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MICROPARTÍCULAS, CITOCINAS E LEPTINA: POSSÍVEIS BIOMARCADORES DO COMPROMETIMENTO COGNITIVO NO ENVELHECIMENTO?

Carolina Antunes Magalhães

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Tese apresentada ao Programa de Pós-Graduação em Análises Clínicas e Toxicológicas da Faculdade de Farmácia da Universidade Federal de Minas Gerais como requisito parcial à obtenção do grau de Doutora.

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FOLHA DE APROVAÇÃO

Micropartículas, citocinas e leptina: possíveis biomarcadores do comprometimento cognitivo no envelhecimento?

CAROLINA ANTUNES MAGALHÃES

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Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
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Lista de siglas, abreviaturas e símbolos

AAN	*American Academy of Neurology*
Aβ	Peptídeo beta-amiloide
AchEIs	Inibidores da acetilcolinesterase
AC	*Abdominal circumference*
AD	*Alzheimer's disease*
ApoA	Apolipoproteína A
ApoB	Apolipoproteína B
ApoC	Apolipoproteína C
ApoD	Apolipoproteína D
ApoE	Apolipoproteína E
ApoJ	Apolipoproteína J
APP	Proteína precursora amiloide
AUC	*Area Under the Curve*
BCSB	*Brief Cognitive Screening Battery*
BMI	*Body Mass Index*
CA	Circunferência abdominal
CCL	Comprometimento cognitivo leve
CID	Classificação Internacional de Doenças
CSF	*Cerebrospinal fluid*
DA	Doença de Alzheimer
DCS	Declínio cognitivo subjetivo
DM	*Diabetes mellitus*
DNA	Ácido desoxirribonucleico
DSM IV	*Diagnostic and Statistical Manual of Mental Disorders IV*
ε1	Epsilon 1
ε2	Epsilon 2
ε3	Epsilon 3
ε4	Epsilon 4
ε5	Epsilon 5
ε7	Epsilon 7
ELISA	*Enzyme-Linked Immunosorbent Assay*
EMPs	Micropartículas derivadas de endotélio
FAST	*Functional Assessment Staging Test*
FDG-PET	Fluordesoxiglicose-PET
HDL	*High density lipoprotein*
IATI	*Innotest Amiloide Tau Index*

IL-1β	Interleucina 1 beta
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-8	Interleucina 8
IL -10	Interleucina 10
IL-12	Interleucina 12
IL-18	Interleucina 18
IFN-Y	*Interferon gama*
IMC	Índice de massa corpórea
LMPs	Micropartículas derivadas de leucócitos
LCR	Líquido cefalorraquidiano
LSD	*Least Significance Difference*
MAPKs	Proteínas cinases ativadas por mitógenos
MCI	*Mild cognitive impairment*
MEEM	Mini-exame do estado mental
MMSE	*Mini-Mental State Examination*
MPs	Micropartículas
microRNA	Micro ácido ribonucleico
mRNA	Ácido ribonucleico mensageiro
NMPs	Micropartículas derivadas de neurônio
NIA	*National Institute on Aging*
NINCDS-ADRDA	*National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association*
NO	Óxido nítrico
OMS	Organização Mundial de Saúde
PBS	*Phosphate buffered saline*
PCR	*Polymerase chain reaction*
PCR-us	Proteína C reativa ultra-sensível
PET	Tomografia por Emissão de Pósitrons
PMPs	Micropartículas derivadas de plaquetas
PS1	Presenilina 1
PS2	Presenilina 2
RMN	Ressonância magnética nuclear
ROC	*Receiver Operating Characteristc Curve*
SCD	*Subjective cognitive decline*
SNC	Sistema Nervoso Central
SNP	*Single Nucleotide Polymorphism*
SPSS	*Statistical Package of the Social Sciences*
TFMPs	Micropartículas derivadas de fator tissular

Tau-P	Proteína Tau fosforilada
TGF-β1	*Transforming growth factor beta 1*
TNF-α	Fator de necrose tumoral alfa
WHO	World Health Organization
VLDL	*Very low density lipoprotein*

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RESUMO

As alterações clínicas decorrentes da Doença de Alzheimer (DA) afetam a função cognitiva do indivíduo, além de ocasionar mudanças na personalidade e comportamento. Já no comprometimento cognitivo leve (CCL), o indivíduo mantêm suas atividades cotidianas relativamente preservadas. No declínio cognitivo subjetivo (DCS), há um comprometimento auto-declarado, mas nenhuma evidência de declínio cognitivo em testes neuropsicológicos padrões. No entanto, esta condição pode preceder o CCL e a DA. As micropartículas (MPs) estão associadas à ativação celular durante a inflamação, coagulação, lesões vasculares e homeostase. O processo inflamatório faz parte da fisiopatologia da DA e a leptina, demonstrou um papel neuroprotetor contra a patologia da DA. Este estudo teve como objetivo investigar os níveis de MPs, das citocinas, IL-1β, IL-6, TNF-α e IL-10, de leptina e proteína C reativa ultrassensível (PCR-us), em pacientes com comprometimento cognitivo (DA e CCL) e indivíduos sem comprometimento cognitivo objetivo (DCS e controles). Avaliamos os níveis de MPs derivadas de plaquetas (PMPs), de leucócitos (LMPs), de fator tecidual (TFMPs), de endotélio (EMPs) e de neurônios (NMPs) no plasma por citometria de fluxo. As citocinas e a leptina foram determinadas no soro por ensaio de imunoenzimático (ELISA). E a PCR-us no soro por ensaio imunoturbidimétrico. Observamos que os níveis de TFMPs, LMPs e NMPs foram significativamente aumentados e a IL-6, IL-1β, TNF-α e PCR-us foram significativamente menores no grupo DA quando comparados aos controles. A regressão logística múltipla mostrou que TNF-α e NMPs foram associados independentemente com DA, o que resultou em uma curva ROC com área sob a curva de 0,957 (sensibilidade = 100% e especificidade = 70%). Além disso, encontramos níveis elevados de MPs em indivíduos com comprometimento funcional e cognitivo e níveis elevados de TNF-α em indivíduos sem comprometimento cognitivo. Não encontramos diferença significativa nos níveis de leptina entre os grupos. No entanto, as mulheres demonstraram níveis mais elevados de leptina do que os homens. E também foram observados níveis mais altos de leptina em indivíduos > 79 anos. Os resultados sugerem que MPs e TNF-α são biomarcadores potenciais para o diagnóstico de DA. E que os níveis de leptina não estão envolvidos na evolução para o estado de comprometimento cognitivo, mas está associado à inflamação ao sexo e a idade.

Palavras-chave: Doença de Alzheimer. Comprometimento cognitivo. Biomarcadores. Micropartículas. Citocinas. Leptina.

ABSTRACT

The clinical impairment established on Alzheimer's Disease (AD) compromises the cognitive fuction, accompanied by personality and behavioral changes. On Mild Cognitive Impairment (MCI), the daily activities are preserved. In Subjective Cognitive Decline (SCD), there is a self-perceived decline, but without evidence of cognitive decline on standard neuropsychological tests. However, this condition may precede frequently the MCI and AD. Microparticles (MPs) are associated to cellular activation during inflammation, coagulation, vascular injuries and homeostasis. An inflammatory process is part of AD pathophysiology. And leptin has been demonstrated to have a neuroprotective role against AD pathology. This study aimed to investigate the MPs and cytokines levels, IL-1β, IL-6, TNF-α and IL-10; leptin and high sensitivity C Reactive protein (hsCRP) levels on patients with cognitive impairment (AD and MCI) and subjects with no objective cognitive impairment (SCD and controls). We evaluated platelets-derived MPs (PMPs), leukocytes-derived MPs (LMPs), tissue factor-derived MPs (TFMPs), endothelium-derived MPs (EMPs) and neuron-derived MPs (NMPs) levels in plasma, by flow cytometer assay. The cytokines and leptin levels were determined using enzyme linked immunosorbent assay (ELISA). And the hsCRP levels by immunoturbidimetric assay. We observed that TFMPs, LMPs and NMPs levels were significant increased, and IL-6, IL-1β, TNF-α and hsCRP were found to be significant lower in the AD group when compared to controls. The multiple logistic regression showed that TNF-α and NMPs were independently associated to AD, which resulted in a ROC curve with an area under the curve of 0.957 (sensitivity = 100% and specificity = 70%). Moreover we found higher MPs levels in sbjects with functional cognitive impairment and lower IL-6, IL-1β, TNF-α and hsRCP and the functional and cognitive impairment, and lower levels of TNF-α in subjects with no cognitive impairment. We found no difference in leptin levels between the cognitive impairment groups and SCD group. However, women demonstrated higher leptin serum levels than men. Higher leptin levels were observed in individuals ≥ 79 years old. The results suggest that MPs and TNF-α have a promising potential as biomarkers for AD diagnosis, but that leptin levels is not involved in the evolution for the cognitive impairment state but it is associated with inflammation, sex and age.

Keywords: Alzheimer's disease. Cognitive impairment. Biomarkers. Microparticles. Cytokines. Leptin.

1 INTRODUÇÃO

Países em desenvolvimento como o Brasil apresentam atualmente taxas de envelhecimento populacional bastante acentuadas que refletem o crescimento rápido das faixas etárias acima de 65 anos. Esta mudança tem lançado grandes desafios para a sociedade, devido ao seu impacto socioeconômico, político e na saúde pública. Todo este movimento traz um aumento na frequência das doenças crônico-degenerativas, dentre elas a demência (World Health Organization - WHO, 2015).

Demência é um termo geral que indica a ocorrência de comprometimento cognitivo adquirido, causando limitação da autonomia funcional e da qualidade de vida (Alzheimer's Association, 2017). A causa mais comum de demência em idosos é a doença de Alzheimer (DA), que leva a perda de funções cognitivas como atenção, memória, percepção, linguagem, capacidade de julgamento, orientação espacial, planejamento e execução de tarefas (Fratiglioni *et al*., 1999; Cacabelos, 2008, Alzheimer's Association, 2017). Na senescência, processo natural de envelhecimento, a capacidade de resposta do organismo a desafios tende a diminuir. No entanto, este processo difere da senilidade, onde ocorre o envelhecimento patológico, entendido como o dano à saúde associado ao tempo, porém causado por doenças ou maus hábitos de vida (Paixão *et al*., 1998). O Comprometimento Cognitivo Leve (CCL) precede a demência devido a DA (Petersen *et al*., 1999). Entre as alterações cognitivas normais do envelhecimento e o CCL foi descrito um estado intermediário definido como declínio cognitivo subjetivo (DCS) que corresponde ao processo de declínio auto-reportado, mas sem evidências de comprometimento em testes neurofisiológicos. Esta condição pode representar a fase pré-sintomática do CCL, que pode ocorrer até 15 anos antes da identificação do estado de CCL (Eckerström *et al*., 2017; Prichep *et al*., 2006).

De acordo com a Alzheimer's Association, nos Estados Unidos, uma em cada 10 pessoas com 65 anos ou mais têm DA, o que corresponde a uma estimativa de 5,3 milhões de pessoas (Alzheimer's Association, 2017). De acordo com dados americanos, entre os indivíduos que sofrem com a DA ou outra demência, menos de 50% recebem o diagnóstico (Alzheimer's Association, 2015).

Diversos estudos sugerem a relação da DA com a obesidade, hipertensão arterial, dislipidemia, *diabetes mellitus* (DM) e fatores genéticos (Tezapsidis *et al*., 2009; Ríos *et al*., 2014). Além disso, na última década, pesquisas têm sido conduzidas no sentido de validar biomarcadores no sangue e no líquido cefalorraquidiano (LCR), objetivando, principalmente,

facilitar o diagnóstico precoce da DA (Caramelli *et al*., 2011, Buchhave *et al*., 2012, Dubois *et al*., 2014).

O presente trabalho objetivou avaliar os níveis de micropartículas, citocinas, leptina, proteínas C reativa ultrassensível (PCR-us) e biomarcadores liquóricos de pacientes com o diagnóstico de DA, a fim de identificar correlações entre os analitos pesquisados e a doença. Dessa forma, busca-se biomarcadores que possam no futuro contribuir para a identificação de indivíduos assintomáticos, em risco de desenvolvimento e em estágios pré-clínicos da DA, além da elucidação de casos de DA na rotina clínica.

2 REVISÃO DA LITERATURA

2.1 A dinâmica demográfica mundial e suas consequências

Desde a antiguidade o número de jovens sempre foi muito maior que o número de idosos em todo o mundo. Entretanto, essa relação tem mudado nos últimos anos devido a queda nas taxas de fertilidade e ao aumento na expectativa de vida. A Organização Mundial da Saúde (OMS) considera o idoso, sob o ponto de vista cronológico, como aquele indivíduo que possui 65 anos ou mais de idade em países desenvolvidos, enquanto que, em países em desenvolvimento, como o Brasil, prevalece a idade de 60 anos ou mais. Estima-se que a população acima dos 65 anos passará dos 524 milhões de indivíduos, em 2010, para 1,4 bilhões em 2030 e 2,1 bilhões em 2050, podendo chegar a 3,2 bilhões de idosos em 2100, principalmente nos países em desenvolvimento (WHO, 2015) (Figura 1).

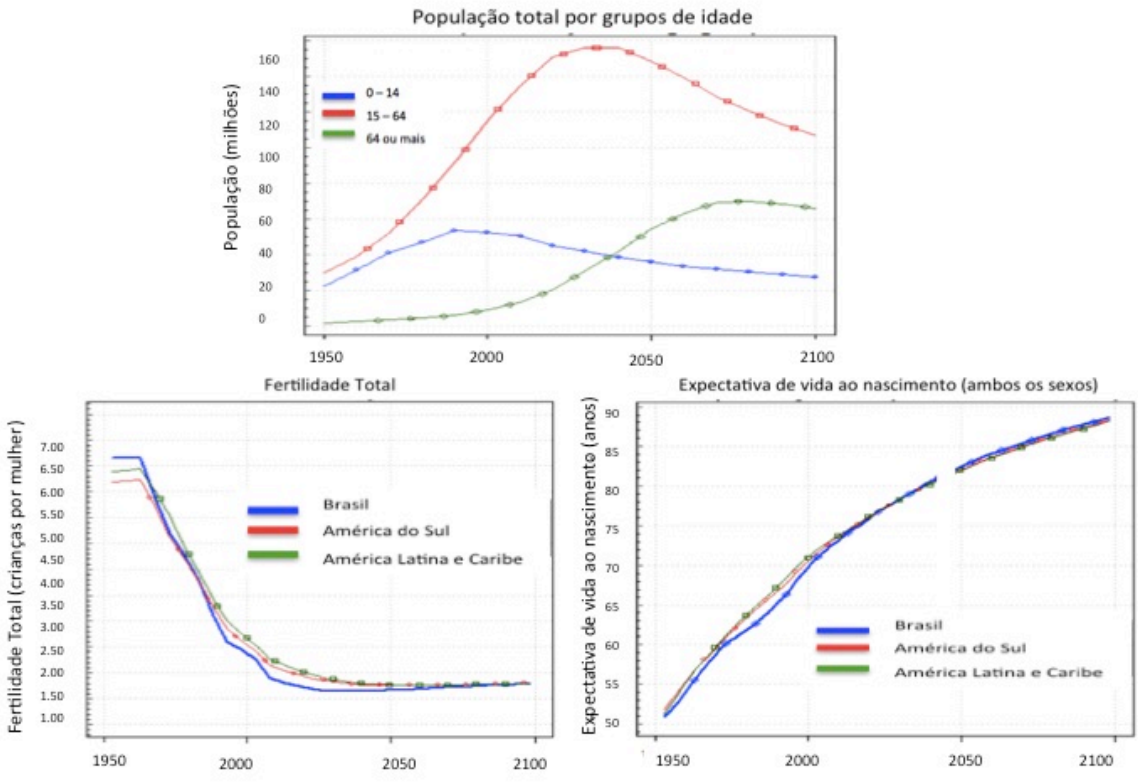


Figura 1: Número populacional por grupos de idade, níveis de fertilidade e expectativa de vida. Fonte: United Nations, Department of Economic and Social Affairs, Population Division (2015). World Population Prospects: The 2015 Revision.

Aliada a essa transição demográfica observa-se também uma transição epidemiológica. As baixas taxas de mortalidade e fertilidade que acompanharam o desenvolvimento socioeconômico dos países trouxeram também uma mudança nas causas de doença e morte.

Observa-se um aumento da ocorrência de doenças crônico-degenerativas, dentre elas, transtornos neuropsiquiátricos, como declínio cognitivo e demência (WHO, 2013).

2.2 Demência

A função cognitiva envolve a atenção, percepção, memória, raciocínio, juízo, imaginação, pensamento, linguagem, função executiva (habilidade de planejar e de executar tarefas) e aprendizado, que, juntamente com o componente emocional, caracterizam a saúde mental de um indivíduo (Hendrie *et al*., 2006). Um declínio constante em muitos processos cognitivos é visto normalmente ao longo da vida, e particularmente a partir dos 30 anos de idade (WHO, 2015).

