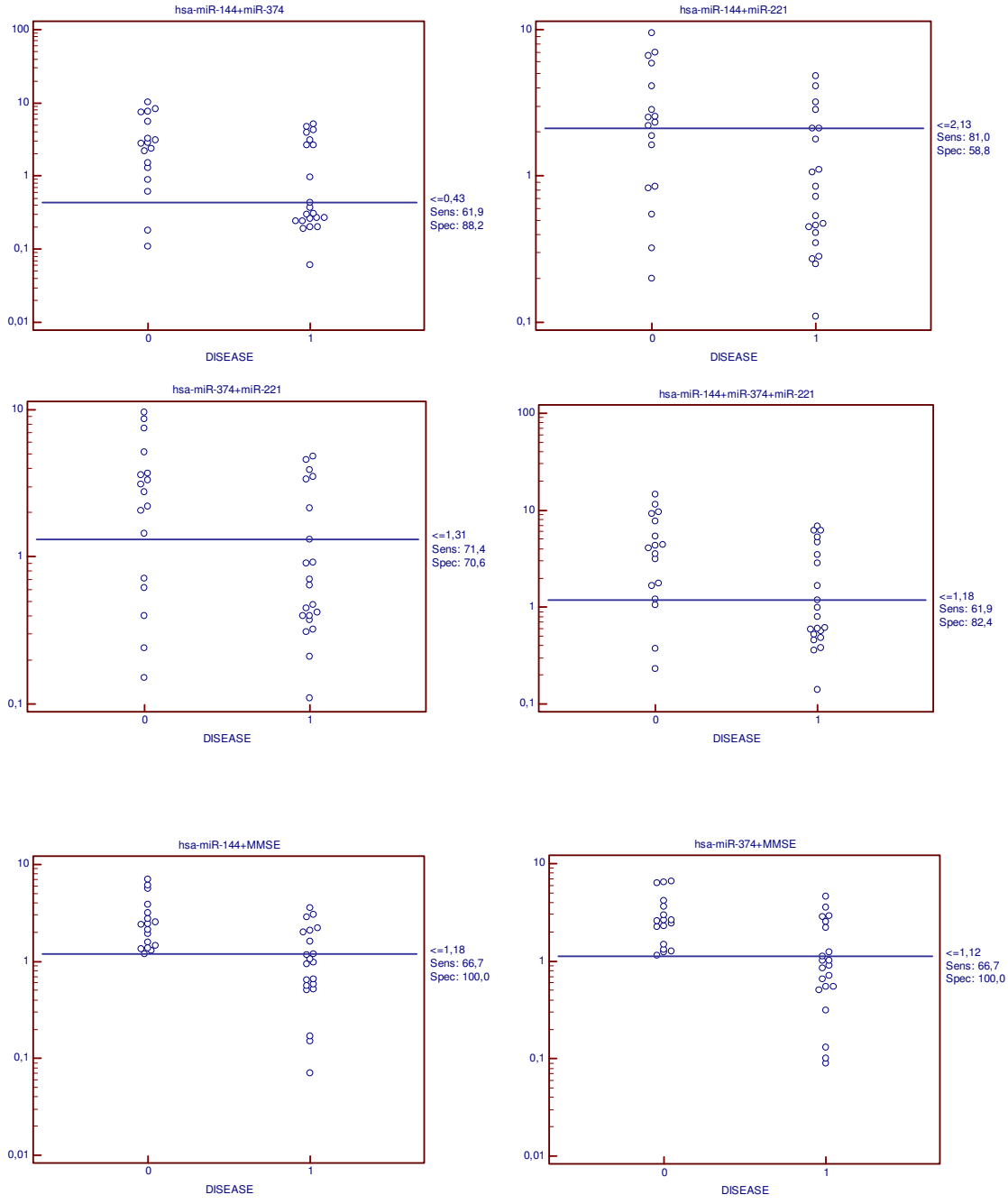
Supplementary Fig. 1. The receiver-operating characteristic (ROC) plots for the 21 miRNAs individually. The Sensitivity is plotted as a function of Specificity. These miRNAs are all characterized with area under the curve (AUC) values ranging from 0.50 to 0.70. Medcalc 14.8.1



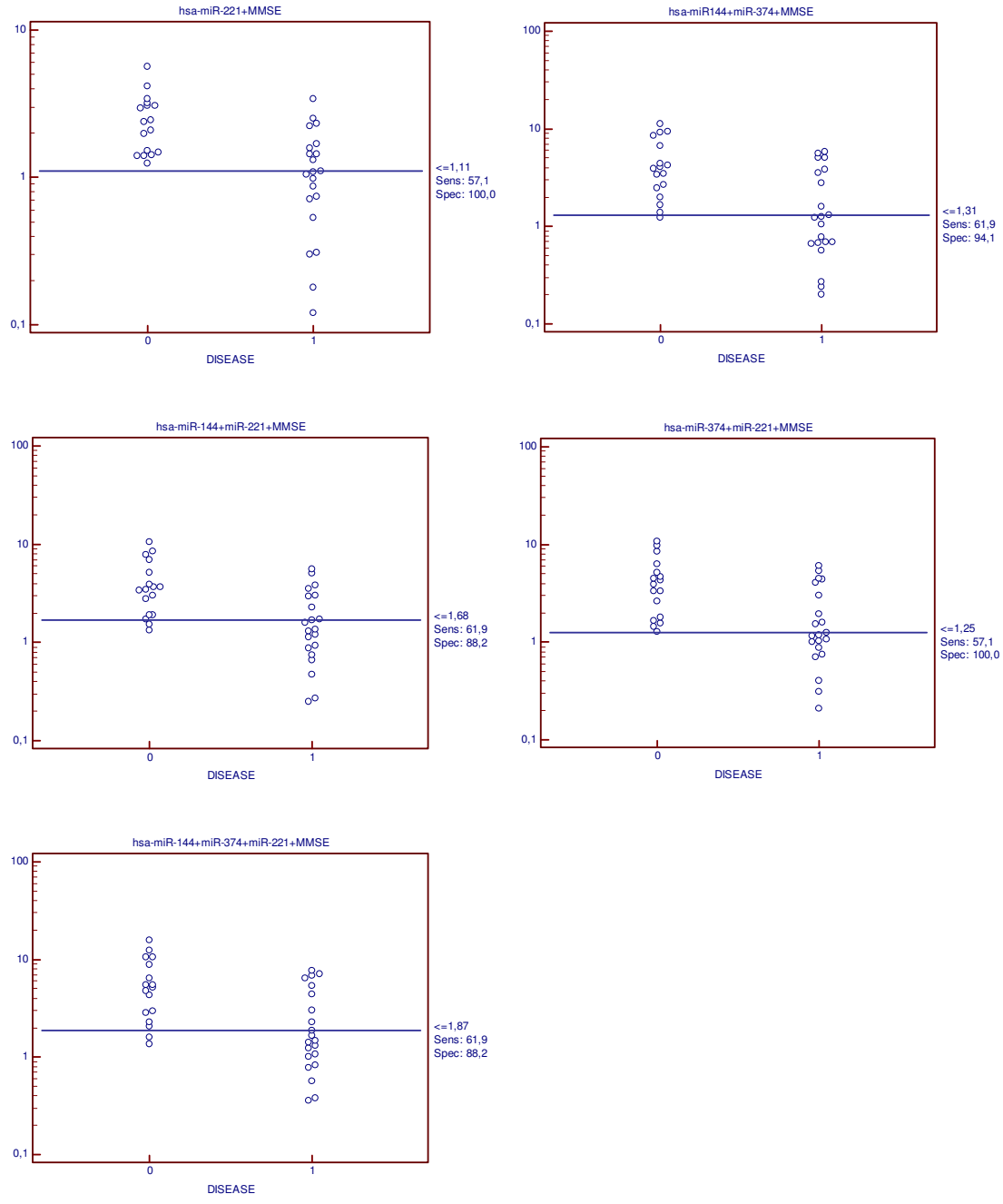
Supplementary Fig. 1 Continuation



Supplementary Fig. 2. Interactive dot diagrams for selected combination of miRNAs and in association with MMSE. The presence of AD disease (1) and non-AD (0) is plotted as a function of associated criterion (best Youden index J). Medcalc 14.8.1



Supplementary Fig. 2 Continuation



CONCLUSÕES

5. CONCLUSÕES

Com base nos resultados do presente estudo, foi possível concluir que:

- Não há diferença significativa da expressão gênica da ADAM10 no sangue total ou em plaquetas de sujeitos com DA ou com TNCL em comparação com sujeitos controles, mesmo com o avançar da doença.

- A diminuição dos níveis proteicos da ADAM10 plaquetária na DA não é ocasionada por alterações gênicas da ADAM10 em nível transcricional (mRNA) sugerindo, portanto, que mecanismos pós-transcricionais ou de tráfego proteico podem estar envolvidos.

- 21 miRNAs no sangue total estão sub expressos na DA, entretanto, apenas os miRNAs miR-144-5p, miR-374 e miR-221 apresentaram redução significativa na DA em comparação com sujeitos controles.

- Não foi observado super expressão de nenhum miRNA de ação direta sobre a ADAM10 que possa responder ou esclarecer nossos achados de sua redução proteica em plaquetas de sujeitos com DA, ao contrário, todos os miRNAs analisados mostraram-se sub expressos na DA. Estratégias compensatórias podem estar envolvidas na tentativa de aumentar a expressão da ADAM10, o que não ocorre na DA devido a presença de algum mecanismo complicador presente nesta patologia. Ou ainda processos que envolvem a regulação indireta da ADAM10 podem estar presentes. Estudos de regulação do tráfego intracelular ou de vias de sinalização poderiam ajudar a esclarecer esta hipótese.

- Os miRNAs miR-144-5p, miR-374 e miR-221 regulam os genes da APP, BACE1 e ADAM10, os quais atuam em diferentes caminhos para formação do Aβ. A sub expressão destes miRNAs corrobora com achados proteicos da APP, BACE1 e ADAM10 e direciona tais miRNAs como potenciais biomarcadores sanguíneos para auxílio no diagnóstico da DA.