O comprometimento cognitivo é detectado e diagnosticado por meio da combinação de histórico do paciente e uma avaliação cognitiva objetiva, que pode ser uma avaliação do estado mental ou um teste neuropsicológico. A dificuldade comportamental ou cognitiva envolve pelo menos dois dos seguintes domínios: a dificuldade de adquirir ou lembrar de novas informações, a dificuldade de lidar com tarefas complexas e julgamento pobre, a dificuldade nas habilidades visuospaciais, a dificuldade nas funções da linguagem (fala, leitura e escrita), ou mudanças na personalidade ou no comportamento (McKhann *et al*., 2011).

Já a demência, segundo McKhann *et al*. (2011), é diagnosticada quando há sintomas cognitivos e comportamentais que interferem na habilidade funcional do trabalho e de atividades usuais; quando representa um declínio no nível de trabalho e performance; e quando não é explicado por *delirium* ou desordem psiquiátrica maior.

Existem outras condições clínicas que imitam os sintomas da demência, entre elas: depressão, efeitos adversos de medicamentos, doenças da tireóide, deficiência vitamínica e uso excessivo de álcool. Estas condições podem ser revertidas com tratamento, ao contrário da demência propriamente dita. Cerca de 9% das pessoas com sintomas de demência apresentam condições clínicas reversíveis (Clarfield, 2003). No entanto, a DA, condição irreversível, é responsável por 60 a 80% dos casos de demência (*Alzheimer's Association*, 2015).

2.3 Declínio Cognitivo Subjetivo

O Declínio cognitivo subjetivo (DCS), uma queixa auto-declarada e sem um comprometimento cognitivo objetivo, detectado na avaliação do estado mental ou em testes neuropsicológicos, pode ser um estágio pré-sintomático do CCL e DA (Prichep *et al*., 2006; Reisberg e Gauthier, 2008). Várias evidências sugerem o DCS como um fator de risco para a demência (Glodzik-Sobanska *et al*., 2007; Reisberg *et al*., 2010). Já foi reportado estar presente até 15 anos antes do CCL (Prichep *et al*., 2006).

2.4 Comprometimento Cognitivo Leve

Segundo Petersen *et al*. (1999), nas últimas décadas, diferentes termos têm sido usados por diversos autores para se referir às alterações cognitivas que ocorrem durante o processo de envelhecimento, como por exemplo: "Esquecimento Senil Benigno", por Kral em 1962; "Comprometimento de Memória Associada à Idade", por Crook *et al*. em 1986; "Declínio Cognitivo Relacionado à Idade", em 1994 pelo Manual de Diagnóstico de Transtornos Mentais Americano - DSM IV; "Transtorno Cognitivo Leve" pela Classificação Internacional de Doenças- CID 10 em 1992; e finalmente "*Mild Cognitive Impairment*" ou Comprometimento Cognitivo Leve, em 1999 por Petersen *et al*.

As primeiras definições propostas objetivaram caracterizar o distúrbio somente dentro dos limites do processo fisiológico do envelhecimento normal. Posteriormente, outros sistemas de classificação diagnóstica surgiram, tentando identificar indivíduos com maior risco de desenvolver um processo demencial. Todos estes conceitos referem-se a condições entre o normal e o patológico, e esta diversidade talvez se deva ao fato de ainda não se dispor de recursos diagnósticos eficientes para se delimitar a fronteira entre as modificações observadas no envelhecimento normal e aquelas que ocorrem nos estágios iniciais da demência (Petersen *et al*., 2001b).

O termo "*Mild Cognitive Impairment*", (MCI), proposto por Petersen *et al*. (1999), aqui traduzido como CCL, é o recomendado pela *American Academy of Neurology* (AAN), e trata-se de um conceito em evolução. O CCL é definido como o estado intermediário entre as alterações cognitivas normais do envelhecimento e a demência da DA (Petersen *et al*., 1999; McKhann *et al*., 2011). Pode também ser caracterizado como uma síndrome cujo declínio cognitivo é maior que o esperado para a idade e para o nível educacional do indivíduo, mas que não interfere, de forma significativa, nas atividades diárias (Gauthier *et al*., 2006). Refere-

se a alterações cognitivas, em particular de memória episódica para eventos recentes, em sujeitos que mantêm suas atividades cotidianas relativamente preservadas e não preenchem os critérios clínicos para o diagnóstico de demência (Gauthier *et al*., 2006). Estudos indicam que 10% a 20% dos indivíduos com 65 anos ou mais têm CCL (Hanninen *et al*., 2002; Roberts *et al*., 2008) e que o risco de indivíduos com CCL desenvolverem DA é de 10% a 15% ao ano (Petersen *et al*., 2001a).

O CCL é uma condição heterogênea caracterizada por mudanças cognitivas leves que pode estar associada a etiologias subjacentes (Dubois *et al*., 2014). A identificação do estado de CCL é algo relevante, na medida em que sujeitos com essa condição têm maior conversão para a demência em comparação com indivíduos sem CCL. O CCL tende a seguir várias trajetórias: pode representar uma condição transitória na qual o sujeito retorna à sua condição de normalidade cognitiva; a alteração cognitiva pode se manter estável, sem retorno à normalidade e sem piora das funções cognitivas; ou o CCL pode progredir para um padrão de declínio cognitivo que se caracteriza pela evolução para a demência (Ward *et al*., 2012).

Diante do quadro de CCL, busca-se investigar se essa condição representa ou não uma fase inicial de demência, sobretudo de DA. Desta forma, novos biomarcadores que possam prever esta evolução, bem como novos medicamentos com propriedades de mudança do processo neurodegenerativo antes da instalação da demência, certamente representarão uma contribuição inestimável para o conhecimento e tratamento desta doença.

2.5 Doença de Alzheimer

A DA foi descrita pela primeira vez pelo médico alemão Aloysius Alzheimer, em 1906. Em sua conferência, intitulada "Uma Doença Peculiar dos Neurônios do Córtex Cerebral", Alzheimer definiu sua descoberta como uma doença neurológica, não reconhecida, que cursava com demência, destacando os sintomas de déficit de memória, alterações de comportamento, evoluindo para a incapacidade em atividades rotineiras. Em seguida, Alzheimer ainda viria a descrever os aspectos anatomopatológicos da doença, cujas principais características eram o acúmulo de placas senis, de emaranhados neurofibrilares e a perda neuronal. Quatro anos mais tarde, na oitava edição do "*Handbook of Psychiatry*", Emil Kraepelin, após estudar casos semelhantes aos descritos por Alzheimer, propôs nomear a enfermidade como "doença de Alzheimer" em homenagem ao pesquisador que a descreveu pela primeira vez (Möller *et al*., 1998).

2.5.1 Diagnóstico

Em 2011, o *National Institute on Aging* (NIA) e a *Alzheimer's Association* propuseram critérios e diretrizes revisados para o diagnóstico da DA (Sperling *et al*. 2011; Jack *et al*., 2011). Nessas diretrizes foram identificados três estágios da DA: a DA pré-clínica, onde o indivíduo não apresenta os sintomas, o CCL devido à DA e a demência devida à DA (Sperling *et al*., 2011; Jack *et al*., 2011).

Um consenso realizado pelo Departamento Científico de Neurologia Cognitiva e do Envelhecimento da Academia Brasileira de Neurologia, em 2011, recomendou novos critérios para o diagnóstico de demência e DA no Brasil. Neste consenso, foram revisados os critérios recomendados em 2005 que seguiam aqueles do DSM IV e do *National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association* (NINCDS-ADRDA). Foram incorporados o uso de biomarcadores liquóricos e os exames de imagem, os quais incluem a Tomografia por Emissão de Positrons (PET), onde se pode identificar as placas senis e o aumento da proteína Tau fosforilada; o Fluordesoxiglicose-PET (FDG-PET), que permite identificar o hipometabolismo de glicose no sistema nervoso central (SNC) e a ressonância magnética nuclear (RMN), que possibilita a visualização da atrofia hipocampal.

Com base nos novos critérios (Academia Brasileira de Neurologia, 2011), pode-se classificar os pacientes como tendo Demência da DA provável, que tem início insidioso, história clara ou observação de piora cognitiva, com tomografia ou ressonância magnética do crânio que exclua possibilidades diagnósticas ou comorbidades, principalmente a doença vascular cerebral. Outra classificação é a Demência da DA possível, que tem início abrupto, uma apresentação mista (com evidência de outras etiologias ou outros tipos de demência) e detalhes de história insuficientes sobre instalação e evolução da doença. Já a Demência da DA definida, é assim classificada quando há o preenchimento dos critérios clínicos e cognitivos para DA provável com comprovação através de exame neuropatológico de tecido cerebral por biópsia ou autópsia.

2.5.2 Epidemiologia

Os últimos dados publicados estimaram que em 2015, 5,5 milhões de americanos apresentavam a DA, sendo 5,3 milhões indivíduos com 65 anos ou mais. Para 2025, este número pode quase triplicar, passando para 13,8 milhões (Hebert *et al*., 2013) (Figura 2).

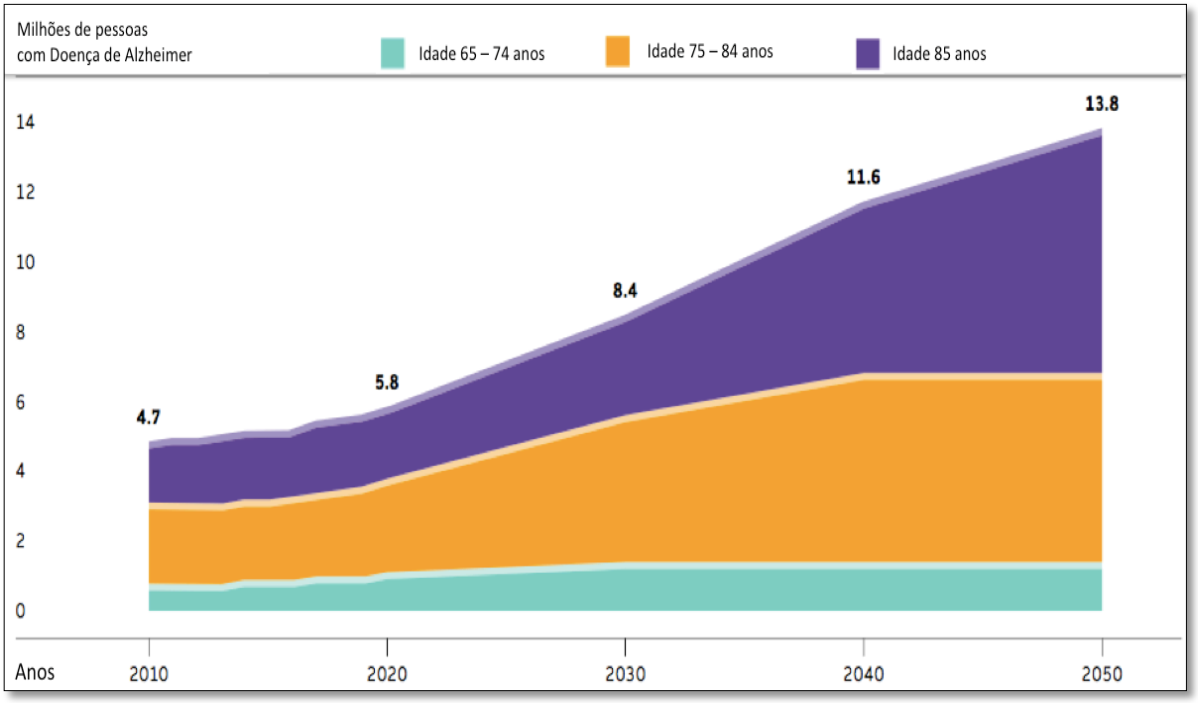


Figura 2: Projeção do número de pessoas com 65 anos ou mais na população americana com a Doença de Alzheimer entre 2010 a 2050. (Adaptado de Alzheimer's Association, 2017).

As mortes por outras causas principais diminuíram, mas registros oficiais indicam que mortes devido a DA têm aumentado significativamente. Entre 2000 e 2014, as mortes por DA aumentaram 89% (Alzheimer's Association, 2017) (Figura 3).

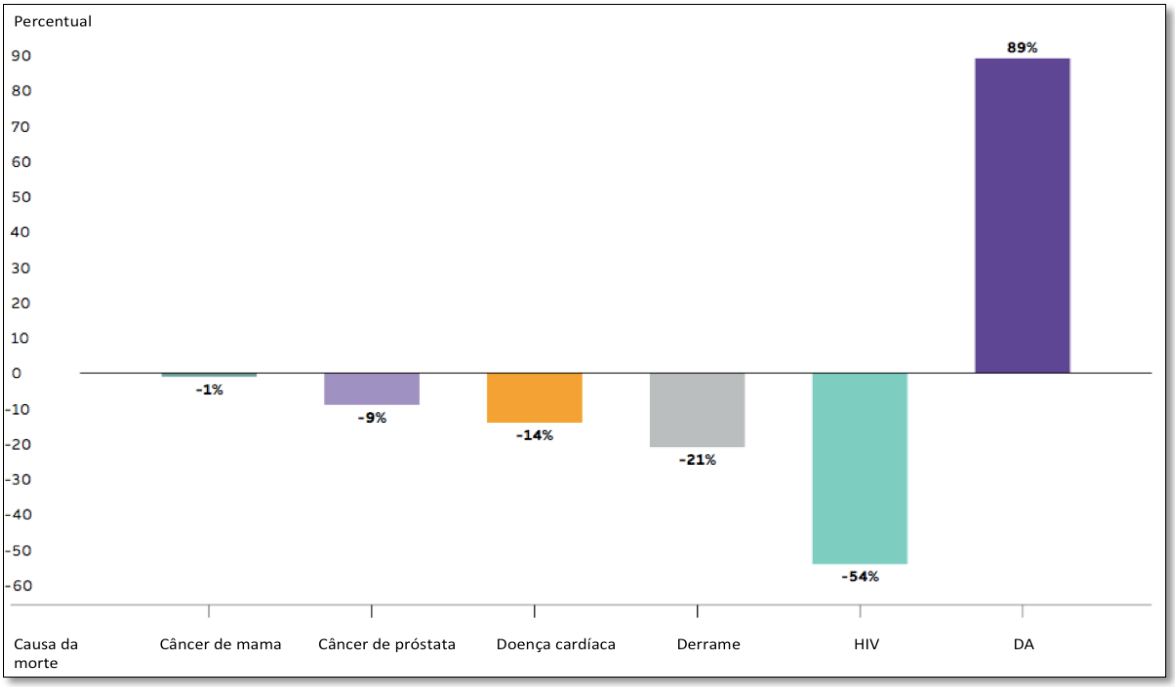


Figura 3: Mudanças percentuais por causas de morte entre 2000 e 2014. (Adaptado de Alzheimer's Association, 2017).

No Brasil, os estudos populacionais sobre demência são escassos, e ainda não existem estimativas precisas da sua incidência e prevalência. Herrera *et al*. (1998) avaliaram 1.660 pessoas com idade igual ou superior a 65 anos, residentes na cidade de Catanduva, estado de São Paulo, correspondendo a aproximadamente 25% da população idosa. Após exames realizados, foram diagnosticados 118 casos de demência, correspondendo à prevalência de 7,1%, sendo a DA responsável por 54,1% desses casos. Lopes e Bottino (2002) identificaram que a prevalência média de demência, acima dos 65 anos de idade, variou entre 2,2% na África, 5,5% na Ásia, 6,4% na América do Norte, 7,1% na América do Sul e 9,4% na Europa.

Nitrini *et al*. (2009) encontraram uma prevalência de demência de 7,1% em idosos com 65 anos ou mais, nos países da América Latina. Já Fagundes *et al*. (2011), em uma revisão sistemática, identificaram que a prevalência de demência variou de 5,1 a 19,0% em idosos brasileiros com idade igual ou superior a 60 anos.

2.5.3 Manifestações clínicas e patogênese

Os sintomas comuns da DA são perda de memória, dificuldades para planejar e resolver problemas, executar tarefas domésticas, do trabalho ou lazer, confusão com locais e horários, problemas de linguagem oral e escrita, perda da capacidade de julgamento, mudança de humor e personalidade, apatia e depressão (Alzheimer's Association, 2015).

Entre os eventos fisiopatológicos da DA estão: deposição extracelular do peptídeo beta-amiloide (Aβ) em placas senis, hiperfosforilação de proteína Tau com formação de novelos neurofibrilares intracelulares, déficit colinérgico, extensa perda neuronal e alterações sinápticas no córtex cerebral, no hipocampo e nas áreas cerebrais essenciais para a memória e para as demais funções cognitivas (Parihar e Taruna, 2004).

Hoje existem várias hipóteses que tentam explicar a causa da DA, como a hipótese colinérgica, a hipótese Aβ, a hipótese da Tau e a da inflamação. A hipótese colinérgica é a mais antiga e que não tem sido bem aceita devido a resultados clínicos e experimentais sem sucesso (Nelson *et al.,* 2009; Comim *et al*., 2012). Segundo ela, ocorre degeneração dos neurônios colinérgicos no prosencéfalo basal e déficit dos marcadores colinérgicos, devido à inibição da liberação de acetilcolina, redução da captação da colina, diminuição da concentração intracelular de acetilcolina e da atividade da colina acetiltransferase. O declínio da neurotransmissão colinérgica resultante desses fatores causa deterioração da função cognitiva e manifestações neuropsiquiátricas da doença (Auld *et al*., 2002).

A hipótese da Aβ inclui a proteína precursora amiloide (APP), uma proteína transmembrana, que sofre clivagem por α-, β- e γ-secretases, produzindo o fragmento Aβ, cuja forma mais comum é um fragmento de 40 aminoácidos. A neurodegeneração na DA é causada pela clivagem proteolítica anormal da APP, determinada por β- e γ-secretases, que favorecem a produção de um fragmento de 42 aminoácidos. Esse fragmento apresenta maior capacidade de agregação e deposição na parte extracelular dos neurônios, levando consequentemente à formação de fibras amilóides ou placas senis, que prejudicam o funcionamento das sinapses (Lucatelli *et al*., 2009; Bekris *et al*., 2010).

Já na hipótese da Tau, ocorre a formação de emaranhados neurofibrilares, que consistem em filamentos helicoidais da proteína Tau hiperfosforilada que se acumulam no citoplasma neuronal (Munoz e Feldman, 2000). A função dessa proteína de estabilizar os microtúbulos neurais, estaria prejudicada na DA, além de haver comprometimento do transporte de nutrientes e moléculas essenciais ao neurônio (Parihar e Taruna, 2004; Rudy *et al*., 2010). Contudo, estas hipóteses não são suficientes para explicar todas as características da DA.

Há mais de duas décadas, dois estudos descreveram um dano nos pequenos vasos sanguíneos, resultante da ação de radicais livres do oxigênio, do acúmulo do peptídeo Aβ e de reações inflamatórias na DA (Fischer *et al.,* 1990; Buée *et al*., 1994). Dessa forma a primeira lesão está localizada em pequenos vasos sanguíneos e essa é capaz de estimular a resposta imune (Marchesi *et al*., 2011). Na hipótese inflamatória, as diversas características da DA são

explicadas pela ativação da micróglia, presença de astrócitos reativos e aumento dos níveis de citocinas pró-inflamatórias. Análises em amostras de cérebro humano de indivíduos com DA revelaram altos níveis de expressão de citocinas pró-inflamatórias durante a fase inicial da DA (Sudduth *et al.,* 2013). Sabe-se que o processo inflamatório é normalmente controlado pela chamada "fase resolutiva" que promove o retorno à homeostase, mas a neuroinflamação crônica presente na DA indica uma disfunção nesse processo resolutivo. Foi demonstrado que o processo resolutivo presente no cérebro humano, composto por mediadores, receptores e enzimas, se encontra alterado na DA (Wang *et al*., 2015).

Um por cento ou menos dos casos de DA ocorre devido a mutações genéticas em algum dos três genes associados à forma precoce da doença: gene da APP, ou genes da Presenilina 1 e 2 (*PS1* e *PS2)* (Bettens *et al*., 2010). Estas mutações alteram o metabolismo da APP, estimulando a produção da forma amiloidogênica, por meio de um efeito direto na γ-secretase (Hardy e Selkoe, 2002). Indivíduos com a mutação nos genes *APP* ou *PS1* desenvolverão a DA. Aqueles que apresentarem a mutação da *PS2* têm 95% de chances de desenvolver a doença (Goldman *et al*., 2011).

2.5.4 Fatores de risco

A DA, com exceção das formas precoces associadas às mutações genéticas, é considerada uma doença multifatorial. Entre os principais fatores de risco estão, a idade, a história familial, a presença do alelo ε4 no gene da Apolipoproteína E (ApoE), os fatores de risco para doenças cardiovasculares, a escolaridade, a ocupação social e o traumatismo craniano.

O maior fator de risco para a DA é a idade, uma vez que a maioria dos indivíduos diagnosticados possuem 65 anos ou mais. Uma entre nove pessoas com 65 anos tem DA, um terço das pessoas com 85 anos ou mais têm DA e 81% das pessoas que têm DA apresentam 75 anos ou mais (Hebert *et al*., 2013).

O risco associado à história familial não é ainda bem elucidado, mas sabe-se que indivíduos que têm pais, irmãos ou irmãs com DA (ou seja, familiares de primeiro e segundo grau), têm maior predisposição para desenvolver esta doença do que aqueles sem histórico da doença (Loy *et al*., 2014).

A ApoE é uma apolipoproteína com 317 aminoácidos sintetizada em uma grande variedade de tecidos além do fígado, órgão responsável pela produção de cerca de três quartos da ApoE circulante no plasma. Constitui uma das muitas classes diferentes de apolipoproteínas, incluindo a ApoA, ApoB, ApoC, ApoD e ApoJ, as quais transportam

lipídios no plasma e em outros fluidos corpóreos. Ela está presente em todas as frações lipoprotéicas do plasma, constituindo de 10% a 20% das moléculas de *very low density lipoprotein* (VLDL) e 1% a 2% das moléculas de *high density lipoprotein* (HDL) (Andrade *et al*., 2000). Nos humanos, o cérebro é o segundo sítio de maior síntese de ApoE, sendo produzida principalmente por astrócitos e pela microglia (Davignon *et al*., 1988). Estudos sugerem que os neurônios humanos podem sintetizar ApoE em quantidades significativas. Sua função seria transportar colesterol principalmente dos astrócitos para os neurônios, e também coordenar a mobilização e a redistribuição do colesterol da mielina e das membranas neuronais (Holtzman *et al*., 2012). Além disso, estaria envolvida no reparo sináptico em resposta à injúria tecidual, na manutenção da estrutura neuronal e na função colinérgica (Munoz e Feldman, 2000).

O gene *ApoE* humano está localizado no braço longo do cromossomo 19 (19q13.2). Existem três principais alelos do gene ApoE, denominados ε2, ε3 e ε4. As isoformas proteicas produzidas por esses alelos diferem na composição de aminoácidos nas posições 112 e/ou 158. O alelo ε2 possui o aminoácido cisteína nas duas posições da proteína (aminoácidos 112 e 158), o ε3 tem a cisteína na 112 e uma arginina na 158 (sendo este o alelo mais comum), enquanto que o alelo ε4 possui argininas nestas duas posições. As outras variantes da ApoE são chamadas de ε1, ε5 e ε7, mas elas são extremamente raras. As várias combinações possíveis de dois dos três alelos principais podem dar origem a seis possíveis genótipos: ε2/ ε2, ε3/ε3, ε4/ε4, ε2/ ε3, ε3/ε4 e ε2/ε4 (Holtzman *et al*., 2012).

O papel do alelo ε4 na patogênese da DA ainda é incerto, mas as hipóteses são: atuação no metabolismo da APP e no acúmulo do peptídeo Aβ como placas senis; hiperfosforilação da proteína Tau e formação de emaranhados neurofibrilares; redução da função colinérgica cerebral; aumento de processos oxidativos, inflamatórios ou de apoptose neuronal; e alteração do metabolismo e do transporte lipídicos, assim como da biossíntese de membranas neuronais (Cacabelos, 2008). Contudo, o alelo ε4 não é suficiente para causar a DA; ele apenas aumenta o risco de o indivíduo desenvolver a doença, indicando que existem outros fatores de risco ambientais e genéticos atuando no desenvolvimento da doença (Chouraki e Seshadri *et al*., 2014).

Evidências sugerem que a saúde cerebral se relaciona com a saúde do nosso sistema cardiovascular. O coração saudável fornece, através dos vasos sanguíneos, o oxigênio e os nutrientes necessários para o funcionamento cerebral. Além disso, fatores como tabagismo, obesidade na meia-idade, hipertensão arterial e diabetes mellitus estão associados ao maior

risco de desenvolvimento de demência (Pendlebury *et al*., 2009; Wu *et al*., 2008; Ronnemaa *et al*., 2011; Gudala *et al*., 2013).

Indivíduos com menor escolaridade têm maior risco de desenvolver DA ou outras demências do que indivíduos com maior escolaridade (Nitrini *et al*., 2005; Sando *et al*., 2008). A hipótese para tal fato seria que indivíduos com menor nível de educação formal geralmente têm ocupações que estimulam menos a mente, ou apresentam menor *status* socioeconômico que pode resultar em nutrição deficiente e menor cuidado com a saúde. Acredita-se que indivíduos com maior escolaridade tenham uma "reserva cognitiva" que possa compensar as mudanças cerebrais que ocorrem nas demências (Stern, 2012).

Alguns estudos sugerem que permanecer socialmente e mentalmente ativo ao longo da vida, auxilia a saúde cerebral e pode reduzir o risco de demência (Saczynski *et al*., 2006; Di Marco *et al*., 2014).

O traumatismo craniano moderado está associado com o risco duas vezes maior de desenvolver DA ou outras demências, já o traumatismo craniano grave aumenta o risco de demência em 4,5 vezes (Plassman *et al*., 2000). No trauma moderado, a lesão craniana resulta em perda de consciência e amnésia com duração de até 30 minutos. Já no trauma grave, ocorre perda de consciência e amnésia com duração maior que 24 horas. Indivíduos que experimentaram repetidas lesões cranianas como boxeadores, veteranos de guerra e jogadores de futebol apresentam alto risco de desenvolver desordens cognitivas (Crawford *et al*., 2002; Lehman *et al*., 2012; McKee *et al*., 2013).

2.5.5 Tratamento da DA

Não existe ainda um tratamento capaz de reverter a perda neuronal ou de curar a DA. Dessa forma, o tratamento farmacológico consiste no uso de medicamentos que podem retardar a evolução natural da doença e aliviar os déficits cognitivos e alterações comportamentais, para que com isso o paciente alcance uma melhora em seu estado funcional, em sua qualidade de vida e da sua família (Bottino *et al*., 2002; Forlenza, 2005, Forlenza *et al*., 2009).

Dentre os fármacos utilizados estão os inibidores da acetilcolinesterase (AchEIs), rivastigmina, donepezila e galantamina, que inibem as enzimas que degradam a acetilcolina, aumentam a capacidade do neurotransmissor de estimular seus receptores cerebrais e, consequentemente, facilitam a neurotransmissão colinérgica, que está prejudicada na doença (Sereniki e Vital, 2008). Outro fármaco utilizado é a memantina, um antiglutamatérgico capaz de oferecer benefícios adicionais aos AchEIs (Engelhardt *et al*, 2005). Existem também fármacos para uso complementar, entre eles antipsicóticos, antidepressivos, ansiolíticos,

sedativos e estabilizadores de humor, com o objetivo de tratar os distúrbios comportamentais da demência (Engelhardt *et al*, 2005; Forlenza, 2005).

2.6 Biomarcadores no líquido cefalorraquidiano no diagnóstico da DA

Diversos estudos têm sido conduzidos, nos últimos dez anos, buscando a validação de biomarcadores no líquido cefalorraquidiano (LCR) ou líquor para o diagnóstico da DA, objetivando assim, facilitar o diagnóstico precoce dessa síndrome demencial. Os trabalhos dedicam-se principalmente à dosagem no LCR das duas principais proteínas envolvidas na doença: o Aβ ($A\beta_{1-42}$), principal componente das placas senis, que se encontra diminuído no LCR de pacientes com DA em virtude de sua deposição no parênquima cerebral, e as proteínas Tau e Tau-fosforilada (Tau-P), que estão aumentadas no LCR dos pacientes devido à degeneração neuronal associada ao acúmulo intracelular dos emaranhados neurofibrilares (Caramelli *et al*., 2011; Dubois *et al*., 2014).

Apesar das vantagens da utilização de biomarcadores no líquor para auxiliar no diagnóstico de DA, alguns desafios ainda são encontrados na implementação dessas ferramentas na rotina laboratorial, sobretudo em relação à dificuldade na coleta, reprodutibilidade dos resultados e ao estabelecimento de valores de *cutoff* ideais. Tal limitação é exemplificada pelo fato de que em estudos multicêntricos, incluindo diferentes laboratórios que utilizam o mesmo ensaio bioquímico, tem-se encontrado uma grande variabilidade nos níveis destes biomarcadores. Diversas razões podem explicar as variações interlaboratoriais nessas dosagens, dentre elas estão as características demográficas dos pacientes, a origem do recrutamento, a gravidade da doença e os critérios de seleção e diagnóstico. Podem ainda estar envolvidos outros fatores como o processo de obtenção do LCR, a coleta e transporte da amostra, metodologias de ensaio, além das condições de armazenamento (Dumurgier *et al*., 2013).

O exame destes biomarcadores no LCR pode aumentar a precisão do diagnóstico na prática clínica, tanto na fase demencial quanto no CCL. Além disso, estes biomarcadores têm sido correlacionados com a intensidade das lesões neuropatológicas e têm demonstrado sensibilidade e especificidade em torno de 85% a 90% para o diagnóstico da DA (Caramelli *et al*., 2011; Dumurgier, *et al*., 2013). A análise de biomarcadores no LCR pode ter aplicação na identificação de indivíduos assintomáticos em risco de desenvolvimento e em estágios pré-clínicos da DA, no acompanhamento da progressão da doença e na avaliação de resposta ao tratamento (Babic *et al*., 2014).

Estes biomarcadores isoladamente são insuficientes para conclusão do diagnóstico da DA. Estudos mostram que os resultados dos biomarcadores devem sempre ser avaliados em conjunto para a caracterização da assinatura patológica da DA (Caramelli *et al*., 2011; Dubois *et al*., 2014), o que confere força diagnóstica ao achado laboratorial. O IATI - *Innotest Amiloide Tau Index* - obtido pela relação entre os valores de Aβ e Tau: ($A\beta_{1-42}$/(240 + 1.18 x Tau), assim com a relação Tau Total/Aβ, parecem apresentar melhor desempenho diagnóstico para a DA do que o resultado isolado de cada biomarcador (Mattsson *et al*., 2013).

2.7 A inflamação na DA

O processo inflamatório é também um fator fisiopatológico associado à DA, conforme hipótese discutida anteriormente. A teoria inflamatória propõe um mecanismo autoimune como um gatilho para esta doença. Esta teoria envolve a barreira hemato-encefálica, neurônios, micróglia, astrócitos e várias citocinas (Parpura *et al*., 2012; Martorana *et al*., 2012; Rubio-Perez e Morrilas-Ruiz, 2012; Heneka *et al*., 2015, Su *et al*., 2016; Calsolaro e Edison, 2016; White *et al*., 2017).

As células da glia são células não neuronais do sistema nervoso central, que proporcionam suporte e nutrição aos neurônios. Estima-se que haja no SNC cerca de 10 células gliais para cada neurônio. Elas diferem em forma e função: oligodendrócitos, astrócitos, células de Schwann, células ependimárias e micróglia. Os oligodendrócitos, responsáveis pela produção da bainha de mielina, possuem a função de isolante elétrico para os neurônios do SNC. Já os astrócitos desempenham funções como a sustentação e a nutrição dos neurônios. As células de Schwann produzem a mielina que envolve os axônios. As células ependimárias revestem partes do cérebro. E finalmente, as micróglias são células pequenas e alongadas, que representam o sistema mononuclear fagocitário do SNC, participam da inflamação e reparação do SNC, secretam diversas citocinas, regulam o processo imunitário e removem os restos celulares que surgem nas lesões do SNC (Junqueira e Carneiro, 2013). Estas células fagocíticas constituem cerca de 10% de todas as células do sistema nervoso e representam a primeira linha de defesa contra patógenos invasores, servindo também de sensores especiais para a ocorrência de injúria tecidual no cérebro (Streit *et al*., 2005; Conde e Streit, 2006). Numa situação patológica, como a neurodegeneração, acidente vascular encefálico, tumores ou dano por trauma, estas células são ativadas, migram e fagocitam células mortas ou danificadas, promovendo a eliminação destes fragmentos celulares da área afetada, de modo semelhante ao que ocorre nos macrófagos fagocíticos no sistema imune

periférico. Uma vez ativada, a microglia libera uma série de mediadores pró-inflamatórios, incluindo citocinas, fatores do sistema de complemento, várias espécies radicalares, produtos secretórios tóxicos e óxido nítrico (NO), todos capazes de contribuir para a disfunção e morte neuronal (Fetler e Amigorena, 2005). Alterações inflamatórias são observadas no cérebro, principalmente nas regiões com depósito amiloide, que são ricas em células microgliais ativadas (Wash *et. al*., 2002b; Walsh e Selkoe, 2004).