ANEXOS

6. ANEXOS

6.1 MANUSCRITO III – Em fase de análise

MANZINE, P.R.; SOUZA, M.S.; COMINETTI, M.R. BACE1 levels are increased in plasma of Alzheimer's disease patients compared to matched cognitively healthy controls. *Cognitive Neuropsychiatry.* IF: 1.91

BACE1 levels are increased in plasma of Alzheimer's disease patients compared to matched cognitively healthy controls

Patrícia Regina Manzine, MSc[1]; Matheus da Silva Souza, BSc[1]; Márcia Regina Cominetti, PhD[1*]

*To whom correspondence should be addressed:
[1]Departamento de Gerontologia, Rodovia Washington Luís, Km 235, CEP 13565-905, São Carlos, SP, Brazil. Tel: +55-16-3306-6663; Fax: +55-16-3351-9628; E-mail: mcominetti@ufscar.br

Acknowledgements

This work was supported by the Sao Paulo Research Foundation (FAPESP) under Grant number 2013/06879-4. P.R. Manzine and M.S. Souza had scholarships sponsored by FAPESP (grants 2012/08654-7 and 2014/06580-1, respectively).

We are grateful to the nurse team for the biological material collection and to all family members and elderly who accepted to participate in this research. There are no conflicts of interest according to the authors.

Running head: BACE1 levels in plasma of Alzheimer's disease.

Keywords: Alzheimer disease; BACE1 protein; beta-site APP-cleaving enzyme 1; biomarkers; dementia.

Abstract

Introduction: The amyloidogenic pathway in Alzheimer's disease (AD) results in the production of amyloid-β (Aβ) peptide from amyloid-β protein precursor (AβPP) after two successive proteolysis by enzymes bearing β- and γ-secretase activities. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is the secretase that operates towards βA production in AD. Here we investigated both mRNA expression in total blood and plasma protein levels of BACE1, in AD patients compared to cognitively healthy subjects. **Methods:** Elderly patients with probable AD (n=47), diagnosed by the NINCDS-ADRDA criteria, grouped according to Clinical Dementia Rating (CDR), and a non-AD control group (n=32), matched by age, gender and education level were evaluated for mRNA expression for BACE1 using RT-qPCR. A subsample of n=21 AD and n=20 non-AD subjects had their plasma BACE1 levels analyzed, using ELISA (Enzyme-linked Immunosorbent Assay). Spearman correlation coefficient and logistic regression between BACE1 and MMSE scores were obtained. AUCs were used to compare the ROC curves. **Results:** We observed no significant differences on BACE1 mRNA expression between AD subjects and age, sex and scholarity-matched cognitively healthy controls, however higher levels of BACE1 were detected in plasma of AD patients. **Conclusions:** Blood-based diagnostic tools are highly desired in the clinics, in order to improve AD diagnosis. BACE1 plasma levels could provide an additional diagnostic tool for AD in association with neuropsychological tests.

Introduction

In Alzheimer's disease (AD), the amyloidogenic pathway results in the production of amyloid-β (Aβ) peptide from amyloid-β protein precursor (AβPP). Aβ has an hydrophobic nature and aggregates extracellularly, forming senile plaques (Selkoe, 1994). In turn, neurofibrillary tangles are intracellular fibrillar aggregates of the hyperphosphorylated microtubule-associated Tau protein. Together, these entities are the key microscopic neuropathological hallmarks of AD (Hardy & Selkoe, 2002). In AD, the predominant route of APP cleavage is headed initially by β-secretases, specially β-site APP cleaving enzyme 1 (BACE- 1), resulting into a large secreted APP fragment (sAβPPα) and a small membrane-bound fragment (c-99), which is further cleaved by γ-secretases resulting in Aβ generation (Mattson, 2004). In the non-amyloidogenic route, AβPP is cleaved by α-secretases (mainly ADAM10) in the middle of Aβ, preventing its liberation and consequent aggregation (Schroeder, Fahrenholz, & Schmitt, 2009).

BACE1 is a 70kDa type I transmembrane aspartyl protease formed by 501 amino acids with an extra cellular domain containing two active aspartyl residues at amino acid positions 93 and 289 (Kandalepas & Vassar, 2014). Several reports indicate an increase in BACE protein levels and activity in CSF of brain tissue of AD patients, as compared to control subjects, both in human or experimental models (Borghi et al., 2007; Ewers et al., 2011; Fukumoto, Cheung, Hyman, & Irizarry, 2002; Grimmer et al., 2012; Hampel & Shen, 2009; Johnston et al., 2005; Li et al., 2004; Perneczky, Alexopoulos, & Alzheimer's Disease euroimaging, 2014; Song et al., 2015; Stockley & O'Neill, 2007). In several cases, this increase appears to be correlated with amyloid load (Fukumoto et al., 2002; Grimmer et al., 2012; Johnston et al., 2005; Li et al., 2004; Stockley & O'Neill, 2007).

Regardless the immense financial, physical and emotional burden of AD, there is still no cure, effective treatment or specific early diagnosis tools for AD. Despite that, biomarkers have recently been included as evidence for AD pathology in the new research criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) work groups (McKhann et al., 2011). The introduction of AD biological markers reflects their importance in the scenario. Attempts to identify AD biomarkers have included cerebrospinal fluid (CSF) and imaging studies, with a number of candidate markers showing significant potential. However, lumbar puncture remains a relatively invasive procedure and may not be practical for conducting large-scale studies on AD. In addition, image methods of diagnosis,

such as Positron-Emission Tomography (PET) and Magnetic Resonance Imaging (MRI) are expensive and not readily available in many places, requiring specialized facilities to be obtained.

On the other hand, blood-based biomarkers are receiving increasing attention in research centers as objectively measurable diagnostic tools (Chiam, Dobson, Kiddle, & Sattlecker, 2015). Plasma or serum measurements are the gold standard in clinics (Humpel, 2011). Advantages of blood-based AD biomarkers include (1) their cost- and time-effective collection, (2) the existence of a blood protein signature, and possibly a transcript signature, that might act to increase confidence in diagnosis; and (3) the fact that CSF could be absorbed into blood every day, which results in some exchange of peptides, albeit at low levels, meaning that a protein fragment of sufficiently small size may be able to cross the blood-brain barrier, potentially allowing detection in serum or plasma.

We have been dedicated to the study of blood-based biomarkers for AD. Here, we investigated the plasma levels of BACE1, the main β-secretase involved in the formation of βA in AD, and observed no significant differences on BACE1 mRNA expression, however higher levels of BACE1 protein in plasma of AD patients, compared to cognitively healthy subjects were found, using a relatively inexpensive, sensitive and commercially available ELISA kit. Despite more studies using a higher number of subjects must be performed, our results indicate that plasma BACE1 has potential as a surrogate AD biomarker serving as a complementary diagnostic tool to be used in clinics.

Material and Methods

Subjects

This research received the approval of the Ethics Committee from the Federal University of São Carlos, São Paulo, Brazil, under protocol number (CAAE: 02760312.0.0 000.5504/112.543). This study was conducted on 47 elderly with probable AD and 32 subjects without the disease or other dementias (non-AD) matched according to education level, age and gender, recruited from municipal outpatient clinics. Probable AD diagnosis was made following the criteria of the National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINDS-ADRDA) (McKhann et al., 2011). AD sample was further subdivided according to the dementia degree based on the Clinical Dementia Rating (CDR1, mild; CDR2, moderate; and CDR3, severe) (Morris, 1993). For evaluation of BACE1 plasma levels, we selected 21 AD patients and 20 cognitively healthy subjects from total sample, also matched according to education level, age and

gender. All participants were submitted to the following exclusion criteria: major depressive disorder; bipolar disorder; schizophrenia; mental retardation and substances related disorder; following the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders); hydrocephalus and/or intracranial mass detectable on computed tomography or magnetic resonance imaging in the last 18 months; significant cerebrovascular disease; clinically significant alterations in vitamin B12 and syphilis serology; cranial trauma; clinically significant non-correctable visual and auditory deficits; non-compensated and clinically significant hypertension, diabetes, hypothyroidism, neoplasias, and hepatic, renal, cardiac and pulmonary diseases; and use of medication that may interfere with platelet functions (antiplatelet drugs, anticoagulants, corticosteroids). All subjects included had a standardized clinical workup based on neurological examinations, laboratory blood and urine analysis, a neuroimaging study (Head Computed Tomography and/or Magnetic Resonance Imaging) and a neuropsychological assessment, including a Mini Mental State Examination (MMSE) and CDR. All patients, or their caregivers when necessary, gave their written informed consent. Data were analyzed anonymously.

MMSE

In this work we used a Brazilian version of MMSE (Brucki, Nitrini, Caramelli, Bertolucci, & Okamoto, 2003). The cutoff values used for educational level were: 20 for illiterates; 1-4 years, 25; 5-8 years, 26; 9-11 years, 28; more than 11 years of formal studies, 29.

Blood collection

Personnel carrying out blood preparation, as well as subsequent analysis, were blind for diagnosis and treatment of subjects. For the isolation of total RNA, 2.5 ml of blood was collected in total RNA extraction tubes (PAXgene Blood RNA - Becton & Dickinson), according to the product manual. After collection, tubes were inverted 10 times and kept for two hours at room temperature in an upright position as manufacturer's instructions and were subsequently frozen at -80°C until use. For plasma isolation, blood was collected in sodium citrate tubes and kept at 4°C. Blood drawings were always taken in the morning, fasted and without tourniquet. After centrifugation at 3,000 rpm for 10 min at room temperature plasma aliquots were stored frozen at -80°C in polypropylene tubes until biochemical analysis. The time interval between blood drawing and centrifugation was never longer than 20–25 min.

qRT-PCR (Real-Time Quantitative Reverse Transcription PCR)

Total RNA isolation from blood samples was performed using PAXgene Blood RNA Isolation kit (Qiagen) according to the manufacture's manual. From the extracted total RNA, samples (1µl) were quantified with a Nanodrop (Thermo Scientific) to obtain absorbance values and their ratios (A260/A280 and A260/A230). For the reverse transcription, cDNA was prepared using Enhanced Avian RT First Strand Synthesis (Sigma-Aldrich) kit according to the manufacturer's manual with the proportion of 700ng of RNA/µL. cDNA dilutions series were performed in order to find the best reaction efficiency for each primer.

Primer sequences for qRT-PCR analysis were designed using NetPrimer (Premier Biosoft). Specificity of the primers was confirmed by BLAST searching. The length of the resulting amplicons was verified by agarose gel electrophoresis. For RT-qPCR, samples were amplified in a thermocycler (RotorGene RG6000 - Corbett Life Sciences) with SYBR Green Jump Start (Sigma-Aldrich), using specific primers for BACE1 (Gene bank reference NM_138973.3; Forward: 5'TTA CCA ACC AGT CCT TCC GC3'; Reverse: 5'ACA GCT CCC ATA ACA GTG CC3') designed to spam exons 7 and 8 junction in BACE1 DNA sequence. Endogenous controls were β-actin (Gene bank reference NM_001101.3; Forward: 5'GAC GGC CAG GTC ATC ACC ATT G3'; Reverse: 5'AGC ACT GTG TTG GCG TAC AGG3') and GAPDH (Gene bank reference NM_002046.4; Forward: 5'GAC TTC AAC AGC GAC ACC CAC3'; Reverse: 5'CAC CAC CCT GTT GCT GTA G3').

Conditions for the annealing temperature were optimized, and analyses of PCR products were performed on agarose (2%) ethidium bromide gel. PCR conditions were as follows: 10.0µl DEPC water, 12.5µl SYBR Green, 0.5µl pure cDNA, 1.0µl Primers Forward/Reverse [10nM], BACE1 Tm 63°C; β-actin and GAPDH Tm 69°C. The qRT-PCR reactions used were standardized to a final volume of 25µl. Cycling conditions were: Hold 94°C, 2min; Cycling (40 repeats) – Step 1: 94°C, 15 sec and Step 2: Tm (x)°C, 1 min; Melt 72-95°C, 45 sec on the 1st step. The melting curves showed a single amplified product and the absence of primer–dimer formation. Non-template controls were included for each primer pair reactions. The amplification efficiency (E) was determined on a cDNA dilution series on threshold 0.05. BACE1: E = 1.07, M = -3.14, R = 0.99; β-actin: E = 0.94, M = -3.47, R = 0.99 and GAPDH: E = 0.90, M = -3.55, R = 0.99. The internal calibrator used as a basis to standardize the results of expression was the control group ΔCts average. Calibration was determined by ΔΔCt = ΔCt (sample) - ΔCt (calibrator). Gene expression was assessed by relative quantification, using the formula $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

ELISA (Enzyme-linked Immunosorbent Assay)

Sandwich ELISA assays were performed using the kit Human Beta-site APP-Cleaving Enzyme 1 (catalog number E01B0315, Blue Gene Biotech Co.). Briefly, plasma samples (50µL) and standards corresponding to the curve points at the following concentrations: 0, 50, 100, 250, 500 and 1000pg/ml were applied in duplicate in an ELISA 96-well plate. Phosphate buffered saline (PBS, 50µl) was used as blank. Next, horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for BACE1 (100µl) was added to each well and plates were covered to protect from light and mixed under mild agitation for 1 hour at 37°C. After the incubation time, plate wells were thoroughly washed to remove any unbound components and substrate solutions were added to each well. Plates were incubated for 15 min at 25°C and then 50µl of stop solution were added to each well. Finally, the absorbance reading was performed using a microplate reader MCL-2100C with $\lambda = 450$ nm.

Statistical Analysis

Blind analysis of the subjects was used for the results quantification. In order to detect the sample normality level, thus ensuring greater reliability to the proposed analysis, this study employed the Kolmogorov-Smirnov test (KS). The statistic tests used in the analyses were the non-parametric Mann-Whitney U test and ANOVA One Way (Kruskal-Wallis test) between the groups and in the subgroups, respectively. This procedure was performed in two distinct steps. For BACE1 mRNA analyses (non-AD, n = 32; AD – CDR 1, 2 and 3, n = 47), and for plasma analyses separately (non-AD, n=20 and AD – CDR1, 2 and 3, n = 21). Spearman's correlation tests were subsequently performed between MMSE and BACE1 plasma levels.

In order to verify possible determinant factors for the increased AD occurrence probability, logistic regression analysis was employed. This analysis considered BACE1, age and education level in terms of its natural logarithms in order to allow the estimated coefficients to be interpreted in terms of elasticity, and in addition, reduce a potential heteroscedasticity, which was also addressed at the maximum likelihood estimator with a robust variance-covariance matrix. In addition, the marginal effects were calculated at means.

Sensitivity and specificity were calculated using the receiver operating characteristic (ROC) curve analysis. To compare the ROC curves in isolated BACE1 and its association with MMSE, the method of DeLong et al. (DeLong, DeLong, & Clarke-Pearson, 1988) (implemented in MedCalc), which evaluates the areas under the curves (AUCs), was

employed. The cutoff with highest Younden index (sensitivity plus specificity -1) was chosen (DeLong et al., 1988). Data were analyzed and the figures were built using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA), Stata MP12 (StataCorp LP, College Station, Tex., USA) and MedCalc 11.5.1 (MedCalc Software, MariaKerke, Belgium) softwares. A 5% significance level was chosen as standard.

Results

Real time quantitative PCR analyses for BACE1 mRNA expression were performed using total blood of 32 non-AD and 47 AD subjects. ELISA assays for BACE1 protein levels measures were accomplished using plasma of 20 non-AD and 21 AD subjects. Subjects were mostly female, with ages ranging from 60 to 90 years and a mean of 6 years of scholarity (Tables 1 and 2). As expected, MMSE scores were significantly lower for AD patients, compared to non-AD. The most prevalent comorbidities were hypertension and diabetes mellitus. These comorbidities were more pronounced in AD group, compared to non-AD subjects. For qRT-PCR analyses using total blood, according to the CDR, the majority of AD patients belonged to CDR1 (50%) and 2 (30%) (Table 1). MMSE scores were significantly different ($p < .05$) between patients and also along the disease's progression in both groups of patients, i.e. those whose blood was evaluated for BACE1 gene expression and protein content, as shown in Figure 1AB.

(Tables 1 and 2 and Figure 1AB about here)

BACE1 mRNA expression show no significant differences between groups with values ranging from 0.42 to 1.79 (non-AD) and 0.33 to 1.98 (AD) ($p = .51$). According to CDR, there were also no differences between subgroups for BACE1 gene expression. CDR1 patients had mRNA for BACE1 ranged from 0.67 to 1.82, 0.52 to 1.98 (CDR2) and 0.70 to 1.30 (CDR3) ($p = .92$) (Figure 2).

(Figure 2 about here)

On the other hand, BACE1 protein levels in plasma were increased in AD compared to non-AD group ($p = .02$), as shown in Figure 3AB. In this analysis we found a mean of 35pg/ml, ranging from 31 to 45.5 (non-AD) and a mean of 40pg/ml, ranging from 30 to 55 (AD). The analysis according to CDRs showed a significant difference only between the non-AD and CDR1 ($p = .01$). Among the subgroups (CDRs) there was no difference in BACE1 protein levels (Table 2, Figure 3B).

(Figure 3AB about here)

To verify the correlation between MMSE and BACE1 plasma levels, Spearman's correlation test was used. It was found a significant and negative correlation between these variables ($r = -0.25$, $p = .05$), which means that the lower the MMSE value, the higher the plasma BACE1 levels (Figure 4). Logistic regression analysis demonstrated that factors such as gender, age and scholarity did not present statistical significance and, therefore, were not determinant variables for the increased AD occurrence probability (Table 3). It can be observed in Table 3 that only the p-value referring to the BACE1 variable is statistically significant ($p = .01$). In terms of elasticity, the coefficient was -1.644, which means, for example, that a 1% increase in BACE1 expression would cause a 1.6% increase in the AD probability of occurrence.

(Figure 4 and Table 3 about here)

The sensitivity and specificity of BACE1 and MMSE association are shown in Table 4. The AUC of BACE1 plasma levels was 0.70 (95% CI 0.54 -0.87; $p = .01$). However, when BACE1 is associated with MMSE scores, a higher AUC value was observed (0.80, 95% CI 0.65 -0.95; $p = .0001$). The combination of BACE1 and MMSE at a cutoff ≤ 56.9 presented sensitivity of 71%, and specificity of 95%, which was significantly better for predicting AD, compared to the AUC of BACE1 plasma levels separately (Figure 5). The association of MMSE and BACE1 expression resulted in an increase of 14%, compared to BACE1 expression alone, in correct classifications of the AD diagnosis (no false positives).

(Table 4 and Figure 5 about here)

Discussion

The 2014 Report on the Milestones for the US National Plan to Address Alzheimer's Disease points out that AD biomarkers should not only be minimally invasive, but also portable and inexpensive to enable large studies in diverse populations (Alzheimer's Association National Plan Milestone et al., 2014). Following this recommendation, we investigated whether total blood BACE1 mRNA expression or BACE1 plasma protein levels would be altered in Alzheimer's disease patients, compared to age, sex and scholarity-matched cognitively healthy controls. The findings of this work show no significant differences on total blood BACE1 mRNA expression, as evaluated by qRT-PCR, however elevated protein levels of BACE1 in plasma samples of AD patients, compared healthy controls, were found using an ELISA assay.

Previous reports used mainly brain tissues or cerebrospinal fluid (CSF) to evaluate BACE1 expression. Borghi and co-workers (Borghi et al., 2007) have shown a

significant increase of BACE1 activity and products of lipid peroxidation in brain tissue of AD cases, with normal gene expression, and non-significant elevation of protein levels. Similarly, other groups have demonstrated an elevation of BACE1 activity in brain tissue of sporadic AD cases, particularly temporal cortex, hippocampus and medial temporal and superior parietal gyri (Coulson et al., 2010; Fukumoto et al., 2002; Holsinger, McLean, Beyreuther, Masters, & Evin, 2002; Yang et al., 2003). On the other hand, other authors reported no differences on BACE1 mRNA expression levels in brain tissues from AD compared to non-demented brains (Gatta, Albertini, Ravid, & Finazzi, 2002).