Os astrócitos participam da retirada e degradação de Aβ, além de formarem uma barreira entre os depósitos de Aβ e os neurônios (Wyss-coray *et al*., 2003). Evidências também sugerem que os astrócitos são capazes de fagocitar peptídeos Aβ, um processo que pode ser dependende do *status* de ApoE, corroborando com a teoria de que polimorfismos de ApoE são fatores de risco para DA, uma vez que podem afetar a fagocitose de peptídeos Aβ pelos astrócitos (Koistinaho *et. al.,* 2004).

Além disso, enquanto acreditava-se que os neurônios eram agentes passivos num processo de neuroinflamação, evidências sugerem que eles são capazes de produzir mediadores inflamatórios. Portanto é possível que os neurônios possam exacerbar a reação inflamatória e contribuir para a sua própria destruição (Heneka e O'Bannion, 2007).

Nas placas senis, a micróglia ativada e astrócitos liberam mediadores, entre eles, citocinas pró e anti-inflamatórias que têm um papel crítico no desenvolvimento e progressão da DA (Parpura *et al.,* 2012). Citocinas são pequenas proteínas secretadas por células ativadas. Elas podem afetar outras células-alvo, ou até mesmo as células que as secretaram. Uma vez ligadas à sua célula alvo, elas coordenam uma série de eventos que podem resultar em mudança de padrão de expressão gênica, alteração da organização do citoesqueleto ou liberação de vesículas. As citocinas fazem a comunicação entre as células e apresentam um papel importante em processos fisiopatológicos (Sabat, 2010).

No processo inflamatório crônico da DA, ainda não está claro se esta inflamação é uma reação de proteção à patologia da DA ou uma contribuição para o surgimento e progressão dessa doença, e nem mesmo quando se inicia ou termina (Combarros *et al.,* 2009; Calsolaro e Edison, 2016). Associações entre a DA e biomarcadores inflamatórios, incluindo interleucina 1 beta (IL-1β), interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF-α) e proteína C reativa (PCR) têm sido registradas com resultados controversos (Sheng *et al*., 2001, Combarros *et al.,* 2009, Swardfager *et al.,* 2010, Chakrabarty *et al*., 2010).

Um estudo recente do nosso grupo corrobora com a hipótese de que o processo inflamatório está associado com o declínio cognitivo. Foi investigado o polimorfismo codon 10 T>C no gene do TGF-β1 em uma amostra populacional de individuos com idade de

75 anos ou mais e observou-se maior frequência de carreadores do alelo T (associado com menor expressão do gene) entre aqueles que mostraram maior declínio cognitivo no estudo prospectivo desta população (Fraga *et al*., 2015). O TGF-β1 já foi descrito como uma citocina que possui propriedades neuroprotetoras e neurotróficas, capaz de limitar neuroinflamação e reduzir a toxicidade das placas amiloides (Caraci *et al*., 2012).

Ghosh *et al*. (2013) induziram um aumento da expressão de IL-1β em modelos murinos de DA e um a três meses depois evidenciaram aumento de Tau hiperfosforilada. Sheng *et al*. (2001) demonstraram que a ativação da micróglia e o aumento da expressão de IL-1β fazem parte de uma cascata onde a amplificação da expressão e ativação de proteínas cinases ativadas por mitógenos (MAPKs) resulta em hiperfosforilação de Tau, levando à patologia neurofibrilar da DA em cérebro de ratos.

Estudos sugerem que a DA é acompanhada por um aumento da expressão de IL-6 no LCR, principalmente em locais próximos às placas senis (Bauer *et al*., 1991; Blum-Degen *et al*., 1995; Hüll *et al*., 1996. Segundo Huell *et al*. (1995), este aumento de expressão de IL-6 precede as mudanças neuropatológicas da DA, indicando que não seria uma consequência da neurodegeneração. Van Duijn *et al*. (1990) e Angelis *et al*. (1998) verificaram que os níveis de IL-6 não estão elevados em pacientes com DA. Dessa forma, vem se tentando estabelecer uma relação entre os níveis de IL-6 no soro e no LCR como um marcador da DA (Bermejo *et al*., 2008; Ciaramella *et al*., 2010; Galimberti *et al*., 2008; Kaplin *et al*., 2009), porém os resultados ainda são inconclusivos (Anoop *et al*., 2010). Segundo Spooren *et al*. (2011), IL-6 induz a expressão da APP e também leva à hiperfosforilação da proteína Tau. Estudos *in vitro* sustentam um papel da IL-6 na DA baseado em sua influência na Aβ e Tau, porém o mesmo não se comprovou em estudos *in vivo* (Chakrabarty *et al*., 2010). Bermejo *et al*. (2008) e Singh e Guthikonda (1997) encontraram níveis elevados de IL-1, IL-6 e TNF-α no sangue periférico de paciente com DA. Savas *et al*., (2016) encontraram níveis elevados de IL-6, mas não para IL-1β ou TNF-α, na DA.

Swardfager *et al*. (2010) realizaram uma meta-análise que incluiu 40 estudos que avaliaram os níveis de citocinas no sangue periférico e 14 estudos que avaliaram os níveis de citocina no LCR de pacientes com DA e controles cognitivamente saudáveis. No sangue periférico, 14 estudos demonstraram níveis elevados de IL-6 e TNF-α e 10 estudos demonstraram níveis elevados de IL-1β na DA comparados com os controles. Não foi observada diferença nos níveis de IL-10 e PCR-us entre os grupos. Para as dosagens no LCR, 5 estudos encontraram níveis elevados de TGF- β na DA e nenhuma diferença nos níveis de IL-1β, IL-6 e TNF-α.

A interleucina 10 (IL-10), uma citocina anti-inflamatória, foi apontada como a principal citocina associada com a ocorrência de DA (Combarros *et al.,* 2009). Ela se opõe à ação pró-inflamatória de outras citocinas ao suprimir a produção das mesmas pelas células T. A expressão de IL-1β, que tem um papel central na inflamação e morte celular, é negativamente controlada pela ação imunomoduladora da IL-10 (Combarros *et al.,* 2009). A IL-10 se apresenta como um supressor de resposta imunoproliferativa e inflamatória no cérebro por três mecanismos: redução de síntese de citocinas pró-inflamatórias, supressão da expressão de receptor de citocina e inibição da ativação desse receptor (Combarros *et al.,* 2009). De acordo com Combarros *et al*. (2009), determinadas combinações de variantes genéticas em regiões regulatórias de dois genes, como IL-6 -174G/C e IL-10 -1082A/G contribuem para a inflamação crônica em idosos, aumentando o risco de DA. Segundo Ma *et al.,* (2005), um desequilíbrio entre citocinas pró-inflamatórias e anti-inflamatórias pode ser um importante fenômeno na DA. Esta hipótese é reforçada por estudos que descrevem um aumento de 7 a 10 vezes na produção de IL-1β do que IL-10 em pacientes com DA quando comparados com controles. Contudo, Rota *et al*. (2006) não encontraram níveis alterados de IL-10 no LCR ou no soro de pacientes com DA.

2.8 Leptina e DA

O estilo de vida, incluindo o comportamento nutricional e o estresse, têm um papel crítico na demência (Lara *et al*., 2013). A relação entre o comportamento nutricional e a DA inclui a obesidade, hipertensão arterial, dislipidemia e níveis elevados de glicose no desenvolvimento da doença ou progressão. Alguns estudos clínicos reforçam a associação entre síndrome metabólica e desenvolvimento de DA (Tezapsidis *et al*., 2009; Ríos *et al*., 2014).

Em concordância com estas hipóteses, alguns estudos têm sugerido que o sobrepeso ou a obesidade na meia-idade são importantes fatores de risco para o desenvolvimento da DA (Whitmer *et al*., 2005; Beydoun *et al*., 2008). Além disso, observou-se que indivíduos com DA, apesar de não mudarem os hábitos alimentares, começam a perder peso anos antes do início dos sintomas clínicos, sugerindo uma relação entre o metabolismo do tecido adiposo e a DA (Kiliaan *et al*., 2014; Gu *et al*., 2014; Arnoldussen *et al*., 2014). Estudos prospectivos mostraram que a obesidade precede a demência e as adipocinas têm sido relatadas em estudos epidemiológicos associadas com o declínio cognitivo (Barrett-connor *et al*., 2007).

Gustafson *et al*. (2010) sugeriram que o esclarecimento da função do tecido adiposo na saúde cerebral é essencial para o completo entendimento do processo demencial. Os mesmos autores, em 2012, também demonstraram que a obesidade pode aumentar o risco de demência após os 32 anos de idade. O tecido adiposo é responsável por produzir moléculas reguladoras denominadas adipocinas. As adipocinas podem agir de forma autócrina, parácrina e endócrina e podem afetar processos no SNC e periférico (Gomes *et al*., 2005; Buchman *et al*., 2008). A liberação de adipocinas pode estar desregulada na obesidade e no envelhecimento, possivelmente devido a um prejuízo de função dos adipócitos nesta última condição. O termo "adiposopatia" pode ser usado para descrever uma desregulação do tecido adiposo e dos níveis de adipocinas com excessiva hipertrofia dos adipócitos (Buchman *et al*., 2008).

A leptina é uma adipocina composta por 167 aminoácidos, que foi relatada pela primeira vez em 1994 por Zhang *et al*. (1994). O nome leptina é derivado do grego "*leptos*", que significa fino, magro, esbelto (Auwerx *et al*., 1998). Trata-se de um hormônio peptídico anorexígeno sintetizado e secretado pelos adipócitos. Este hormônio é transportado ativamente pela barreira hematoencefálica e atua no hipotálamo, modulando o comportamento alimentar e o gasto de energia. A leptina, adipocina mais estudada, está associada com estrutura e função cerebral e tem diversos efeitos no cérebro relacionados com a cognição e envelhecimento. Estudos recentes indicam o efeito marcante desse hormônio no funcionamento e desenvolvimento do hipocampo, principalmente em processos de aprendizagem e memória (Bigalke *et al*., 2011; Theodoropoulou *et al*., 2012).

Foi demonstrado que a leptina tem um papel neuroprotetor contra a patologia da DA (Harvey *et al*., 2006; Bednarska-Makaruk *et al*., 2017). Estudos *in vitro e in vivo* já demonstraram a performance da leptina no equilíbrio da fosforilação da Tau e da proteína β-amiloide (βA). Estudos têm proposto que a leptina apresenta atividades neuroprotectivas que podem ser explicadas pela inibição do processo amiloidogênico, redução dos níveis de fosforilação da Tau e melhora da função cognitiva (Fewlass *et al*., 2004; Greco *et al*., 2008; Greco *et al*., 2009; Greco *et al*., 2010; Greco *et al*., 2011; Folch *et al*., 2012, Guo *et al*., 2016, Malekizadeh *et al*., 2016). Dessa forma, esse hormônio contribuiria para diminuir dois importantes eventos da DA. Khemka *et al*. (2014) propuseram que os níveis séricos de leptina na DA se correlacionam negativamente com a grau de demência.

2.9 Micropartículas

Micropartículas (MPs) são pequenas vesículas de 0,1 μm a 1 μm originadas de uma variedade de células como as plaquetas, endotélio, leucócitos, fator tissular e neurônios (Hargett *et al*., 2013; Schindler *et al*., 2014).

Peter Wolf (1967), utilizando microscopia eletrônica e um processo de ultracentrifugação, foi o primeiro a identificar as micropartículas como um produto das plaquetas e as nomeou como "*platelet dust*" - "poeira das plaquetas". Estudos têm demonstrado que as MPs são liberadas não só por plaquetas ativadas, mas também por várias outras células.

Inicialmente consideradas um lixo celular, após décadas de estudos, hoje sabe-se que elas não representam apenas produtos inertes dos debris celulares. As MPs podem conter uma variedade de moléculas bioativas como proteínas, lípides e ácidos nucleicos. Estas vesículas foram identificadas no plasma humano, urina, saliva e LCR (Koppler *et al*., 2006; Daniel *et al*., 2006; Cerri *et al*., 2006).

As MPs são hoje consideradas uma forma universal de comunicação entre células, de transferência cito/nuclear de proteínas (anexina, citocinas, fatores de crescimento e proteínas angiogências), DNA, mRNA e microRNA (Pap *et al*., 2009; Mause e Weber, 2010; Barteneva *et al*., 2013; Marques *et al*., 2013; Patz *et al*., 2013; Barile e Vassali, 2017; Maas *et al*., 2017).

MPs estão envolvidas na ativação de células envolvidas no processo de coagulação, sinalização celular, injúria vascular e homeostasia (Burnier *et al*., 2009; Pap *et al*., 2009; Marques *et al*., 2013). Alguns trabalhos do nosso grupo mostraram um aumento de MPs em várias doenças, como síndrome do ovário policístico e pré-eclâmpsia, resultados estes que auxiliaram no entendimento da fisiopatologia destas diversas condições clínicas (Marques *et* al., 2012; 2013; Carvalho *et al*., 2017).

Já foi reportado que fatores inflamatórios e vasculares estão alterados no cérebro ou no LCR de pacientes com DA (Zamolodchikov e Strickland, 2016; Bagyinszky *et al*., 2017). Também já foi demonstrado que MPs derivadas de plaquetas podem carrear a APP e a Aβ, podendo estar associada à DA (Matsubara *et al*., 2002). Kumar *et al*. (2017) observaram que MPs carreadoras de moléculas pró-inflamatórias liberadas pela microglia após um trauma podem contribuir para uma resposta neuroinflamatória progressiva em modelos animais, assim como estimular a resposta imune sistêmica.

Muito embora já seja conhecido que as MPs estão associadas à injúria microvascular, processos inflamatórios crônicos e disfunção da barreira hematoencefálica (Attems and

Jellinger, 2004; 2005), poucos estudos avaliaram o papel das MPs na fisiopatologia da DA (Matsubara *et al*., 2002; Xue *et* al., 2012; Schindler *et al*., 2014).

Desse modo, este estudo objetiva avaliar os níveis de MPs, de leptina e marcadores inflamatórios em idosos que apresentam comprometimento cognitivo, com fins de aplicação futura desses possíveis marcadores no diagnóstico e prognóstico dessas condições clínicas.

3 OBJETIVOS

3.1 Objetivo geral

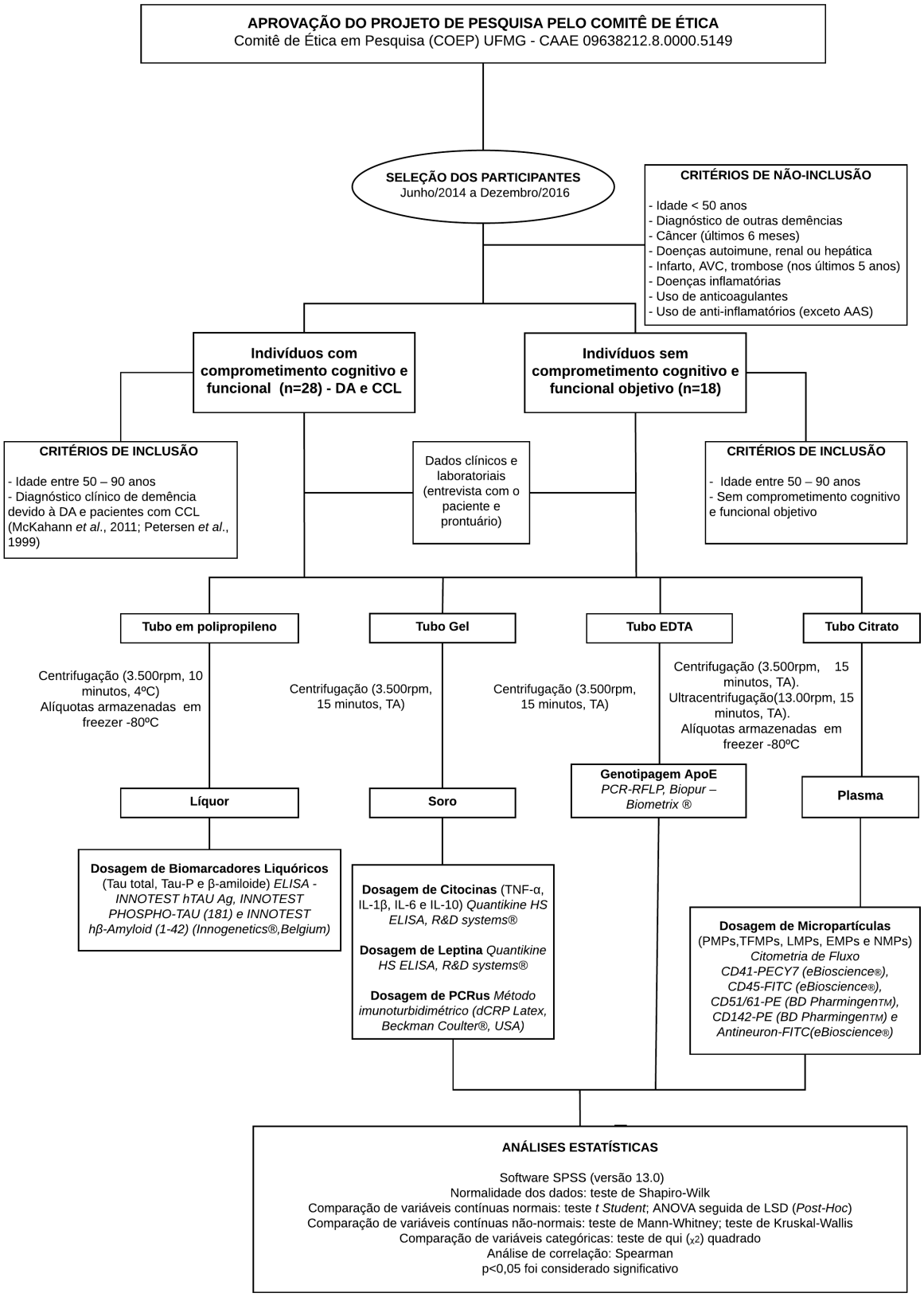
Avaliar os níveis de micropartículas, citocinas e leptina a fim de identificar a associação destes com o comprometimento cognitivo no envelhecimento.