Since the discovery that BACE1 holoprotein and ectodomain can be released from cultured neurons into the milieu (Murayama, Kametani, & Araki, 2005) and the first detection of soluble form of BACE1 in CSF (Holsinger, Lee, Boyd, Masters, & Collins, 2006; Verheijen et al., 2006) new perspectives for blood-based BACE1 biomarkers and AD diagnosis were open. Nevertheless, great variability was found among different studies. Several reports have indicated increasing BACE1 levels and/or activity in CSF of MCI (Hampel & Shen, 2009; Zhong et al., 2007) or AD (Barao et al., 2013; Ewers et al., 2011; Ewers et al., 2008; Grimmer et al., 2012; Mulder et al., 2010; Zetterberg et al., 2008) patients.

On the other hand, some reports demonstrated a significant decline in age-adjusted CSF BACE1 activity in AD patients, compared to controls (Rosen et al., 2012; Wu et al., 2008) and other authors however, have found no significant differences for BACE1 activity in CSF between controls and MCI or AD patient groups. They found, though, significant correlations with BACE1 activity for CSF APPβ and total Tau (Perneczky et al., 2014; Savage et al., 2015).

In regard to BACE1 plasma levels and activity in patients with Alzheimer's disease, very little information is available. Wu and collaborators (Wu et al., 2008) have developed assays to evaluate BACE1 in CSF and more recently, applied these assays to measure BACE1 activity in plasma. They demonstrated a significant increase (32%) in plasma BACE1 activity in AD patients compared to age-matched controls (Wu et al., 2012). We had 14.4% increases of BACE1 plasma protein levels in AD patients, compared to non-AD controls. Their results are consistent with ours, except that we assessed the quantity and not the protein activity, albeit these parameters are most of the time, positively related.

When subjects were separated in different subgroups (CDRs), we found a significantly increase in BACE1 plasma levels only for CDR1 subgroup, when compared to

non-AD. This result is in line with reported by Rosen and colleagues (Rosen et al., 2012) that suggested that BACE1 activity may be elevated in CSF of early-stage AD patients.

Johnston and co-workers (Johnston et al., 2008) analyzed platelet β-secretase activity in 86 AD subjects and compared with 115 age-matched healthy controls. They found 17% elevation of β-secretase activity in AD group and no significant correlation between platelet β-secretase activity and MMSE scores. On the contrary, our results demonstrated a significant and negative correlation between BACE1 and MMSE. It should be noted that results of Johnston and colleagues represent the activity of β-secretases in general and not BACE1 specifically, since they used a synthetic peptide that could be cleaved by a range of membrane-associated platelet proteases (Johnston et al., 2008).

We finally evaluated the ability of plasmatic BACE1 to identify AD subjects compared with controls using ROC AUC analysis. We found that the association of MMSE and BACE1 levels resulted in an increase of 14%, compared to BACE1 expression alone, in correct classifications of the AD diagnosis. Wu and co-workers (Wu et al., 2012) found exactly the same results with ROC AUC of 0.7.

Limitations of this work include mainly the limited number of participants, although we have been able to match all the subjects in the control and those with AD, by age and sex education.

This study demonstrated no significant differences between BACE1 gene expression in AD, compared to non-AD subjects. On the other hand, plasma protein BACE1 levels were increased in AD. The association of MMSE and BACE1 expression was significantly better as a diagnostic tool compared with BACE1 expression separately. Therefore, plasma-based detection of BACE1, preferably in association with MMSE, appears to be a potential tool to improve the early AD clinical detection. To the best of our knowledge, this is the first time that a relatively inexpensive, sensitive and commercially available ELISA kit was used to detect BACE1 in plasma samples and to effectively differentiate non-AD from AD patients.

References

Alzheimer's Association National Plan Milestone, W., Fargo, K. N., Aisen, P., Albert, M., Au, R., Corrada, M. M., Carrillo, M. C. (2014). 2014 Report on the Milestones for the US National Plan to Address Alzheimer's Disease. Alzheimers Dement, 10(5 Suppl), S430-452. doi: 10.1016/j.jalz.2014.08.103

Barao, S., Zhou, L., Adamczuk, K., Vanhoutvin, T., van Leuven, F., Demedts, D., De Strooper, B. (2013). BACE1 levels correlate with phospho-tau levels in human cerebrospinal fluid. Curr Alzheimer Res, 10(7), 671-678

Borghi, R., Patriarca, S., Traverso, N., Piccini, A., Storace, D., Garuti, A., Massimo, T. (2007). The increased activity of BACE1 correlates with oxidative stress in Alzheimer's disease. Neurobiol Aging, 28(7), 1009-1014. doi: 10.1016/j.neurobiolaging.2006.05.004

Brucki, S. M., Nitrini, R., Caramelli, P., Bertolucci, P. H., & Okamoto, I. H. (2003). [Suggestions for utilization of the mini-mental state examination in Brazil]. Arq Neuropsiquiatr, 61(3B), 777-781

Chiam, J. T., Dobson, R. J., Kiddle, S. J., & Sattlecker, M. (2015). Are blood-based protein biomarkers for Alzheimer's disease also involved in other brain disorders? A systematic review. J Alzheimers Dis, 43(1), 303-314. doi: 10.3233/JAD-140816

Coulson, D. T., Beyer, N., Quinn, J. G., Brockbank, S., Hellemans, J., Irvine, G. B., Johnston, J. A. (2010). BACE1 mRNA expression in Alzheimer's disease postmortem brain tissue. J Alzheimers Dis, 22(4), 1111-1122. doi: 10.3233/JAD-2010-101254

DeLong, E. R., DeLong, D. M., & Clarke-Pearson, D. L. (1988). Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics, 44(3), 837-845

Ewers, M., Cheng, X., Zhong, Z., Nural, H. F., Walsh, C., Meindl, T., Hampel, H. (2011). Increased CSF-BACE1 activity associated with decreased hippocampus volume in Alzheimer's disease. J Alzheimers Dis, 25(2), 373-381. doi: 10.3233/JAD-2011-091153

Ewers, M., Zhong, Z., Burger, K., Wallin, A., Blennow, K., Teipel, S. J., Hampel, H. (2008). Increased CSF-BACE 1 activity is associated with ApoE-epsilon 4 genotype in subjects with mild cognitive impairment and Alzheimer's disease. Brain, 131(Pt 5), 1252-1258. doi: 10.1093/brain/awn034

Fukumoto, H., Cheung, B. S., Hyman, B. T., & Irizarry, M. C. (2002). Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol, 59(9), 1381-1389

Gatta, L. B., Albertini, A., Ravid, R., & Finazzi, D. (2002). Levels of beta-secretase BACE and alpha-secretase ADAM10 mRNAs in Alzheimer hippocampus. Neuroreport, 13(16), 2031-2033

Grimmer, T., Alexopoulos, P., Tsolakidou, A., Guo, L. H., Henriksen, G., Yousefi, B. H., Perneczky, R. (2012). Cerebrospinal fluid BACE1 activity and brain amyloid load in Alzheimer's disease. ScientificWorldJournal, 2012, 712048. doi: 10.1100/2012/712048

Hampel, H., & Shen, Y. (2009). Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) as a biological candidate marker of Alzheimer's disease. Scand J Clin Lab Invest, 69(1), 8-12. doi: 10.1080/00365510701864610

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, 297(5580), 353-356. doi: 10.1126/science.1072994

Holsinger, R. M., Lee, J. S., Boyd, A., Masters, C. L., & Collins, S. J. (2006). CSF BACE1 activity is increased in CJD and Alzheimer disease versus [corrected] other dementias. Neurology, 67(4), 710-712. doi: 10.1212/01.wnl.0000229925.52203.4c

Holsinger, R. M., McLean, C. A., Beyreuther, K., Masters, C. L., & Evin, G. (2002). Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. Ann Neurol, 51(6), 783-786. doi: 10.1002/ana.10208

Humpel, C. (2011). Identifying and validating biomarkers for Alzheimer's disease. Trends Biotechnol, 29(1), 26-32. doi: 10.1016/j.tibtech.2010.09.007

Johnston, J. A., Liu, W. W., Coulson, D. T., Todd, S., Murphy, S., Brennan, S., Passmore, A. P. (2008). Platelet beta-secretase activity is increased in Alzheimer's disease. Neurobiol Aging, 29(5), 661-668. doi: 10.1016/j.neurobiolaging.2006.11.003

Johnston, J. A., Liu, W. W., Todd, S. A., Coulson, D. T., Murphy, S., Irvine, G. B., & Passmore, A. P. (2005). Expression and activity of beta-site amyloid precursor protein cleaving enzyme in Alzheimer's disease. Biochem Soc Trans, 33(Pt 5), 1096-1100. doi: 10.1042/BST20051096

Kandalepas, P. C., & Vassar, R. (2014). The normal and pathologic roles of the Alzheimer's beta-secretase, BACE1. Curr Alzheimer Res, 11(5), 441-449

Li, R., Lindholm, K., Yang, L. B., Yue, X., Citron, M., Yan, R., Shen, Y. (2004). Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. Proc Natl Acad Sci U S A, 101(10), 3632-3637. doi: 10.1073/pnas.0205689101

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4), 402-408

Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. Nature, 430(7000), 631-639. doi: 10.1038/nature02621

McKhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R., Jr., Kawas, C. H., Phelps, C. H. (2011). The diagnosis of dementia due to Alzheimer's disease:

recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement, 7(3), 263-269. doi: 10.1016/j.jalz.2011.03.005

Morris, J. C. (1993). The Clinical Dementia Rating (CDR): current version and scoring rules. . Neurology, 43, 2412-2414

Mulder, S. D., van der Flier, W. M., Verheijen, J. H., Mulder, C., Scheltens, P., Blankenstein, M. A. Veerhuis, R. (2010). BACE1 activity in cerebrospinal fluid and its relation to markers of AD pathology. J Alzheimers Dis, 20(1), 253-260. doi: 10.3233/JAD-2010-1367

Murayama, K. S., Kametani, F., & Araki, W. (2005). Extracellular release of BACE1 holoproteins from human neuronal cells. Biochem Biophys Res Commun, 338(2), 800-807. doi: 10.1016/j.bbrc.2005.10.015

Perneczky, R., Alexopoulos, P., & Alzheimer's Disease euroimaging, I. (2014). Cerebrospinal fluid BACE1 activity and markers of amyloid precursor protein metabolism and axonal degeneration in Alzheimer's disease. Alzheimers Dement, 10(5 Suppl), S425-S429 e421. doi: 10.1016/j.jalz.2013.09.006