3.2 Objetivos específicos

Comparar a concentração de micropartículas derivadas de plaquetas, leucócitos, fator tissular, endotélio e neurônio em indivíduos com e sem comprometimento cognitivo, bem como associá-las aos parâmetros clínicos e demográficos nos dois grupos.

Comparar os níves de leptina, IL-1β, IL-6, TNF-α, IL-10 e PCR-us entre indivíduos com e sem comprometimento cognitivo, bem como associá-los aos parâmetros clínicos e demográficos nesses grupos.

4 DELINEAMENTO EXPERIMENTAL



5 CAPÍTULO 1

Microparticles and TNF-α: Potential Biomarkers in Alzheimer's Disease

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ABSTRACT

Inflammatory process is part of Alzheimer's disease (AD) pathophysiology. Microparticles (MPs), a member of the extracellular vesicle family, are associated to cellular activation during inflammation, coagulation, vascular injuries and homeostasis. This study aimed to investigate the MPs and cytokines levels in AD patients compared to controls, and to propose a diagnostic algorithm for AD. Thirty-nine participants were included in this study, of whom 21 with probable dementia due to AD and 18 cognitively healthy volunteers. We evaluated platelets-derived MPs (PMPs), leukocytes-derived MPs (LMPs), tissue factor-derived MPs (TFMPs), endothelium-derived MPs (EMPs) and neuron-derived MPs (NMPs) levels in plasma, by flow cytometer assay. IL-1β, IL-6, TNF-α and IL-10 were determined using enzyme linked immunosorbent assay (ELISA). We observed that TFMPs, LMPs and NMPs levels were significant increased in the AD group when compared to controls. IL-6, IL-1β, TNF-α (Tumor Necrosis Factor-α) and hsCRP (high-sensitivity C Reactive Protein) were found to be significant lower in AD patients than in controls. The multiple logistic regression showed that TNF-α and NMPs were independently associated to AD, which resulted in a ROC curve with an area under the curve of 0.957 (sensitivity = 100% and specificity = 70%). The results suggest that MPs and TNF-α have a promising potential as biomarkers for AD diagnosis.

KEYWORDS: Alzheimer's disease, microparticles, cytokines, platelets, leukocytes, endothelial cells, tissue factor, neuron and TNF-α.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia. According to Alzheimer's Association (2017), 60% to 80% of dementia cases are due to AD. Patients with this condition exhibit as common features the limitation of autonomy and quality of life. Intrinsically related to aging, AD has been highlighted in social contexts and public health all over the world (Alzheimer's Association, 2017).

AD is histologically characterized by the accumulation of amyloid-β protein (Aβ) on extracellular plaques and deposition of hyperphosphorylated tau protein (Phospho-Tau) in intracellular neurofibrillary tangles (Hardy *et al*., 2002; Hartmann *et al*., 2004; Nitrini *et al*., 2005; Souza *et al*., 2014). The inflammatory process is also a physiological factor associated to AD, which evolves blood-brain-barrier, neurons, microglia, astrocytes and several cytokines, such as IL-6, IL-1β, TNF-α and IL-10 (Parpura *et al*., 2012). An increased number of studies have been conducted in order to validate reliable biomarkers that allow an early diagnosis of AD (Van Duijn *et al*., 1990; Bermejo *et al*., 2008; Hesse *et al*., 2016; Savas *et al*., 2016; Calsolaro & Edison *et al*., 2016; White *et al*., 2017; Bagyinszky *et al*., 2017; Popp *et al*., 2017).

Microparticles (MPs) are small vesicles released from the cell membrane during its activation and apoptosis. They are defined as particles ranging from 0,1 and 1 μm, which typically exhibit phosphatidylserine on their outer leaflet of the plasma membrane. Various types of cells have been shown to be capable of producing MPs by membrane blebbing and fission (Piccin *et al*., 2007; Hargett *et al*., 2013; Schindler *et al*., 2014).

Using electron microscopy, Wolf in 1967 was the first to identify MPs as the pro-coagulant component of plasma that was removed by high-speed centrifugation. These particles were apparently derived from activated platelets and he described them as platelets 'dust' (Wolf, 1967). Studies have shown that MPs are not only released by activated platelets, but from most cell types. Although initially these particles were dismissed as cellular 'trash', in the past decade several studies have expanded the knowledge about MPs function. They are now considered a universal form of cell–cell communication, transferring cyto/nuclear proteins (annexins, cytokines, growth factors, angiogenic proteins), DNA, mRNA and miRNA (Mause and Weber, 2010; Barteneva *et al*., 2013; Patz *et al*., 2013; Barile and Vassali, 2017;

Maas *et al*., 2017), associated to cellular activation in process as coagulation, cellular signaling, vascular injury and homeostasis (Burnier *et al*., 2009; Pap *et al*., 2009; Marques *et al*., 2013).

MPs were identified in human plasm, urine, salive and cerebrospinal fluid (CSF) (Koppler *et al*., 2006; Daniel *et al*., 2006; Cerri *et al*., 2006). Several MPs subtypes have been associated with different disorders, such as cancer (Goubran *et al*., 2015), inflammatory/infectious (Campos *et al*., 2010; Mooberry *et al*., 2016), thromboembolic (Campello *et al*., 2016), and autoimmune diseases (Burbano *et al*., 2015), as well as preeclampsia (Marques *et al*., 2013) and polycystic ovary syndrome (Carvalho *et al*., 2017).

Kumar *et al*. (2017) observed that MP loaded with pro-inflammatory molecules, released by microglia after trauma, could contribute to progressive neuroinflammatory response in an animal model of traumatic brain injury, as well as stimulate systemic immune responses. Moreover, it has been demonstrated that MPs derived from platelets may carry amyloid precursor protein (APP) or Aβ (Matsubara *et al*., 2002). However, few studies have evaluated the role of MPs in AD pathophysiology (Matsubara *et al*., 2002; Xue *et al*., 2012; Schindler *et al*., 2014), although it is known that the disease is associated with microcirculatory injury, chronic inflammatory processes and disruption of the blood-brain barrier (Attems and Jellinger, 2004; 2005).

Considering that MPs are involved in neuroinflammation and hemostasis, and that levels of several vascular and inflammatory factors were reported to be increased in patients with AD (Zamolodchikov & Strichland, 2016; Bagyinszky *et al*., 2017), we hypothesized that plasmatic levels of MPs may be altered in patients with AD. Therefore, we evaluated the association of MPs levels (platelet, leukocyte, endothelial, neuron cell-derived MPs and MPs that express TF) with clinical and laboratory parameters in AD dementia patients. Moreover, we evaluated the accuracy of the main parameters for AD diagnosis.

MATERIAL AND METHODS

Subjects

The AD group (n=21; aged 66.9 ± 9.2 years) was recruited at the Neurology Outpatient Clinic, from the Hospital das Clínicas of the Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. The control group, composed by cognitively healthy volunteers (n=18; aged 73.3 ± 7.5 years), was recruited at the Geriatric Outpatient Clinic at the same university hospital. Both groups were selected from June 2014 to December 2016. The diagnosis of AD was based on the clinical criteria for probable dementia due to AD according to recommendations from the National Institute on Aging and Alzheimer's Association - NIA-AA (McKahann *et al*., 2011).

Patients and controls were submitted to clinical and neurological examinations, including functional and cognitive evaluations. The *Functional* Assessment Staging Test (*FAST*) and the Mini-mental State Examination (MMSE) were administered to patients and controls (Folstein *et al*., 1975; Reisberg, 1998). MMSE scores were normalized according to educational level (Brucki *et al*., 2003; Caramelli *et al*., 2007). The AD patients also were submitted to the Brief Cognitive Screening Battery (BCSB; Nitrini *et al*., 1994) and the Pfeffer Functional Activities Questionnaire (Pfeffer *et al*., 1982). Importantly, all patients with AD diagnosis showed CSF biomarker compliant with the disease, with the IATI index < 1.0 pg/mL [($A\beta_{1-42}$/(240 + 1.18 x Tau)] (Riemenschneider *et al*., 2000). Controls had no history of neurological, presented performance in the MMSE above education-adjusted cut-off scores and FAST index < 3.

Exclusion criteria for both groups were: age < 50 years, autoimmune disease, kidney and liver disease, cancer (past six months), acute inflammatory disease, acute myocardial infarction or stroke (past five years), other dementias, use of anticoagulants, steroidal and non-steroidal anti-inflammatory medications, except acetylsalicylic acid.

This study was approved by the Ethics Committee of Federal University of Minas Gerais (Minas Gerais, Brazil) - CAAE 09638212.8.0000.5149 - according to the guidelines from the Declaration of Helsinki. All participants provided written informed consent prior to entering the study.

Biochemical analysis

Venous blood samples were obtained after 8 hours fasting using tubes with sodium citrate and EDTA as anticoagulant or tubes anticoagulant-free (Vacuette®). The samples were centrifuged at 1,500 xg for 20 min at 4°C to obtain the plasma or serum. Aliquots were immediately processed or stored at −80 °C until the use.

The concentrations of glucose, total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C) were determined by using commercially available colorimetric method kits (Gold Analisa Diagnostica®, Brazil). The low-density lipoprotein-cholesterol (LDL-C) was estimated by Friedwald equation (Friedewald *et al*., 1972). The serum high sensitivity - C Reactive Protein (hsCRP) levels were performed using the immunoturbidimetric method (dCRP Latex, Beckman Coulter®, USA).

Arterial hypertension was defined as systolic blood pressure ≥ 140mmHg and/or diastolic blood pressure ≥ 90mmHg at the time of evaluation, or regular use of antihypertensive medication (Chobanian *et al*., 2003). We considered dyslipidemic subjects as those currently using lipid lowering medication or with altered lipid profile, according to the III Brazilian Guidelines on Dyslipidemia and Atherosclerosis Prevention (TC > 240 mg/dL, LDL-C > 160 mg/dL, HDL-C < 40 mg/dL, or TG > 201 mg/dL) (Santos *et al*., 2001). Body Mass Index (BMI) was measured by weight in kilograms divided by the square of the height in meters (kg/m^2) (WHO, 2000), and the individuals categorized as normal - BMI < 25 kg/m^2, overweight - BMI between 25 to 29 kg/m^2, and obese - BMI ≥ 30 kg/m^2. The abdominal circumference (AC) was measured and values for men ≥ 102 cm and women ≥ 88 cm were classified as increased (WHO, 2000).

Using a commercially available kit (Biopur - Biometrix®, Brazil), genomic DNA were extracted from blood cells. The presence of the allele ε4 on Apolipoprotein E gene were evaluated by Polymerase Chain Reaction (PCR-RFLP), using the primers and methodology described by Main *et al*. (1991).

Cytokines analysis

The serum concentrations of IL-1β, IL-6, TNF-α and IL-10 were determined using enzyme linked immunosorbent assay (ELISA) (Quantikine HS ELISA, R&D systems®, USA). The lower detection limits ranged from (0.023-0.140), (0.016-0.110), (0.038-0.191), (0.03-0.17) pg/mL, respectively. The samples were analyzed in duplicate, with an intra-assay variations <5%. An internal quality control was used in all assays.

CSF biomarkers

Lumbar puncture was performed to collect the CSF in AD group. The samples were centrifuged at 1,500 xg for 10 minutes, at 4ºC, maximum 4 hours after collection. Aliquots were immediately stored at –80 °C until the analysis. The biomarkers were measured using the INNOTEST hTAU Ag, INNOTEST PHOSPHO-TAU (181) and INNOTEST hβ-Amyloid (1-42) (Innogenetics®, Belgium) kits by ELISA. The manufacturer's instructions were strictly followed. The samples were analyzed simultaneously for the three biomarkers, and intra-assay variations based on duplicates was <5%. In all assays was used an internal quality control.

MPs Flow Cytometry Assay

The purification of the MPs was performed according to Campos *et al*. (2010). In order to obtain platelet-free plasma, the plasma samples were centrifuged at 13,000 xg for 5 minutes. The supernatant was aspirated and diluted 1:3 in phosphate buffered saline (PBS) containing heparin, and again centrifuged at 14,000 xg for 90 min at 15°C. The subsequent MPs pellet was resuspended in 1X annexin V binding buffer (BD Pharmingen®, USA).

MPs were determined in the LSR Fortessa cytometer (BD Biosciences®, USA) and gated on basis of their forward (FSC) and side (SSC) scatter distribution of synthetic 0.7–0.9 μm SPHEROTM Amino Fluorescent Particles (Spherotech®, USA). The presence of phosphatidylserine residues on the MP surfaces was assessed for their positive staining with monoclonal antibodies against annexin V (BD Pharmingen®, USA) labeled with allophycocyanin (APC).

Cell-specific monoclonal antibodies were used to identify the source of the MPs. CD41-PECY7 (eBioscience®, USA), CD45-FITC (eBioscience®, USA), CD51/61-PE (BD Pharmingen®, USA) and CD142-PE (BD Pharmingen®, USA) and Antineuron-FITC were used to label platelet-derived MPs (PMPs), leukocyte-derived MPs (LMPs), endothelium cell-derived MPs (EMPs), MPs that express TF (TFMPs) and neuron-derived MPs (NMPs), respectively. The antibodies were used in concentrations according to manufacturer's instructions. The specific monoclonal antibody was corrected for isotype-matched control antibodies. FACSDIVA 6.2 software (BD Pharmingen®, USA) was used for data acquisition and the analysis was performed using the FlowJo software (Tree Star®, USA).

In order to determine the absolute MPs plasma levels, the cytometer was set to operate with a high setting flow for 60 seconds for each sample. The MPs/μL of plasma was calculated as described by Campos *et al*. (2010): MPs/μL = (N x 400) / (100 × 60), in which N is equal to the number of events, 400 is the total volume of sample into the tube prior to analysis, 60 is the volume of the analyzed sample, and 100 is the original volume of MPs suspension. This formula was validated using Trucount tubes (BD PharmingenTM) in five samples, randomly selected.

Statistical analyses

Statistical analyses were performed using Statistical Package of the Social Sciences (SPSS) 13.0 version. The results are expressed as median (interquartile ranges, 25th–75th percentiles) or mean (± standard deviation), when appropriate. We performed the Student t test for normal variables and the Mann-Whitney test for non-normal variables. For categorical variables, we used the chi-square test. Correlation was assessed using the Spearman rank correlation method. For all analyses, we considered $p<0.05$ statistically significant.

We performed a univariate logistic regression with the clinical and laboratorial variables considering as outcome the AD diagnosis compared with control. After univariate analysis, the variables with p value under than 0.2 were selected to multiple logistic regression. The adequacy to the model was confirmed through Hosmer-Lemeshow test.

The variables independently associated with AD ($p<0.05$) were included in the construction of an algorithm, through the logistic regression formula, which provided π values for each patient,

$$\pi = 1/[1 + e^{-(\beta 0+\beta 1X1+\beta 2X2+\beta 3X3)}]$$

where β0 is the constant of the model, β1…β3 are the estimated coefficient for each variable, and x1…x3 are the values for each patient corresponding to the variable.

A Receiver Operator Characteristic (ROC) curve was constructed with the π values obtained from each subject, considering the AD group as disease and the control group as non-disease. The best cut-off for π value was established by the highest value of the Youden index based on ROC curve.

RESULTS

AD patients were significantly younger than controls (p=0.024). Since educational level showed difference between the groups, with controls presenting higher frequency of individuals with ≤ 4 years of study, the cognitive evaluation was normalized according to schooling. We observed that 95.2% of AD patients and none of controls, presented MMSE alterated (p<0.001). ApoE ε4 carriers were more frequent in AD group (p=0.029). No difference regarding arterial hypertension, diabetes mellitus, dyslipidemia, BMI and AC was observed between the groups (all p>0.05) (Table 1).

AD patients presented $A\beta_{(1-42)}$ = 547.89 ± 125.97 pg/mL, Total-Tau (T-Tau) = 671.11 ± 308.88 pg/mL, Phospho-Tau (P-Tau) = 84.34 ± 36.89 pg/mL, and IATI index = 0.60 ± 0.23 pg/mL.

IL-6, IL-1β, TNF-α and hsCRP levels were found to be significant lower in AD patients than in controls (p=0.046, 0.024, 0.001 and 0.027, respectively). However, serum IL-10 levels of AD patients showed no change when compared to the cognitively healthy controls (p=0.940) (Table 2). The correlation between cytokines and hsCRP levels with CSF biomarkers on AD group was evaluated and no correlation was observed (all p>0.05, data not shown).

A higher total number of MPs was observed in AD (1999.91 ± 584.36 MPs/μL) than in the control group (392.12 ± 237.17 MPs/μL, p<0.001). TFMPs (94.50 IQR 110.73 MPs/μL), LMPs (109.74 ± 65.51 MPs/μL) and NMPs (429.95 ± 321.65 MPs/μL) levels in AD were significant increased when compared to controls - TFMPs (38.53 IQR 11.60 MPs/μL,

p=0.029); LMPs (43.82 ± 30.68 MPs/μL, p=0.004); NMPs (95.36 ± 99.74 MPs/μL, p=0.002), respectively. The PMPs levels demonstrated a tendency to be higher in AD group (260.27 IQR 360.97) than in control group (135.47 IQR 225.87, p=0.081). No difference in EMPs levels between the groups (71.10 ± 53.88 for AD and 39.73 ± 62.99 for controls, p = 0.155) was found (Figure 1). All MPs subtypes showed correlation between each other (p<0.01). However, it was not observed correlation between the severity of AD and cytokines or microparticles levels (all p>0.05).

We also evaluated the correlation between MPs and CSF biomarkers in the AD group. We observed a negative correlation between Aβ and LMPs (r: -0.410), Aβ and PMPs (r: -0.367), Aβ and TFMPs (r: -0.364). A negative correlation between IATI index and NMPs (r: -0,362) was also observed. All of them were significant (p<0.05). Besides, we observed that ApoE ε4 presence was correlated with higher levels of T-Tau, PMPs and TFMPS (r: 0.471, r: 0.589, r: 0.495, respectively; p<0.05).

The multiple logistic regression showed that the variables independently associated to AD were TNF-α (OR = 0.793, 95% CI = 0.635-0.991; p=0.041) and NMPs (OR= 1,034, 95% CI = 1.003-1.066; p=0.029). The β_0 constant of the model was -2.527, β1 TNF-α coefficient was -0.232, and β_2 NMPs coefficient was 0.034, which were used in the logistic regression formula to calculate π values. After applying this algorithm for each individual from both groups, the ROC curve was obtained (Figure 2), considering the AD patients as the disease group and controls as the non-disease group. The area under the curve (AUC) was 0.957 (p<0.001). The π value of 0.264 was the best cut-off for balancing sensitivity (100%) and specificity (70%). Consequently, π values higher than 0.264 reinforce the AD diagnosis.

DISCUSSION

This study evaluated the relationship between AD, cytokines and MPs. We observed that total MPs (annexin-positive) level were increased in AD patients when compared to the control group, as well as TFMPs, LMPs and NMPs, which suggests their potentialities as biomarkers in AD. Moreover, TNF-α and NMPs showed independent association with the disease. Younger subjects were more frequent in AD than in control group, although all AD patients presented positive CSF biomarkers for AD and higher frequency of ε4 carriers.

IL-6, IL-1β, TNF-α and hsCRP levels were found to be significant lower in AD patients and serum IL-10 levels of AD patients showed no change when compared to cognitively healthy controls. Although there is evidence of the effect of BMI on cytokines levels (Larsson *et al,* 2015), in our study, BMI and AC did not differ between the groups and no influence on cytokine levels was observed.

Neuroinflammation is a common feature in neurodegenerative disorders. In AD microglia are over-activated, resulting in increased production of pro-inflammatory cytokines. (Martorana *et al*., 2012; Rubio-Perez *et al*., 2012; Heneka *et al*., 2015, Su *et al*., 2016; Calsolaro and Edison; 2016; White *et al*., 2017). However, the debate is ongoing about the neuroinflammation precise role, whether it is protective or harmful, and when it starts and finishes. Some of the measured inflammatory markers might increase steadily during disease progression or temporarily at the pre-clinical and early clinical disease stages (Calsolaro and Edison, 2016).

Increased levels of IL-1, IL-6, or TNF-α have been found in peripheral blood of patients with mild to moderate AD (Bermejo *et al;* 2008; Singh and Guthikonda 1997). However, other studies did not detect different levels of cytokines when compared AD and control groups (Van Duijn *et al*., 1990; Angelis *et al*., 1998). Swardfager *et al*. (2010) included 40 studies assessing cytokines in peripheral blood and 14 assessing cytokines in CSF in patients with AD and cognitively healthy controls in a meta-analysis. They discussed that 14 studies demonstrated elevated levels of IL-6, TNF-α and 10 studies demonstrated elevated levels of IL-1β in peripheral blood on AD compared to controls. They did not find differences between IL-10 and CRP levels in these groups. For those dosages in CSF, five studies found elevated levels of TGF- β on AD and no difference for IL-6, TNF-α and IL-1β.

Different study designs and types of samples lead to conflicting results that hinder the use of cytokines as biomarkers for AD (Hesse *et al*., 2016). Hesse *et al*. (2016) analyzed IL-1β, IL-8 and TNF-α in CSF and serum samples of AD patients and they found decreased IL-8 levels in both samples, besides negative correlation of MMSE and IL-1β. Savas *et al*. (2016) found higher blood levels of IL-6, but not for hsCRP or TNF-α, in AD patients compared to controls.

Popp *et al*. (2017) evaluated inflammatory biomarkers associated to AD and their association with CSF biomarkers (amyloid protein and Tau pathology). IL-1β, IL-6, TNF-α and IL-10

did not show correlation with CSF AD biomarkers. They suggested that inflammation is part of the AD pathology only at early clinical stages. Our results suggest that there is a decrease in cytokine levels with the clinical course of AD. Independent sample validation and longitudinal clinical follow-up are needed to evaluate the usefulness of inflammatory biomarker signatures in clinical settings.

MPs have been implicated in neuronal development, synaptic activity, nerve regeneration, and protective mechanisms (Verderio *et al.,* 2012; Lai and Breakefield, 2012). They are also capable of transferring proteins between cells, which have implications for neurodegenerative disorders, such as AD (Matsubara *et al.,* 2002). Elevated levels of MPs have been detected in the CSF and plasma of individuals suffering from a variety of diseases of the central nervous system (Verderio *et al.,* 2012; Minagar *et al.,* 2001; Combes *et al.,* 2005). We suggest that increased levels of specific types of MPs in plasma and CSF might represent reliable AD biological markers (Verderio *et al.,* 2012; Huttner *et al.,* 2012; Witwer *et al.,* 2013).

It is important to highlight that AD is a condition associated with inflammation, progressive degeneration of neurons, formation of neurofibrillary tangles, development of Aβ plaques, as well as involvement of microcirculation injury, capillary blockade, and disruption of proper functioning of the blood-brain barrier (Yun *et al.,* 2016). Consequently, we characterized MPs according to cell origin associated to these mechanisms. We found that TFMPs, LMPs and NMPs levels in AD patients were significant increased when compared to controls.

TFMPs contribute to coagulation processes and have potential relevance in disorders of hemostasis and thrombosis (Lacroix *et al.,* 2013; Mooberry *et al.,* 2016). TF is the principal physiological initiator of coagulation *in vivo* through its interactions with the coagulation protease Factor VII/VIIa and is constitutively expressed by most vessel component cells other than endothelium (Key *et al.,* 2010). This function may underlie the contribution of TFMPs to the possible presence of a vascular injury on AD.

LMPs originate from neutrophils, monocytes/macrophages, and lymphocytes (Wojta *et al.,* 2015; Angelillo-Scherrer, 2012). In inflammatory diseases, such as arthritis, LMPs are increased in the blood and other fluids, where they stimulate cell release of proinflammatory cytokines (Mause *et al., 2005;* Miller *et al.,* 2016). In our study, we demonstrated higher levels of these MPs in the AD group, which reflects leukocyte activation in the disease.

The PMPs levels demonstrated a tendency to be higher in AD group than in control group. PMPs have influence in innate immunity, inflammation and thrombosis (Kapur *et al.,* 2015; McCarthy *et al.,* 2017). Moreover, PMPs seem to have the capacity to capture and incorporate TF in order to promote coagulation (Del Conde *et al.,* 2005). PMPs also can stimulate the release of cytokines and change the endothelial reactivity (Varon *et al.,* 2015). Considering that platelets play a central role in primary hemostasis (Puddu *et al.,* 2010; Varon *et al.,* 2015), we could hypothesize that PMPs can result from platelet aggregation in AD.

Lee *et al*. (2014) illustrated the role of EMPs in endothelial dysfunction. They observed an increased level of MP generation in response to TNF-α treatment. Xue *et al*. (2012) found that EMPs were significantly higher in the AD patients than in the control subjects, but the exact mechanism underlying this association is unclear. Circulating EMPs are associated with vascular dysfunction and have been shown to be critical for angiogenesis (Yun *et al.,* 2016). A significant difference in EMPs between AD and the control groups was not observed. We hypothesized that lower levels of TNF-α in AD group compared to control group could have prevented the increase of EMPs in AD.

To our knowledge, it is the first study that investigated the NMPs levels in patients with AD when compared to cognitively health individuals. Higher NMPs levels in AD individuals than controls suggest that MPs contribute to the pathophysiology of the disease.

We found significant and inverse correlations between LMPs, PMPs and TFMPs with Aβ, as well as NMPs with IATI index. These results suggest that these MPs can follow the evolution of the disease and reinforces its potential use as a diagnosis marker. Besides, we observed that the presence of ApoE ε4 allele was correlated with higher levels of T-Tau, PMPs and TFMPS, which confirm its importance on AD pathogenesis, although the mechanisms involving ApoE and MPs remains to be elucidated.

In order to investigate the association between the variables and AD diagnosis, a multiple logistic regression was applied and showed that TNF-α and NMPs was independently associated with the disease. These variables were included in the design of an algorithm that generated a ROC curve, in which the AUC was 0.957 for AD diagnosis.

Several studies have been carried out in order to optimize the AD diagnosis and differentiate it from others neurodegenerative disorders. Molinuevo *et al*. (2013) found that CSF Aβ and Tau proteins measurement presents 85% of sensitivity and 74.2% and 80.3% of specificity, respectively, for AD diagnosis, when compared to healthy controls. The ROC curve obtained demonstrated 100% sensitivity and 70% specificity for AD diagnosis. The sum of these values was higher when compared with the isolated analysis of each CSF biomarker. It is important to point out that this mathematical model requires determination of levels of only two biomarkers (TNF-α and NMPs), all measured in peripheral blood samples. As the sample size was the main limitation of this study, we highlight the need of validating this analysis as an algorithm in other larger populations.

CONCLUSIONS

Our results demonstrate an increase in the number of TFMPs, LMPs, NMPs and a decrease on IL-6, IL-1β, hsCRP and TNF-α levels in patients with probable AD dementia when compared to cognitively healthy controls. We also showed that MPs correlates with CSF biomarkers on AD and *APOE* genotype. The multiple logistic regression showed that TNF-α and NMPs were independently associated to AD, which resulted in a ROC curve with an area under the curve = 0.957, sensitivity of 100% and specificity of 70%. Further studies are required for an improved understanding of the pathological mechanisms underlying MPs formation. However, our results suggest that MPs, especially NMPs and TNF-α have a promising potential as biomarkers for AD diagnosis.

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CONFLICTS OF INTEREST

Conflicts of interest: none

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L.C., Alcolea, D., Jessen, F., Sarazin, M., Lamari, F., Balasa, M., Antoneli, A., Rami, L., 2013. The AD-CSF-Index Discriminates Alzheimer's Disease Patients from Healthy Controls: A Validation Study. Journal of Alzheimer's disease, 36, 67-77

Table 1: Demographic and clinical frequency data for Alzheimer's disease and the control groups.

Characteristics		AD group N=21	Control group N=18	P
Age	≤ 59 years	7(33.3%)[b]	0(0%)[a]	0.024*
	60 - 79 years	11(52.4%)	13(72.2%)	
	≥79 years	3(14.3%)	5(27.8%)	
Sex	male	11(52.4%)	4(22.2%)	0.098
	female	10(47.6%)	14(77.8%)	
Education	≤ 4 years	4(19.0%)[b]	12(66.7%)[a]	0.007*
	5 - 8 years	7(33.3%)	4(22.2%)	
	> 9 years	10(47.6%)	2(11.1%)	
AD severity	1	10(47.6%)	-	-
	2	6(28.6%)	-	
	3	5(23.8%)	-	
MMSE	normal	1(4.8%)	18(100%)	<0.001*
	altered	20(95.2%)	0(0%)	
Hypertension	no	9(42.9%)	4(23.5 %)	0.307
	yes	12(57.1%)	13 (76.5%)	
Diabetes mellitus	no	17(81.0%)	14(82.4%)	1.000
	yes	4(19.0%)	3(17.6%)	
Dyslipidemia	no	10(47.6%)	8(47.1%)	1.000
	yes	11(52.4%)	9(52.9%)	
BMI	normal	11(52.4%)	4(25.0%)	0.083
	overweight	8(38.1%)	6(37.5%)	
	obese	2(9.5%)	6(37.5%)	
AC	normal	13(61.9%)	6(35.3%)	0.111
	increased	8(38.1%)	11(64.7%)	
ApoE ε4	not carrier	4(21.1%)	9(64.3%)	0.029*
	carrier	15(78.9%)	5(35.7%)	

AD - Alzheimer's disease, ApoE - apolipoprotein ε4, MMSE - Mini-mental state examination, BMI - Body mass index, AC - Abdominal circumference. a – less frequent by residue test, b - more frequent by residue test. *Significative: P<0.05. Missing data: hypertension=1 control; diabetes mellitus=1control; dyslipidemia=1 control; BMI=2 controls; AC=1 control; ApoE ε4=2 ADs and 4 controls. *Significative: p<0.05.

Table 2: Cytokines and CRP serum levels on Alzheimer's disease and control groups.

Variables	AD	Control	P
IL-6 (pg/mL)	2.18 ± 1.96	3.58 ± 2.19	0.046*
IL-1β (pg/mL)	0.13 (0.30)	0.29 (2.35)	0.024*
TNF-α (pg/mL)	1.08 (0.50)	2.04 (10.94)	0.001*
IL-10 (pg/mL)	0.70 (0.69)	20.46 (1.29)	0.940
hsCRP (mg/dL	0.34 (0.88)	2.29 (4.74)	0.027*

Variables expressed as mean ± standart deviation or median (interquartile range). *Significative: p<0.05.

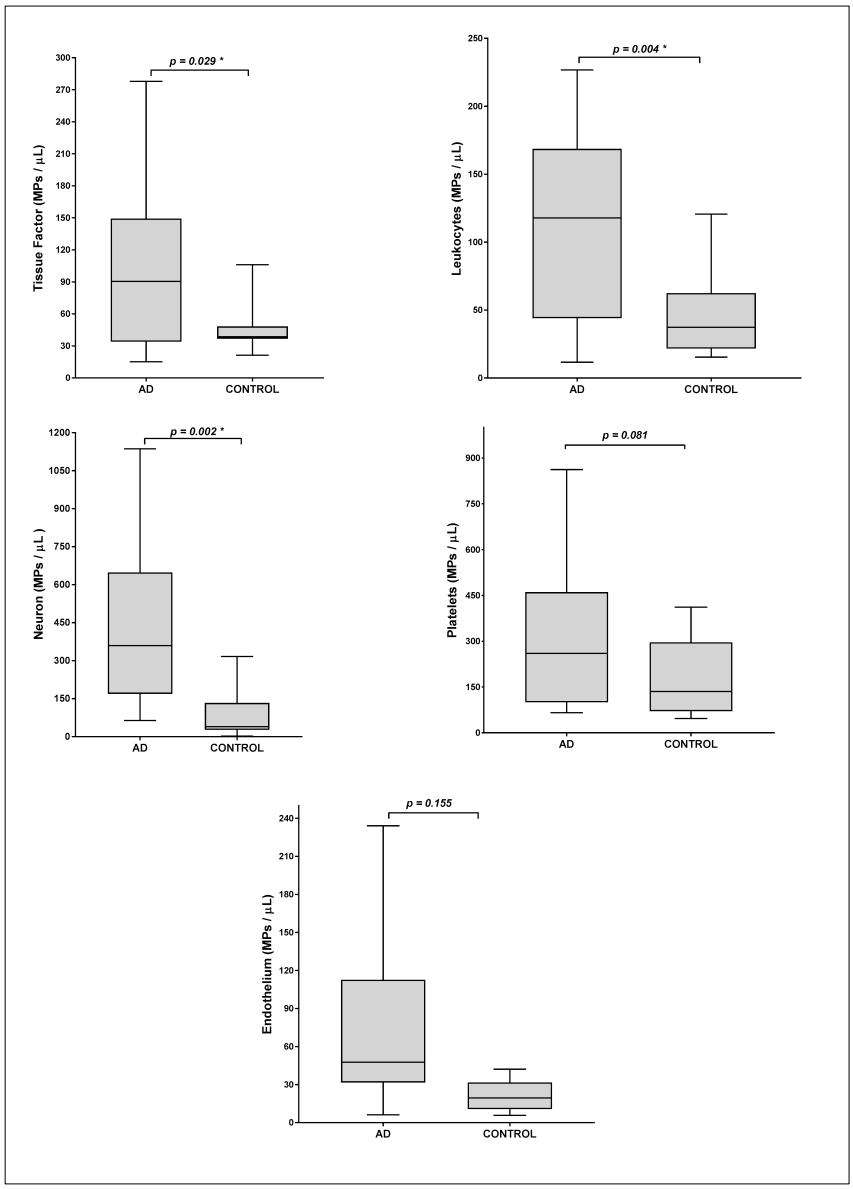

Figure 1: Distribution of microparticles levels (MPs/µL) between AD and control groups. MPs that express tissue factor (TFMPs – annexin V and CD142-PE), Leukocytes-derived MPs (LMPs - annexin V and CD45-FITC), Neuron cell-derived MPs (NMPs - annexin V and antineuron-FITC), Platelets-derived MPs (PMPs - annexin V and CD41-PECY7) and Endothelial cell-derived MPs (EMPs - annexin V and CD51/61-PE). *p<0.05 was considered statistically significant; T-Student and Mann-Whitney test.