Rosen, C., Andreasson, U., Mattsson, N., Marcusson, J., Minthon, L., Andreasen, N., Zetterberg, H. (2012). Cerebrospinal fluid profiles of amyloid beta-related biomarkers in Alzheimer's disease. Neuromolecular Med, 14(1), 65-73. doi: 10.1007/s12017-012-8171-4

Savage, M. J., Holder, D. J., Wu, G., Kaplow, J., Siuciak, J. A., & Potter, W. Z. (2015). Soluble BACE-1 Activity and sAbetaPPbeta Concentrations in Alzheimer's Disease and Age-Matched Healthy Control Cerebrospinal Fluid from the Alzheimer's Disease Neuroimaging Initiative-1 Baseline Cohort. J Alzheimers Dis, 46(2), 431-440. doi: 10.3233/JAD-142778

Schroeder, A., Fahrenholz, F., & Schmitt, U. (2009). Effect of a dominant-negative form of ADAM10 in a mouse model of Alzheimer's disease. J Alzheimers Dis, 16(2), 309-314. doi: 10.3233/JAD-2009-0952

Selkoe, D. J. (1994). Amyloid beta-protein precursor: new clues to the genesis of Alzheimer's disease. Curr Opin Neurobiol, 4(5), 708-716

Song, W. J., Son, M. Y., Lee, H. W., Seo, H., Kim, J. H., & Chung, S. H. (2015). Enhancement of BACE1 Activity by p25/Cdk5-Mediated Phosphorylation in Alzheimer's Disease. PLoS One, 10(8), e0136950. doi: 10.1371/journal.pone.0136950

Stockley, J. H., & O'Neill, C. (2007). The proteins BACE1 and BACE2 and beta-secretase activity in normal and Alzheimer's disease brain. Biochem Soc Trans, 35(Pt 3), 574-576. doi: 10.1042/BST0350574

Verheijen, J. H., Huisman, L. G., van Lent, N., Neumann, U., Paganetti, P., Hack, C. E., Hanemaaijer, R. (2006). Detection of a soluble form of BACE-1 in human cerebrospinal fluid by a sensitive activity assay. Clin Chem, 52(6), 1168-1174. doi: 10.1373/clinchem.2006.066720

Wu, G., Sankaranarayanan, S., Tugusheva, K., Kahana, J., Seabrook, G., Shi, X. P., Simon, A. J. (2008). Decrease in age-adjusted cerebrospinal fluid beta-secretase activity in Alzheimer's subjects. Clin Biochem, 41(12), 986-996. doi: 10.1016/j.clinbiochem.2008.04.022

Wu, G., Sankaranarayanan, S., Wong, J., Tugusheva, K., Michener, M. S., Shi, X., Savage, M. J. (2012). Characterization of plasma beta-secretase (BACE1) activity and soluble amyloid precursor proteins as potential biomarkers for Alzheimer's disease. J Neurosci Res, 90(12), 2247-2258. doi: 10.1002/jnr.23122

Yang, L. B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X. L., Shen, Y. (2003). Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat Med, 9(1), 3-4. doi: 10.1038/nm0103-3

Zetterberg, H., Andreasson, U., Hansson, O., Wu, G., Sankaranarayanan, S., Andersson, M. E., Blennow, K. (2008). Elevated cerebrospinal fluid BACE1 activity in incipient Alzheimer disease. Arch Neurol, 65(8), 1102-1107. doi: 10.1001/archneur.65.8.1102

Zhong, Z., Ewers, M., Teipel, S., Burger, K., Wallin, A., Blennow, K., Shen, Y. (2007). Levels of beta-secretase (BACE1) in cerebrospinal fluid as a predictor of risk in mild cognitive impairment. Arch Gen Psychiatry, 64(6), 718-726. doi: 10.1001/archpsyc.64.6.718

Table1. Descriptive analysis of the variables for control and AD groups, according to CDR using total blood in qRT-PCR analyses.

Variable	CDR0	CDR1	CDR2	CDR3	p-value
Cases (n/%)	32/32	24/51	14/30	9/19	
Age, mean (range)	74 (64-86)	77 (66-86)	79 (67-89)	73 (60-90)	0.40
Gender, female (%)	68	83	64	33	
MMSE, mean ± SD	28 ± 8	18 ± 5	14 ± 4	2 ± 2	*
BACE1 (mRNA), mean ± SD	1.05 ± 0.32	1.08 ± 0.32	1.05 ± 0.44	1.01 ± 0.17	0.92
Scholarity, mean (years)	7	4.7	7.5	9	
Comorbidities (%)					
Hypertension	28	46	71	11	
Diabetes Mellitus	9	25	50	11	
Hypothyroidism	6	17	-	11	
None	50	13	-	67	

CDR, Clinical Dementia Rating; MMSE, Mini Mental State Examination; *<0.0001 CDR0≠1/2/3; CDR1≠3; CDR2≠3; <0.05 CDR1≠2. GraphPadPrism 5.01.

Table 2. Descriptive analysis of the variables for control and AD groups, according to CDR using plasma in ELISA analyses.

Variable	CDR0	CDR1	CDR2	CDR3	p-value
Cases (n)	20	7	7	7	
Age, mean (range)	74 (64-84)	76 (66-84)	79 (69-89)	71 (60-90)	0.72
Gender, female (%)	65	100	58	43	
MMSE, mean ± SD	27 ±1.8	16 ± 5	14 ± 4	2 ± 2.3	*
Plasma BACE1, mean ± SD	35 ± 4	46 ± 4	40 ± 8	38 ± 8	**
Scholarity, mean (years)	6	4	7.4	7	
Comorbidities (%)					
Hypertension	20	43	86	14	
Diabetes Mellitus	-	14	57	14	
Hypothyroidism	10	57	-	14	
None	70	-	-	57	

CDR, Clinical Dementia Rating; MMSE, Mini Mental State Examination; * <0.0001 CDR0≠1/2/3; 1≠3; 2≠3; ** <0.05 CDR0≠1. GraphPadPrism 5.01.

Table 3. Logistic regression between variable presence of AD on the basis of gender, education level, age and BACE1.

Marginal effect at means (in elasticity)	Coefficient (dy/dx)	p-value
BACE1	1.644	0.010
Age	0.804	0.437
Gender	-0.055	0.781
Education level	-0.039	0.745

Stata MP12.

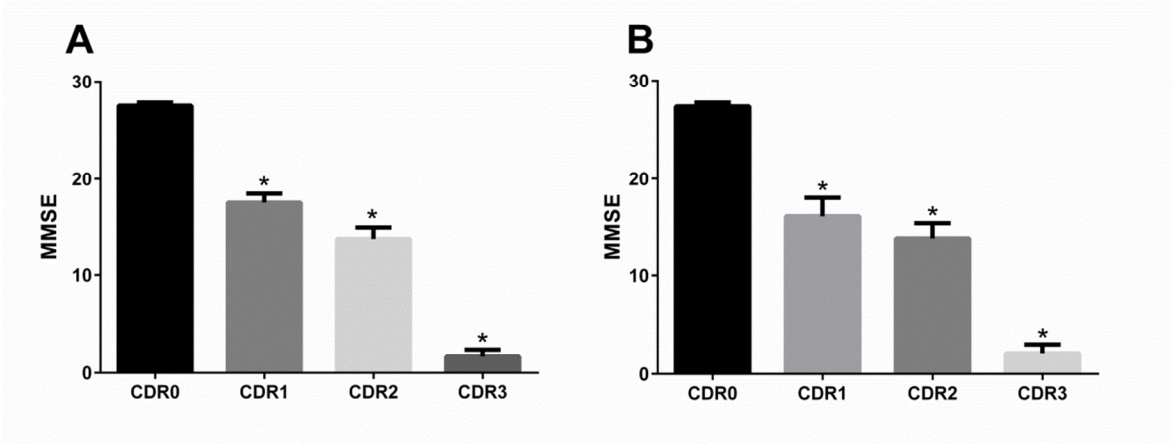
Table 4. Comparison of single and associated variables for AD prediction diagnoses (ROC curve analysis).

AD sample (n = 21) compared with non-AD sample (n = 20)				
Variable	AUC (95% CI)	Cut-off	Sensitivity, % (95% CI)	Specificity, % (95% CI)
BACE1	0.705 (0.54-0.86)	>34.6	76.2 (52.8 - 91.8)	65.0 (40.8 - 84.6)
BACE1 + MMSE	0.802 (0.65-0.95)	≤56.9	71.4 (47.8 - 88,7)	95.0 (75.1 - 99.9)

CI, confidence interval; AUC, area under curve; MMSE, Mini-Mental State Examination. The cut-offs were chosen to yield the highest Youden index. MedCalc 11.5.1.

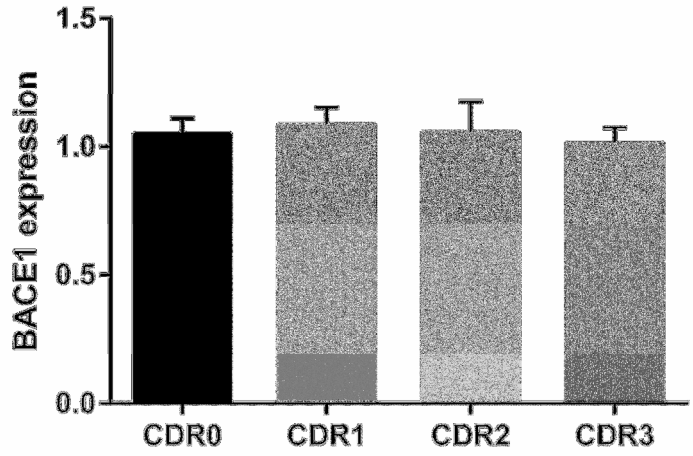
Figures

Figure 1. MMSE score in groups according to CDR in total blood and plasma analysis.