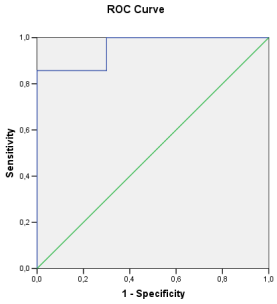

Figure 2 - ROC curve for π values from logistic regression formula considering the AD diagnosis as reference.

6 CAPÍTULO 2

Letter

MICROPARTICLES ARE RELATED TO COGNITIVE AND FUNCTIONAL STATUS EVALUATED FROM NORMAL AGING TO DEMENTIA

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Dear editor,

Dementia is a general term for the occurrence of cognitive impairment that may be caused by a group of different disorders (Alzheimer's Association, 2017). The most frequent cause of dementia is Alzheimer's disease (AD) (Alzheimer's Association, 2017). AD may cause a significant and progressive loss of cognitive and functional performance, often accompanied by personality and behavioral changes (Castellani *et al*. 2010; Heppner *et al*., 2015). The mild cognitive impairment state (MCI) precedes the AD dementia (Petersen *et al*., 1999), and refers to condition in which the subjects maintain preserved function in daily activities (Petersen *et al*., 1999, Gauthier *et al*., 2006). Between normal aging and mild cognitive impairment (MCI) it have been described an intermediate state defined as subjective cognitive decline (SCD), characterized by a self-perceived decline, but without objective evidence of cognitive decline. The Functional Assessment Staging – FAST is a measure of the degree of impairment in functional capabilities, which accompanies the cognitive impairment (Reisberg, 1988; Shankle *et al*., 2013). Indeed, the cognitive screening is frequently evaluated by the Mini-Mental State Examination (MMSE), which allows a good evaluation of global cognitive functioning.

Microparticles (MPs) are small vesicles, ranging from 0,1 and 1 μm, released from the cell membrane during its activation and apoptosis They are involved in universal form of cell–cell communication, transferring cyto/nuclear proteins, DNA, mRNA and miRNA (Mause and Weber, 2010; Barteneva *et al*., 2013; Patz *et al*., 2013; Barile and Vassali, 2017; Maas *et al*., 2017). Considering that MPs are involved in neuroinflammation and hemostasis on AD (Zamolodchikov & Strickland, 2016; Kumar *et al*., 2017; Bagyinszky *et al*., 2017), this study proposed to investigate the correlation between the MPs levels (platelet, leukocyte, endothelial, MPs that express TF and neuron cell-derived MPs), global cognitive performance and functional status in a sample of elderly individuals. We hypothesized that plasmatic levels of MPs may be altered with the onset of MCI and AD.

For this purpose, subjects were recruited among patients attending at the Geriatric and Neurology Outpatient Clinics of the Hospital das Clínicas, Federal University of Minas Gerais (UFMG), in Belo Horizonte, Brazil. Between June 2014 and December 2016, 43 participants were included in this study, of whom 12 with probable AD, 16 with MCI and 15 with no objective cognitive or functional impairment. The diagnosis of AD was based on

from the National Institute on Aging and Alzheimer's Association - NIA-AA (McKhann *et al*., 2011) recommendations and subjects with MCI were selected according to Petersen *et al*. (1999).

Cognitive performance was evaluated according to levels of education (illiterate; 1-3; 4-7; and >7 years of education). For MMSE value, a reference cut-off was set at the 25th percentile, determined by the epidemiological study of dementia in a Brazilian healthy community (Herrera *et al*., 2002) and in accordance with the baseline performance from the participants that remained free of dementia in a 3-year follow-up (Caramelli *et al*., 2007b). The MMSE values were also normalized as z scores, from the standards of performance, according to four levels of formal educational levels obtained from Caramelli *et al*. (2007b) evaluation. Patients with no functional impairment were classified as FAST stage 1. Individuals with subjective cognitive complaints but without objective evidence of impairment were classified as FAST stage 2. FAST stage 3 included MCI patients. Patients with FAST stage 4, 5 and 6 had functional deficits that correspond to mild, moderate and severe dementia respectively (Reisberg, 1988; Shankle *et al*., 2013).

The absolute MPs plasma levels were determined according to Campos *et al*. (2010). Cell-specific monoclonal antibodies were used to identify the source of the MPs by flow cytometer. CD41-PECY7 (eBioscience®, USA), CD45-FITC (eBioscience®, USA), CD51/61-PE (BD Pharmingen®, USA) and CD142-PE (BD Pharmingen®, USA) and Antineuron-FITC were used to label platelet-derived MPs (PMPs), leukocyte-derived MPs (LMPs), endothelium cell-derived MPs (EMPs), MPs that express TF (TFMPs) and neuron-derived MPs (NMPs), respectively.

Statistical analyses were performed using SPSS 13.0 version. The results are expressed as median (interquartile ranges) (all non-parametric). The Mann-Whitney test was applied to compare non-normal two groups. Correlation was assessed using the Spearman rank correlation test. For all analyses, we considered $p<0.05$ statistically significant.

The group was composed by 15 (35%) men and 28 (65%) women, with median age = 72.0 (12.0) years. Educational level, 2 patients (4.6%) were illiterate, 23 (53.5%) had 1-3, 8 (18.6%) had 4-7, and 10 (23.3%) had >7 years of education. The frequencies of FAST stages were: 1 – 6 individuals (8.5%); 2 – 7 (9.9%); 3 – 18 (25.4%); 4 – 6 (8.5%); 5 – 4 (2.8%); and

6 – 2 individuals (2.8%). We observed 17 individuals (23.9%) with MMSE scores below the cut-offs according to educational level. The median z score was -0,102 (2.970).

We found a negative and significant correlation between PMPs, TFMPs, LMPs and EMPs with z score (r: -0.542, p=0.002; r= -0.501, p=0.006; r=-0.456, p=0.013; and r=-0.485, p=0.008, respectively). We also observed a positive and significant correlation between TFMPs, LMPs, EMPs and NMPs with FAST stages (r=0.427, p=0.021; r: 0.532, p=0.003; r: 0.589, p=0.001; and r: 0.655, p<0.001).

In another analysis, the z score was reclassified as 1 (z score negative) or 2 (z score=0 or positive). We observed that in subjects with z score=1, the PMPs [206.13 (158.33) MPs/μL], LMPs [84.07 (77.33) MPs/μL] and TFMPs [70.60 (61.60) MPs/μL] levels were higher when compared to subjects with z score=2: PMPs [102.43 (109.25) MPs/μL], LMPs [40.33 (68.77) MPs/μL] and TFMPs [40.47 (39.08) MPs/μL] (p=0.016, p=0.046 and p=0.023, respectively). The EMPs [34.67 (44.40) MPs/μL] levels demonstrated a tendency to be higher in z score=1 group than z score=2 group [16.47 (25.50) MPs/μL, p = 0.051].

Similarly, we reclassified FAST groups in 1 (stages 1 and 2) and 2 (3 to 6 stages, MCI and AD). We observed that subjects with MCI or AD (FAST 2) demonstrated higher levels of LMPs [85.87 (83.47) MPs/μL] and NMPs [133.87 (486.47) MPs/μL] when compared to subjects with no functional impairment or cognitive complaints (FAST 1); LMPs [40.33 (40.33) MPs/μL] and NMPs [39.30 (90.07) MPs/μL] (p=0.024 and p=0.004, respectively). EMPs demonstrated a tendency to be higher in FAST 2 group [34.67 (38.93) MPs/μL] than in FAST 1 group [19.53 (16.02) MPs/μL] (p = 0.062).

Our study showed that higher MPs levels, especially PMPs, LMPs and TFMPs, were associated with worst cognitive decline in this population. PMPs, LMPs and TFMPs are related to inflammation, endothelial damage and hemostasis complications (Kapur *et al.,* 2015; McCarthy *et al.,* 2017). Moreover, these MPs can also stimulate cell release of proinflammatory cytokines, amplifying the systemic inflammatory process (Varon *et al.,* 2015). Consequently, the results suggest that the platelet aggregation and inflammation are possible mechanisms associated with a decline of cognition. We also observed that higher MPs levels, mainly LMPs and NMP, were associated to lower functional performance (FAST

stages) in the elderly sample. These findings suggest a role of inflammation and neuronal loss on the dementing process that compromises functional capabilities.

To our knowledge, it is the first study that investigated the MPs levels according to cognitive and functional stages, varying from normal to demential stages. The main limitation of our study is the small sample size. Consequently, prospective design with larger samples studies are required in order to better comprehend the role of MPs on the dementing process that compromises functional and cognitive capabilities.

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CONFLICTS OF INTEREST

Conflicts of interest: none

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7 CAPÍTULO 3

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Invited critical review

Leptin in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common cause of progressive dementia in the elderly population. AD is histologically characterized by accumulation of amyloid-β protein (Aβ) on extracellular plaques and deposition of hyperphosphorylated tau protein in intracellular neurofibrillary tangles. Several studies have shown that obesity may precede dementia and that lifestyle factors play a critical role in the onset of AD. Furthermore, accumulating evidence indicates that obesity is an independent risk factor for developing AD. In this scenario, the understanding of the role of adipose tissue in brain health is essential to clarify the establishment of demential processes. The objective of this work was to review studies regarding leptin, an anorexigenic peptide hormone synthesized in adipocytes, in the context of dementia. Some authors proposed that leptin evaluation might be a better predictor of dementia than traditional anthropometric measures. Leptin, once established as a biomarker, could enhance the understanding of late-onset AD risk over the life course, as well as the clinical progression of prodromal state to manifested AD. Other studies have proposed that leptin presents neuroprotective activities, which could be explained by inhibiting the amyloidogenic process, reducing the levels of tau protein phosphorylation and improving the cognitive function.

Contents

1. Introduction

Alzheimer's disease (AD) is the most common cause of progressive dementia in the elderly population [1–3]. This chronic neurodegenerative disorder leads to progressive disturbances of cognitive functions including memory, judgment, decision-making, orientation to physical surroundings and language [4]. AD is histologically characterized by the accumulation of amyloid-β protein (Aβ) on extracellular plaques and deposition of hyperphosphorylated tau protein in intracellular neurofibrillary tangles [5–8]. According to Alzheimer's Association (2015), the disease prevalence is one case in nine elderly with 65 years or more [1].

Recent studies suggest that lifestyle factors, including nutritional behaviors and stress, play a critical role in the onset of dementia and those individuals with AD present a generalized metabolic disorder [9]. The relationship between nutritional behavior and AD includes the role of obesity, hypertension, dyslipidemia and elevated glucose levels in the disease development or progression. Some clinical studies support the association between metabolic syndrome and the onset of AD [10,11]. In agreement with this hypothesis, some studies have suggested that

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overweight or obesity in middle age is considered an important risk factor for later development of AD [12,13]. Additionally, a consistent observation is that individuals with AD, despite unchanged eating habits, begin to lose weight some years before the onset of clinical symptoms, suggesting a relation between adipose tissue metabolism and AD [14–16]. Prospective studies have showed that obesity precedes dementia and adipokines had been reported in epidemiologic studies associated with cognitive decline [17].

Gustafson (2010) have suggested that clarifying the role of adipose tissue in health of the brain is essential to a complete understanding of demential processes [18].

The Gustafson et al. (2012) also demonstrated that high mid-life central adiposity might increase the risk for dementia after 32 years [19].

Accumulating evidence indicates that obesity is an independent risk factor for developing Alzheimer disease (AD). Zeki et al. (2013) proposed that leptin, an adipokine, might be a better predictor of dementia/mild cognitive impairment (MCI) than traditional anthropometric measures [20]. They observed association between higher serum leptin and lower frequency of dementia/MCI in women with normal body mass index than overweight or obese women. Koga et al. (2014) verified that diet-induced obesity enhanced Aβ and tau induced-pathology in wild-type mice hippocampus [21]. They demonstrated that persistent obesity from early life induces tau phosphorylation in the hippocampus accompanied by enhanced astroglial leptin receptor (LEPR) expression that might accelerate pathological processes in neurodegenerative disorders.

The aim of this work was to elaborate a narrative review regarding the role of leptin in the context of dementia. The gathered data suggest that variations in the leptin levels can be a risk factor associated with the occurrence of AD.

2. Material and methods

For this narrative review, a search was done in Pubmed, Cochrane, Science Direct, Scopus and Web of Science database with the terms "dementia", "Alzheimer's disease", "adipokines", "leptin" and studies reporting on "associations between Alzheimer's disease and leptin", with no date or type of study restrictions. We included in this review 69 studies published between 1998 and 2015, in English and Portuguese languages.

3. Leptin

The adipose tissue is responsible for production of regulatory molecules, which may be assorted in a group — the adipokines. The word adipokine or adipocytokine means adipose cell in movement. Adipokines have autocrine, paracrine, and endocrine mechanisms of action and many adipokines affect processes in both the peripheral and central nervous system (CNS) [22,23]. Adipokine release can be dysregulated in both obesity and ageing, possibly due to impaired function. The term "adiposopathy" has been used to describe dysregulated adipose tissue and adipokine levels with excessive hypertrophy of adipocytes [22].

Leptin is an adipokine composed of 167 amino acids, first related in 1994 by Zhang et al. [24]. The name leptin (Lep) is derived from the Greek *leptos*, which means thin. The human leptin is encoded by the LEP gene (also called obese, OB) on chromosome 7q32; its gene spans approximately 20 Kb and contains 3 exons [25].

Leptin is an anorexigenic peptide hormone synthesized and secreted from adipocytes, mainly by white adipose tissue and in very small amounts by brown adipose tissue. This hormone is actively transported across the blood–brain barrier and acts on hypothalamic modulation of feeding behavior and energy expenditure [26,27]. Leptin is also synthetized in other tissues, including placenta, ovaries, skeletal muscle and stomach [28].

Circulating concentrations of leptin exhibit pulsatility and circadian rhythmicity. Unlike other inflammatory mediators, leptin is readily detectable in circulation under normal conditions and fluctuates to regulate the energy status of the body [29]. The levels of plasma leptin vary directly with the body mass index and percentage of body fat. Metabolic hormones, sex, and body energy requirements influence its plasma concentration. Leptin has historically been associated with obesity, since defects in the leptin signaling pathway result in obesity in animal models. Only a few obese humans have been identified with mutations in the leptin gene or in the leptin receptor, however, most cases of obesity in humans are associated with high leptin levels. Thus, human obesity may represent a state of leptin resistance. The fluctuations in peripheral leptin concentrations influence the activity of the hypothalamic–pituitary–ovarian and hypothalamic–pituitary–adrenal axes, indicating that leptin may be a modulator of reproduction, stress-related endocrine function and behavior [30].

Leptin receptors (LEPRs) are present in both hypothalamic and extrahypothalamic neurons, including neurons of the hippocampus and cerebral cortex, brain stem and cerebellum [31,32]. Six different isoforms from the leptin receptor gene are synthesized: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf. They are generally classified into the short (ObRa, c, d, and f), long (ObRb) and soluble (ObRe) forms [33]. The long form is responsible for signaling induced by ligand binding and eliciting an array of subsequent intracellular signaling cascades [33]. The short forms are less involved in leptin activated intracellular signaling. They appear important in mediating the transfer of leptin from the periphery through the blood–brain barrier. It has been demonstrated that leptin is able to bind in megalin-LRP2, a multi-ligand receptor that is expressed in choroid plexus epithelial cells (Fig. 1) [33].