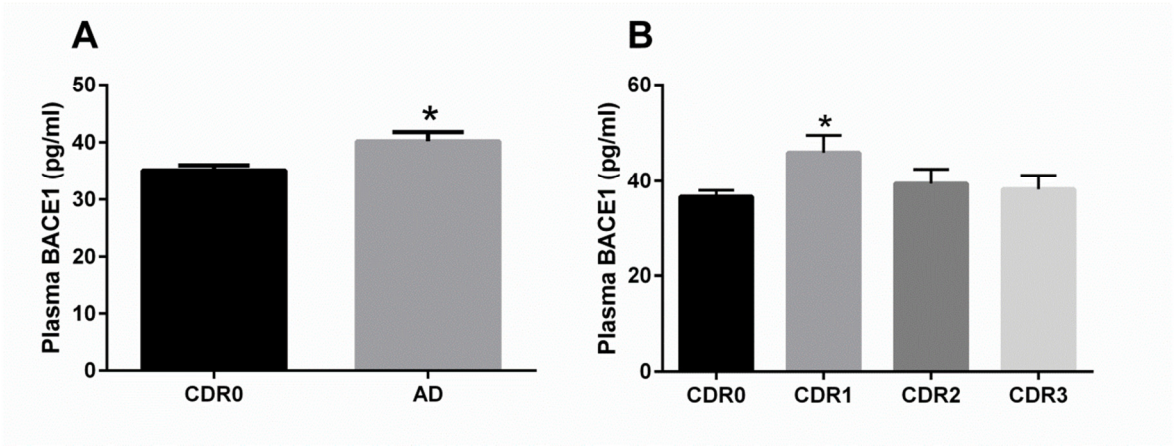
CDR, Clinical Dementia Rating; MMSE, Mini Mental State Examination. **(A)** Total blood MMSE analysis.*<0.05 CDR1≠2; <0.0001 CDR0≠CDR1/2/3; CDR1≠3; CDR2≠3. **(B)** Plasma MMSE analysis. *<0.0001 CDR0≠1/2/3; 1≠3; 2≠3. GraphPadPrism 5.01.

Figure 2. Total blood BACE1 (mRNA) gene expression in the groups, according to CDR.



CDR, Clinical Dementia Rating; MCI, Mild Cognitive Impairment. ANOVA One-way Test (Kruskal-Wallis Test) (p = 0.92). GraphPadPrism 5.01.

Figure 3. Plasma protein BACE1 levels in the groups (A) and according to CDR (B).



CDR, Clinical Dementia Rating. AD, Alzheimer´s disease group. (A)*p=0.02 CDR0≠AD. (B)*p=0.01 CDR0≠1. GraphPadPrism 5.01.

Figure 4. Linear association between plasma BACE1 levels and MMSE.

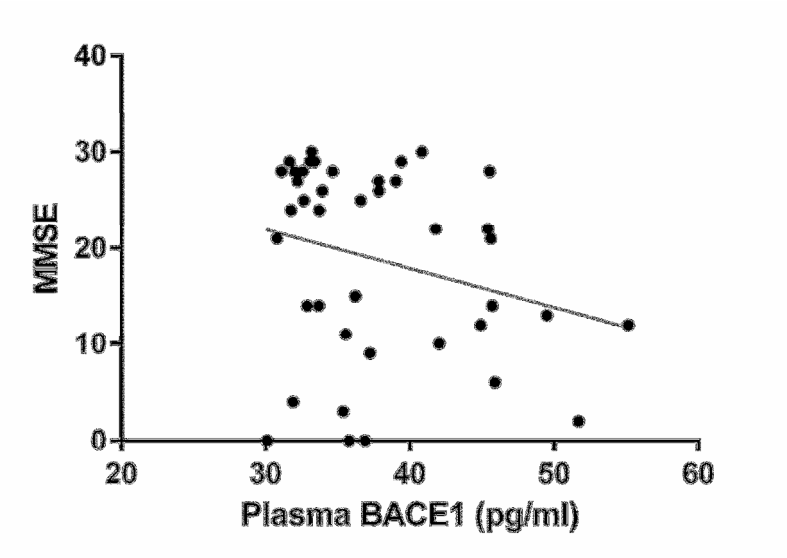
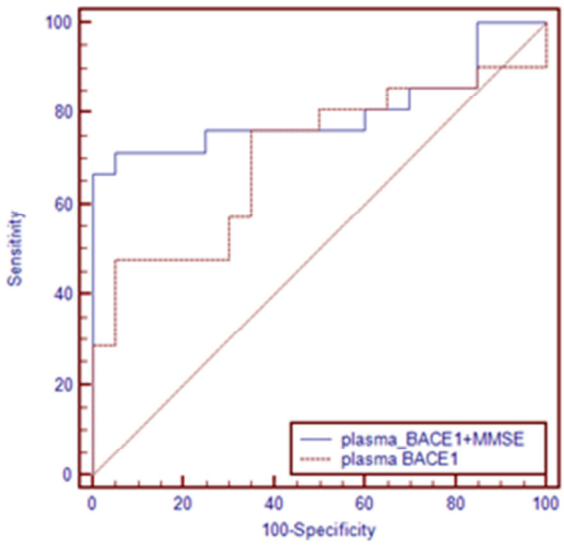


Figure 5. Comparison of receiver operating characteristic curves. Areas under the curves (AUCs) for BACE1 compared with MMSE score combined with BACE1. MedCalc 11.5.1.



6.2 MANUSCRITO IV – Publicado em colaboração

BIANCO, O.A.; **MANZINE, P.R**.; NASCIMENTO, C.M.; VALE, F.A.C.; PAVARINI, S.C.I.; COMINETTI, M.R. Serotoninergic antidepressants positively affect platelet ADAM10 expression in patients with Alzheimer's disease. Int Psychogeriatr. Nov 1, 1:1-6, 2015. IF: 1.93

International Psychogeriatrics: page 1 of 6 © International Psychogeriatric Association 2015
doi:10.1017/S1041610215001842

Serotoninergic antidepressants positively affect platelet ADAM10 expression in patients with Alzheimer's disease

Otávio Augusto Fernandes Marques Bianco[#,1] Patrícia Regina Manzine[#,1] Carla Manuela Crispim Nascimento,[1] Francisco Assis Carvalho Vale,[2] Sofia Cristina Iost Pavarini[1] and Márcia Regina Cominetti[1]

[1] *Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil*
[2] *Department of Medicine, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil*

ABSTRACT

Background: Studies have demonstrated a decreased platelet ADAM10 expression in patients with Alzheimer's Disease (AD), classifying this protein as a blood-based AD biomarker. About 50% of the patients with AD are diagnosed with depression, which is commonly treated with tricyclic and tetracyclic antidepressants, monoaminoxidade (MAO) inhibitors and, more preferably, with selective serotonin reuptake inhibitors (SSRIs). Considering that a large proportion of patients with AD takes antidepressant medications during the course of the disease we investigated the influence of this medication on the expression of platelet ADAM10, which is considered the main α-secretase preventing beta-amyloid (βA) formation.

Methods: Blood was collected for protein extraction from platelets. ADAM10 was analyzed by using western blotting and reactive bands were measured using β-actin as endogenous control.

Results: Platelet ADAM10 protein expression in patients with AD was positively influenced by serotoninergic medication.

Conclusion: More studies on the positive effects of serotonergic antidepressants on ADAM10 platelet expression should be performed in order to understand its biological mechanisms and to verify whether these effects are reflected in the central nervous system. This work represents an important advance for the study of AD biomarkers, as well as for more effective pharmacological treatment of patients with AD and associated depression.

Key words: antidepressant drugs, Alzheimer's disease, biomarkers, dementia, depression, human ADAM10 protein

Introduction

AD pathogenesis is multifaceted and difficult to pinpoint. Current genetic and cell biology research have led to the amyloid hypothesis, which posits that the beta-amyloid peptide (Aβ) plays a pivotal role in AD (Hardy and Selkoe, 2002). Aβ derives from the concerted action of BACE1 (β-secretase) and the γ-secretase complex on the cleavage of amyloid precursor protein (APP). In healthy subjects, the predominant route of APP processing via non-amyloidogenic pathway consists of successive cleavages by α and γ-secretases in the middle of Aβ region, thus releasing sAPPα – a structure with neurotrophic and neuroprotective functions (Bandyopadhyay *et al.*, 2007). Several enzymes in the "a disintegrin and metalloproteinase" (ADAM) family, including ADAM9, ADAM10, and ADAM17, have α-secretase activity *in vitro*, although recent studies have demonstrated that ADAM10 is the major α-secretase that catalyses APP ectodomain shedding in the brain (Bernstein *et al.*, 2014). Several studies have demonstrating the effects of serotonin receptors (5-HTRs) on APP processing and Aβ levels, showing that their activation increases non-amyloidogenic processing of APP *in vitro* (Arjona *et al.*, 2002; Shen *et al.*, 2011) and that chronic administration of SSRIs may reduce brain Aβ levels *in vivo* (Nelson *et al.*, 2007; Cirrito *et al.*, 2011).

Our research group has been dedicated to the study of the platelet ADAM10 expression as a blood-based biomarker for AD patients.

Correspondence should be addressed to: Márcia Regina Cominetti, Departamento de Gerontologia, Universidade Federal de São Carlos, Rodovia Washington Luís, Km 235, São Carlos, SP, 13565-905, Brazil. Phone: +55-16-3306 6663; Fax: +55-16-3351-9628. Email: mcominetti@ufscar.br. Received 15 Jun 2015; revision requested 26 Aug 2015; revised version received 10 Sep 2015; accepted 8 Oct 2015.
[#] Both authors have contributed equally to this work.

In the previous studies we (Manzine *et al.*, 2013b) and other authors (Colciaghi *et al.*, 2002; 2004) have reported a marked reduction in platelet ADAM10 protein levels in AD patients compared to cognitively healthy subjects. We have also demonstrated a positive correlation between ADAM10 levels, Mini Mental State Examination (MMSE) and clock drawing test and that ADAM10 levels decrease as the advance of the disease (Manzine *et al.*, 2013a; 2014). Since ADAM10 is the most important α-secretase involved in cleavage of APP and antidepressant medications are frequently utilized by AD patients, the aim of this research was to investigate whether platelet ADAM10 protein levels would be related with the use of antidepressant drugs in older adults diagnosed with AD.

Methods

Study design and participants

Patients were form a convenience sample, which was recruited in reference (Public Centre of Specialties) and counter-reference (Family Health Units) health services in Brazil. Eligible participants were diagnosed with probable AD according to National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCS-ADRDA) criteria (McKhann *et al.*, 1984). This study was carried out with 26 older adults diagnosed with probable AD, divided in two groups. The control group was composed of 13 AD patients, who were not taking any antidepressant drug and the SSRI group, which was formed of 13 AD patients already taking SSRIs medication, in a regular-basis, under medical prescription. Exclusion criteria for participants were: head trauma, metabolic dysfunctions, haematological diseases, alcohol abuse, drug abuse, delirium, mood disorders, and treatment with medications affecting platelet functions, i.e. anticoagulants, antiplatelet drugs, serotoninergic agonists-antagonists (for control group), and corticosteroids. All included participants were taking proper AD medication and had a standardized clinical workup based on neurological examinations, laboratory blood, and urine analysis, a neuroimaging study (Head Computed Tomography and/or Magnetic Resonance Imaging), and a neuropsychological assessment, including a MMSE according to scholarity levels of Brazilian population (Brucki *et al.*, 2003) and a Clinical Dementia Rating (CDR) (Montano and Ramos, 2005). Before enrolment, subjects or their legal caregivers filled out an informed consent, after the nature and possible consequences of the study were explained. The research project was approved by Brazil Platform Ethics Committee (CAAE: 02760312.0.0000.5504/ No: 112.543).

ADAM10 quantification

ADAM10 quantification was carried out according to our previous studies (Manzine *et al.*, 2013b). Briefly, blood (8.5 mL) was collected in sodium citrate tubes (3.8% containing 136 mM glucose). The interval between the collection and the processing was 20 to a maximum of 30 min. The platelet-rich plasma (PRP) was obtained by centrifugation at 1,200 rpm for 10 min. From the PRP, platelets were collected by centrifugation at 2,400 rpm for 10 min at room temperature, then washed twice in phosphate buffered saline solution (PBS) and finally suspended in lysis buffer (200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP40, 0.1% SDS, and protease inhibitors). Bradford kit (1:4) (BioRad, Hercules, CA, USA) was used for protein measurement.

The necessary volume to obtain a total of 100 μg of protein for each sample was applied into a 10% SDS-PAGE gel (Laemmli, 1970). Page-Ruler Prestained (Fermentas, Burlington, CA, USA) was used as a molecular marker in all gels. After the gel running, the proteins were transferred into nitrocellulose membranes (Sigma, St. Louis, MO, USA) for the period of 1 hour using the Mini Trans-Blot Cell transfer system (Bio-Rad, Hercules, CA, USA). The membranes were incubated with anti-ADAM10 mouse monoclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology). After these procedures, the membranes were developed using Clarity^{TM} Western CCL Substrate (Bio-Rad). The antibody anti-β-actin conjugated to HRP (Santa Cruz Biotechnology) was used to detect β-actin protein, which was the endogenous control. The immunoreaction bands were quantified with ChemiDoc^{TM} MP Imaging System (Bio-Rad).

Statistical analysis

A blind analysis of the participants was used for the ADAM10 sample quantification. In order to detect the sample normality level this study employed the Kolmogorov–Smirnov test (KS). Student's *t* test was used between groups and non-parametric Mann–Whitney U test was employed among subgroups. The figures were made using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA) software. A 5% significance level was chosen as standard.

Table 1. Patients' demographic and clinical variables

VARIABLE	CONTROL	SSRI
Cases (n)	13	13
[a]CDR 1 (n)	6	10
[b]CDR 2 (n)	7	3
Age (mean ± [c]SD)	77.3 ± 5.7	78.0 ± 6.0
Gender (M/F)	4/9	2/11
Scholarity	6.7 ± 6.6	3.8 ± 2.3
[d]MMSE (mean ± SD)	17.2 ± 4.6	14.46 ± 4.7
ADAM10 (mean ± SD)	0.31 ± 0.11	0.53 ± 0.28*
[e]AD medication (%)		
Rivastigmine	46	46
Donepezil	38	46
Galantamine	8	-
Memantine	8	8
[f]SSRI (%)		
Sertraline	-	54
Citalopram	-	23
Fluoxetine	-	8
Paroxetine	-	8
Venlafaxine	-	7
Other psychotropic medications		
Clonazepam	31	23

[a]Mild AD (CDR1), [b]Moderate AD (CDR2), [c]Standard Deviation, [d]Mini-Mental State Examination, [e]Alzheimer's Disease medication, [f]Selective Serotonin Reuptake Inhibitors. *$p < 0.001$ compared to Control, multiple t test.

Results

A total of 26 AD patients took part in this study. Socio demographic, clinical and cognitive data for all participants of this study are presented on Table 1. Patients enrolled in this study were all taking different types and dosages of acetylcholinesterase inhibitors (AChEIs). In both groups (control and SSRI) the most used AChEI (46%) was rivastigmine with a dosage ranging from 6–12 mg/day. The second most used AChEI (38% and 46%, for control and SSRI groups, respectively) was donepezil with dosages ranging from 5–10 mg/day. Memantine, an N-methyl-D-aspartate (NMDA)-receptor antagonist, was used (8% in both groups) at 10–20 mg/day and galantamine (8%, only in control group) at 16–24 mg/day. Among the SSRIs used in this study we found that 54% were sertraline (50 mg-100 mg/day); 23% citalopram (20–40 mg/day); 8% fluoxetine (20–40 mg/day); 8% paroxetine (20–40 mg/day), and 7% venlafaxine (75 mg/day) (Table 1).

Blood was collected and analyzed for platelet ADAM10 expression in control and SSRI groups. Patients in more advanced stages of the disease (according to the CDR scores) had their ADAM10 platelet expression significantly reduced, confirming our previous results (Figure 1A). ADAM10 platelet levels were significant higher in patients taking SSRI medication, compared to controls (Table 1, Figure 1B). The ADAM10 expression measured by western blotting analysis and normalized by the endogenous control β-actin is presented on Figure 1C.

Discussion

Alpha-secretase-mediated cleavage of APP releases the neuroprotective sAPPα fragment and prevents βA formation (Postina, 2012). ADAM10 and ADAM17 are considered the main α-secretases involved in non-amyloidogenic pathway in AD (Fahrenholz, 2010). Our previous studies had demonstrated that platelet ADAM10 levels were reduced in AD patients compared with cognitively healthy subjects (Manzine *et al.*, 2013b) and that this reduction had a significant positive correlation with MMSE (Manzine *et al.*, 2013a) and clock drawing (Manzine *et al.*, 2014) tests. However, we also recently showed that this reduction was not resulting from differences in ADAM10 mRNA levels, suggesting that post-transcriptional or trafficking mechanisms could play a role in the regulation of ADAM10 expression (Manzine *et al.*, 2015). Here we observed the same results, showing that with the advance of the disease, according to CDR, platelet ADAM10 expression is significantly decreased in AD patients. However, the platelet ADAM10 levels were increased in AD patients using SSRIs as antidepressant medication, compared with the control group.

Recent evidence suggests a strong relationship between depression and AD. A lifetime history of depression has been considered a risk factor for later development of AD and depressive symptoms can positively affect the conversion of mild cognitive impairment (MCI) into AD. In addition, neuritic plaques and neurofibrillary tangles are more pronounced in the brains of depressed AD patients, compared to non-depressed ones (Caraci *et al.*, 2010; Chi *et al.*, 2015).

Cochet and co-authors (Cochet *et al.*, 2013) have demonstrated that 5-HTRs constitutively induce APP cleavage by ADAM10 with concomitant release of neuroprotective sAPPα both in HEK-293 cells and cortical neurons. Moreover, Cirrito and co-workers (Cirrito *et al.*, 2011) demonstrated that brain Aβ levels were significantly decreased following the administration of several SSRIs antidepressant drugs or the direct infusion of 5-HT in the brain of PS1APP transgenic mice, with the involvement of extracellular regulated kinase (ERK) signaling pathway, as the treatment with ERK inhibitors reversed the Aβ clearance. These

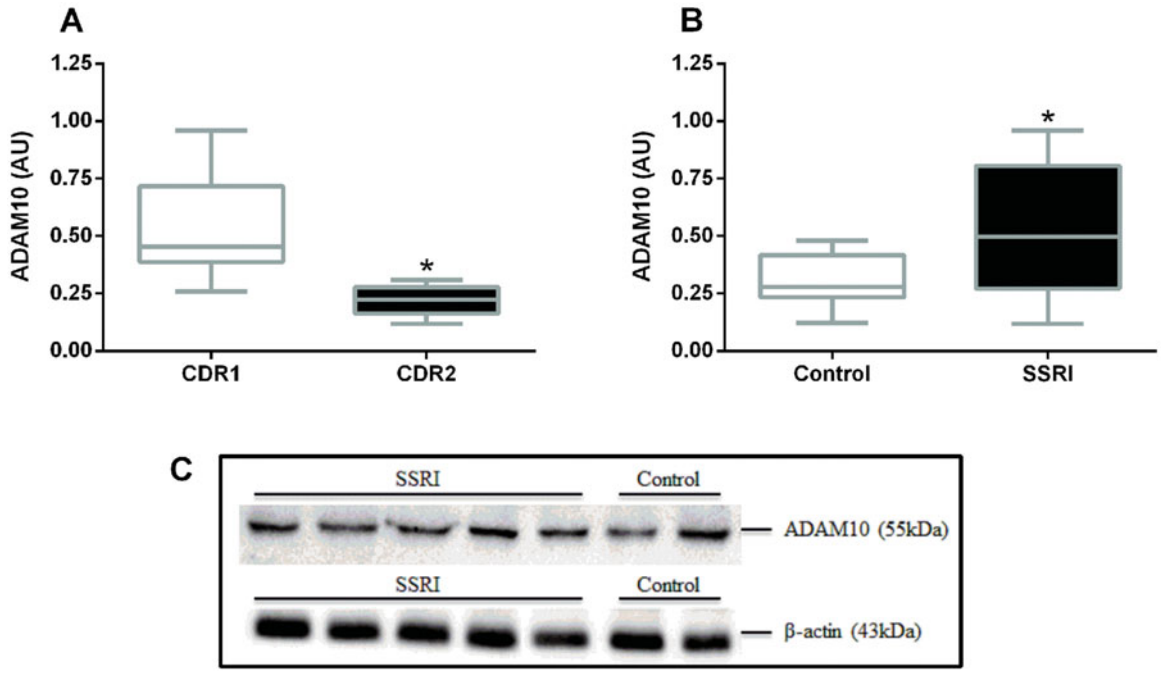


Figure 1. (Colour online) SSRI antidepressants positively influences ADAM10 expression. Platelet ADAM10 expression in AD patients according to CDR (A), control AD patients (B) or patients taking SSRI medication (B). Representative western blotting membranes showing platelet ADAM10 expression in five patients from SSRI, and in two patients from the control group (C). Platelets were collected, lysed and the protein content was applied to a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane followed by the incubation with anti-ADAM10 or anti-β-actin antibodies. The bands were developed with the Clarity™ Western CCL Substrate (Bio-Rad) and then scanned for the measure of the reactive bands. Graph represents quantification of the ADAM10 normalized to β-actin bands; *$p < 0.001$, Mann–Whitney test.

authors also demonstrated an increased ADAM10 α-secretase enzymatic activity in antidepressant-treated mice.

The link between AD and depression could be related to 5-HT neurotransmitter and its receptor. One of the molecular mechanisms of depression is known to be the increased clearance of 5-HT from the synaptic cleft. SSRIs act inhibiting 5-HT reuptake, increasing its synaptic concentration and availability. It was demonstrated that G protein-coupled receptors (GPCRs) can affect Aβ peptide production by either modulating the cellular trafficking of APP or by influencing the activity and trafficking of α-, β-, and γ-secretases. Among GPCRs, 5-HTRs are transmembrane receptors that interact with ADAM10 and enhance sAPPα production by stimulating α-secretase activity (Thathiah and De Strooper, 2011). Moreover, activation of 5-HTRs stimulates acetylcholine release in prefrontal cortex and hippocampus and improves learning and memory in various preclinical paradigms of memory acquisition and retention (Bockaert *et al.*, 2011). These findings suggest that 5-HTR agonists like SSRIs might be used to improve cholinergic function and cognition, which may both be compromised in AD. In fact, it was already demonstrated that in AD patients treated with acetylcholinesterase inhibitors (AChEIs), SSRIs may exert some protection against the negative effects of depression on cognition (Rozzini *et al.*, 2010). On the other hand, there are reports showing that AChEIs activate specific subtypes of muscarinic acetylcholine receptors (mAChRs) that inhibit sAPPα release and aggravate amyloid-β generation (Thathiah and De Strooper, 2011). Our results emphasize the former hypothesis, showing that there is one plausible mechanism that could be related to the influence of these molecules on ADAM10 expression. It is important to mention that all AD patients in our study were using AChEIs. The exact molecular mechanisms underlying this positive effect of SSRIs on ADAM10 expression remain to be elucidated.

In this study the prevalence of patient's CDR was different in each group (control and SSRI), therefore, we analyzed the ADAM10 levels in both groups, classified for CDR scores. We observed that, for CDR1 subjects, ADAM10 levels were higher in SSRI, compared to control subjects. This was not true for CDR2 subjects, where no differences were found for ADAM10 levels between

control and SSRI patients (data not shown). This raises an important question: are the SSRI effects on ADAM10 protein levels only effective for patients in mild stages (CDR1) of the disease? One of the limitations that avoid an immediate answer for this is the small number of the participants in this study, which does not allow subject matching for aging, sex, scholarity, or CDR levels. The lack of a measure for ADAM10 activity is also a limitation, since western blotting analysis only shows the protein quantity and not its activity, although usually these variables are positively related. Moreover, the effects of other antidepressant medications, such as MAO inhibitors or tricyclic drugs on ADAM10 expression were not analyzed in this study and could also be considered as a study limitation.

Conclusion

The results from this study provide relevant clinical implications by showing that serotonergic antidepressants positively influence the α-secretase of ADAM10 platelet expression in patients with AD. Studies on the molecular mechanisms of how platelet ADAM10 expression is positively influenced by SSRIs and whether this expression is reflected in the central nervous system should be carried out using a larger number of patients, and may represent an important step for the study of AD and for its more effective pharmacological treatment.

Conflicts of interest

None.

Description of authors' roles

O.A.F.M. Bianco had contributed to data collection, P.R. Manzine and C.M.C. Nascimento had substantially contributed to data collection, analysis and interpretation of data, S.C.I. Pavarini and F.A.C. Vale substantially contributed to critically revising the paper with important intellectual content; and M.R. Cominetti contributed to drafting the paper and revising it critically for important intellectual content. All authors have given the final approval of the version to be published.

Acknowledgments

The authors thank all the subjects and their families. The authors are grateful for the financial support of Sao Paulo Research Foundation (FAPESP) grants 2010/09497-7 and 2013/06879-4. O.A.F.M. Bianco, P.R. Manzine, and C.M.C Nascimento have scholarships sponsored by FAPESP (grants 2012/08654-7, 2012/01936-7, and 2014/21066-2, respectively).

References

Arjona, A. A., Pooler, A. M., Lee, R. K. and Wurtman, R. J. (2002). Effect of a 5-HT(2C) serotonin agonist, dexnorfenfluramine, on amyloid precursor protein metabolism in guinea pigs. *Brain Research*, 951, 135–140.

Bandyopadhyay, S., Goldstein, L. E., Lahiri, D. K. and Rogers, J. T. (2007). Role of the APP non-amyloidogenic signaling pathway and targeting alpha-secretase as an alternative drug target for treatment of Alzheimer's disease. *Current Medicinal Chemistry*, 14, 2848–2864.

Bernstein, H. G., Steiner, J., Bogerts, B., Stricker, R. and Reiser, G. (2014). Nardilysin, ADAM10, and Alzheimer's disease: of mice and men. *Neurobiol Aging*, 35, e1.

Bockaert, J., Claeysen, S., Compan, V. and Dumuis, A. (2011). 5-HT(4) receptors, a place in the sun: act two. *Current Opinion in Pharmacology*, 11, 87–93.

Brucki, S. M., Nitrini, R., Caramelli, P., Bertolucci, P. H. and Okamoto, I. H. (2003). Suggestions for utilization of the mini-mental state examination in Brazil. *Arquives of Neuropsiquiatry*, 61, 777–781.

Caraci, F., Copani, A., Nicoletti, F. and Drago, F. (2010). Depression and Alzheimer's disease: neurobiological links and common pharmacological targets. *European Journal of Pharmacology*, 626, 64–71.

Chi, S., Wang, C., Jiang, T., Zhu, X. C., Yu, J. T. and Tan, L. (2015). The prevalence of depression in Alzheimer's disease: a systematic review and meta-analysis. *Current Alzheimer Research*, 12, 189–198.

Cirrito, J. R. *et al.* (2011). Serotonin signaling is associated with lower amyloid-beta levels and plaques in transgenic mice and humans. *Proceedings of Natural Academy of Science USA*, 108, 14968–14973.

Cochet, M. *et al.* (2013). 5-HT4 receptors constitutively promote the non-amyloidogenic pathway of APP cleavage and interact with ADAM10. *ACS Chemical Neuroscience*, 4, 130–140.

Colciaghi, F. *et al.* (2002). [alpha]-Secretase ADAM10 as well as [alpha]APPs is reduced in platelets and CSF of Alzheimer disease patients. *Molecular Medicine*, 8, 67–74.

Colciaghi, F. *et al.* (2004). Platelet APP, ADAM 10 and BACE alterations in the early stages of Alzheimer disease. *Neurology*, 62, 498–501.

Fahrenholz, F. (2010). The close link between retinoid signalling and the alpha-secretase ADAM10 and its potential for treating Alzheimer's disease (commentary on Jarvis et al.). *European Journal of Neuroscience*, 32, 1245.

Hardy, J. and Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*, 297, 353–356.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.

Manzine, P. R., Barham, E. J., Vale, F. A., Selistre-de-Araujo, H. S., Pavarini, S. C. and Cominetti, M. R. (2014). Platelet a disintegrin and metallopeptidase 10 expression correlates with clock drawing test scores in Alzheimer's disease. *International Journal of Geriatric Psychiatry*, 29, 414–420.

Manzine, P. R., Barham, E. J., Vale Fde, A., Selistre-de-Araujo, H. S., Iost Pavarini, S. C. and Cominetti, M. R. (2013a). Correlation between mini-mental state examination and platelet ADAM10 expression in Alzheimer's disease. *Journal of Alzheimers Disease*, 36, 253–260.

Manzine, P. R. *et al.* (2013b). ADAM10 as a biomarker for Alzheimer's disease: a study with Brazilian elderly. *Dementia and Geriatriatic Cognitive Disorders*, 35, 58–66.

Manzine, P. R. *et al.* (2015). ADAM10 gene expression in the blood cells of Alzheimer's disease patients and mild cognitive impairment subjects. *Biomarkers*, 20, 196–201.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E. M. (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, 939–944.

Montano, M. B. and Ramos, L. R. (2005). [Validity of the Portuguese version of Clinical Dementia Rating]. *Revista de Saude Publica*, 39, 912–917.

Nelson, R. L. *et al.* (2007). Prophylactic treatment with paroxetine ameliorates behavioral deficits and retards the development of amyloid and tau pathologies in 3xTgAD mice. *Experimental Neurology*, 205, 166–176.

Postina, R. (2012). Activation of alpha-secretase cleavage. *Journal of Neurochemistry*, 120 (Suppl. 1), 46–54.

Rozzini, L. *et al.* (2010). Efficacy of SSRIs on cognition of Alzheimer's disease patients treated with cholinesterase inhibitors. *International Psychogeriatrics*, 22, 114–119.

Shen, F. *et al.* (2011). 5-HT(4) receptor agonist mediated enhancement of cognitive function *in vivo* and amyloid precursor protein processing *in vitro*: A pharmacodynamic and pharmacokinetic assessment. *Neuropharmacology*, 61, 69–79.

Thathiah, A. and De Strooper, B. (2011). The role of G protein-coupled receptors in the pathology of Alzheimer's disease. *Nature Reviews Neuroscience*, 12, 73–87.