LEPR activates several downstream molecules involved in pathways related to cell survival and metabolism such as STAT3, PI3K, AMPK, AKT, SIRTUIN1 and GSK3b. These pathways form a network that is involved in leptin physiological response [34,35].

4. Alzheimer's disease and leptin

Leptin is the most studied adipokine associated with brain structure and function, and has several effects on the brain in relation to cognition and ageing (Table 1). Recent studies indicate a remarkable effect of the leptin on hippocampal development and function, mainly learning and memory processes. Leptin dysfunction has recently been linked to AD [36,37]. Farr et al. (2006) showed that leptin was able to improve memory processing in mice model, which normally develop elevated amyloid-β and memory deficits in advancing age [38]. Warren et al. (2012) suggested that leptin was associated with higher Montreal Cognitive Assessment (MoCA) total scores and delayed recall domain score for white men [39].

Leptin acts in the hippocampus to promote hippocampal synaptic plasticity, including enhancement of neuronal morphology, and increases the neurogenesis and synaptic transmission. Studies using animal models of ageing and AD have shown that achieving energy balance in adipose tissue, through feeding and exercises can improve cognitive function and prevent an age-related decline in learning [40]. In the same sense, Folch et al. (2012) have proposed that leptin presents neuroprotective activities, which could be explained by inhibiting the amyloidogenic process, reducing the phosphorylation levels of tau protein and improving the cognitive function [34].

Lieb et al. (2009) provided evidence for a lower incidence of AD in nonobese individuals with higher leptin levels [41]. Bigalke et al. (2011) have found that AD patients had significantly decreased plasma levels of leptin compared with healthy controls [36]. However, Theodoropoulou et al. (2012) found no difference in leptin level between patients with AD and controls [37].

Data obtained by Hazzouri et al. (2013) corroborated the evidence that obesity may interfere with the neuroprotective effect of leptin on the brain, possibly by leptin resistance [42]. Leptin resistance may result

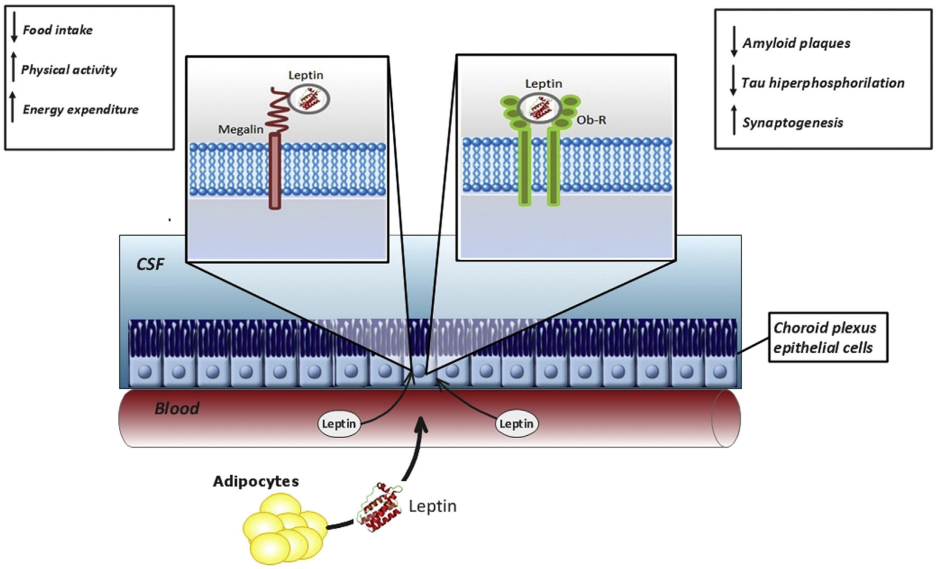

Fig. 1. Putative mechanisms involving the transfer of leptin from the periphery to central nervous system.

in compromised leptin transport across the blood–brain barrier as well as reduced leptin signaling. Akin to this idea, Bonda et al. (2014) observed that leptin concentration in cerebrospinal fluid (CSF) was significantly higher in AD group compared to both MCI and control groups and found lower expression level of Ob-Rb mRNA in AD hippocampal tissue compared to control [26]. These results suggest a neuronal leptin resistance in AD. Indeed, Rajagopalan et al. (2013) associated chronically elevated plasma levels of leptin, due to leptin resistance, with brain volume reduction [43]. In addition, according to Khemka (2014), the serum level of leptin in AD subjects is negatively correlated with the degree of dementia [44]. On the other hand, as subject progresses to AD, Maioli et al. (2015) have observed unchanged leptin levels in CSF [47]. In the same study, the authors have reported that brain leptin signaling was decreased and leptin localization was shifted, being more abundant in reactive astrocytes and less in neurons [45]. These results suggest that leptin signaling is impaired in AD while leptin levels are intact.

However, a very recent study has reported that serum leptin is not altered nor related to cognitive decline in Alzheimer's disease [46]. In addition, these authors have suggested that peripheral leptin levels do not play a role in evolution of AD pathology.

Oania and McEvoy (2015) have examined whether plasma leptin levels in individuals with mild cognitive impairment were related to cognitive function at baseline and whether higher leptin levels were associated with reduced risk of dementia [47]. Their findings suggest that plasma leptin levels are not predictive of dementia in patients with MCI. These investigators have also suggested that plasma leptin, on its own, is unlikely to become a useful clinical biomarker for AD [47].

4.1. Leptin, amyloid-β and tau-phosphorylation

Studies in vitro and in vivo have shown the leptin performance on tau phosphorylation and amyloid-β (Aβ) homeostasis. Leptin reduces tau phosphorylation and Aβ production in neuronal cells and transgenic mice models of AD [48-52].

In the hypothalamus, the activation of the LEPRb boosts a complex and integrated simulation of several signal transduction cascades. The LEPRb activates the constitutively associated tyrosine kinase Jak2, resulting in Jak2 autophosphorylation, as well as phosphorylation of several tyrosine residues within its intracellular domain. Phosphorylation of the LEPRb at Tyr1138 results in the recruitment and phosphorylation of the signal transducer and activator of transcription (STAT3). The autophosphorylation of Jak2 stimulates the phosphatidylinositol 3 kinase (PI3K) cascade and the adenosine monophosphate-activated protein kinase (AMPK), leading to the phosphorylation and activation of protein kinase B or Akt (PKB/Akt), a serine/threonine kinase which inactive glycogen synthase kinase-3b (GSK-3β). GSK-3β is responsible for the phosphorylation of all phospho-epitopes and is the main tau kinase in the brain [53]. This hypothesis corroborates with the findings of Tezapsidis et al. (2009) who demonstrated that leptin treatment could lead to a reduction in tau phosphorylation [54].

The amount of extracellular accumulation of Aβ depends on the antagonizing rates of its production/secretion and clearance. The Aβ's precursor, amyloid precursor protein (AβPP), is cleaved by the protease β-secretase (BACE) releasing the intermediate fragment CAPPβ. The latter is processed by a γ-secretase, a multimeric complex, whose four main components are presenilin 1(PS1), nicastrin, presenilin enhancer 2 (PEN2), and APH1 (anterior pharynx-defective 1), releasing then Aβ [54].

Niedowicz et al. (2013) demonstrated that plasma leptin is strongly and negatively correlated with brain Aβ levels and PS1 expression in mice, concluding that plasma leptin directly controls PS1 expression via transcriptional regulation [55]. AMPK downregulates PS1 expression, one γ-secretase component. Therefore, there is a decrease on γ-secretase activity and consequently in Aβ generation. Low levels of leptin contribute to an insufficient stimulation of AMPK, which, in turn, favors an increase in β-amyloid levels [34,49]. Marwarha et al. (2014) demonstrated that leptin decreases BACE mRNA expression and protein levels by attenuating the transcriptional activity of NF-κB on BACE promoter through SIRT inhibitor (silent mating type information regulation 2 homolog — SIRT 1) (Fig. 2) [56].

Once outside the neuron, Aβ can exert a biological activity or be removed by mechanisms of endocytosis, involving apoE. Leptin is one compound that facilitates the uptake of ApoE/Aβ complexes; in this

Table 1
Major associations between leptin and Alzheimer's disease.

		Publication	Population (n)	Results
Human	In vitro	Greco et al. [52]	Human cell lines: SH-SY5Y and NTera-2	Decrease in tau phosphorylation in cells treated with leptin.
		Martins et al. [68]	Primary cultured hippocampal neurons	Leptin and ghrelin inhibit cell death through a receptor-dependent mechanism. And also prevent GSK3β activation.
		Marwarha et al. [56]	Cultured human neuroblastoma SH-SY5Y cells	Leptin attenuates the activity of NF-κB in a SIRT1-dependent manner and consequently reduces Aβ genesis.
		Bonda et al. [26]	Human AD and non-demented control hippocampal or cortical tissue samples were obtained at autopsy	Leptin concentration in CSF at the time of autopsy was higher in AD compared to both MCI and control cases. CSF leptin was higher in females compared with males only within the AD group.
	In vivo	Utsunomiya et al. [64]	49 patients with AD and 134 normal controls in Japanese population (11 men, 38 women)	No association between the LEPR Gln223Arg polymorphism and AD.
		Bigalke et al. [36]	41 patients with early AD and 37 healthy controls	AD patients had significantly decreased plasma levels of leptin compared with healthy controls.
		Gustafson et al. [19]	1462 women followed from mid-life to late-life age, in Gothenburg, Sweden	Leptin is not a mid-life marker of late-life dementia risk in this population sample of Swedish women born between 1908 and 1930.
		Theodoropoulou et al. [37]	27 patients (10 men, 17 women) with AD, 23 controls (10 men, 13 women)	No difference in leptin level is found between patients with AD and controls.
		Warren et al. [39]	2731 subjects, 50% African Americans	Leptin was associated with lower Montreal Cognitive Assessment total scores and delayed recall domain score for black men. White men demonstrated a reverse relationship.
		Zeki et al. [20]	579 older women from study of osteoporotic fractures, who were dementia-free at year	Higher serum leptin was associated with lower dementia/MCI in women with normal body mass index, but not in overweight or obese women. Leptin may be a better predictor of dementia/MCI than traditional anthropometric measures.
		Rajagopalan et al. [43]	517 elderly individuals (53 healthy controls, 354 MCI, 110 AD)	Higher plasma leptin in women than men, no effect of carrying the APOE4 genotype on leptin levels. Chronically elevated plasma levels of leptin, perhaps due to leptin resistance may be associated with brain volume deficits.
		Johnston et al. [67]	819 subjects: cognitively normal (229), MCI (398) and mild AD (192) from the Alzheimer's Disease Neuroimaging Initiative (ADNI)	70% of both men and women with MCI have plasma leptin levels lower than NC. 50% carry at least one apolipoprotein-E4 allele. Plasma leptin typically reflected the levels of leptin in CSF in all groups (NCI/MCI/AD) in both genders.
		Khemka [44]		The decreased *serum* level of leptin in AD subjects is negatively correlated with the degree of dementia.
		Teunissen et al. [46]	295 non-obese subjects, healthy controls (n = 65), subjective memory complaints (n = 99), AD (n = 100), and vascular dementia (n = 31)	Serum leptin levels show no difference in this population of relatively young AD or vascular dementia patients compared to healthy and clinical control groups and were not related to cognitive decline. Leptin levels were two times higher in females than in males. The authors suggested that peripheral leptin levels do not play a role in evolution of AD pathology.
		Oania [47]	352 MCI (224 men and 128 women)	Leptin levels were higher in women than in men, however plasma leptin level was not associated with cognitive function at baseline, nor did it predict risk of dementia.
Human and animal	In vitro	Fewlass et al. [48]	Human (SY5Y), mouse neuroblastoma cell lines, and transgenic animals	Demonstrated leptin's ability to modify Aβ levels in vitro and in vivo and also able to increase apoE-dependent Aβ uptake in vitro.
		Greco et al. [49]	Leptin-treated human and/or rat neuronal cultures	Leptin increased the phosphorylation of Jak2, AMPK, Akt and GSK-3b.
		Greco et al. [50]	Human neuroblastoma cell line, SH-SY5Y, overexpressing APP. Later, neuronal cells were treated with Leptin in the presence or absence of nicotinamide or compound C	Leptin activates AMPK and SIRT in neuronal cells, which mediates leptin's reducing effects on tau phosphorylation and Aβ production.
		Niedowicz et al. [55]	APPΔNLh-overexpressing H4 neuroglioma cells	Inhibition of Aβ accumulation is due to the downregulation of transcription of the γ-secretase components. β-Secretase expression is either unchanged (BACE1) or increased (BACE2). Presenilin 1 (PS1) is negatively correlated with plasma leptin.
		Maioli et al. [45]	4 brains from patients with definite AD (two males and two females 75–86-year-old) and 4 aged-matched controls (two females and two males, 66–87-year-old)	Downregulation of leptin receptors in brains from individuals suffering severe AD.
	In vivo	Niedowicz et al. [55]	APPΔNLh × PS1P264L knock-in mice	Plasma leptin is a strong negative predictor of Aβ levels in the mouse brain.
		Maioli et al. [45]	278 subjects: 88 controls, 63 with AD, 81 with stable MCI and 46 MCI that converted to AD within 18 month after baseline from the North American multicenter Alzheimer's Disease Neuroimaging Initiative (ADNI)	Found no significant changes in CSF leptin levels among controls, MCI and patients with AD. CSF leptin levels were significantly higher in women than in men, probably due to gender differences in body fat, sex hormones and insulin levels.

manner it may serve as a mechanism for clearing Aβ from the brain interstitium [54].

Aβ can also be degradated by extracellular proteases. Two major endopeptidases associated with Aβ degradation in the brain are zinc metalloendopeptidases, neprilysin (NEP) and insulin degrading enzyme (IDE) [57]. It has been shown Aβ deposition in NEP- and IDE-knockout animals [58-61]. According to Yamamoto et al. (2013; 2014) this degradation may promote Aβ deposition in patients with sporadic late-onset AD [62,63]. They evaluated if leptin is associated with Aβ degradation of astrocytes and observed a decrease on expression of NEP in cultured rat astrocytes treated with leptin. The authors suggest that leptin suppresses Aβ degradation by NEP through activation of extracellular signal-regulated kinase (ERK) in astrocytes.

Fewlass et al. (2004) demonstrated the leptin's ability to modify Aβ levels in vitro and in vivo. Leptin reduces γ-secretase activity in neuronal cells as well as increases apoE-dependent Aβ uptake in vitro [48]. Indeed, leptin signaling is probably related to changes in ApoE gene expression and would exert this effect on the removal of amyloid-β

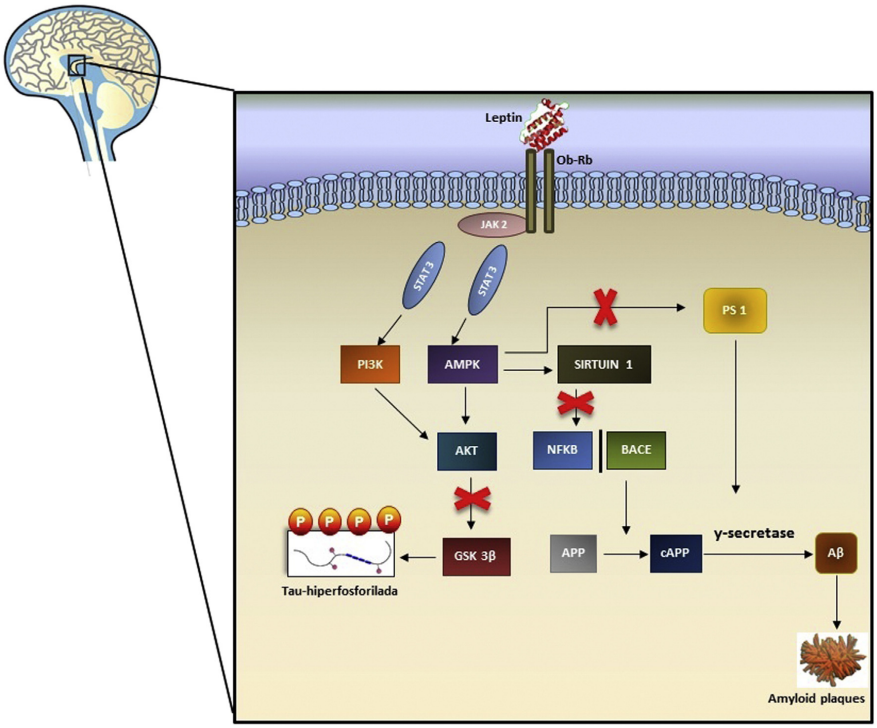

Fig. 2. Activation of LEPRb in the hypothalamus boosts a complex and integrated simulation of several signal transduction cascades, reducing amyloid plaques and tau-hyperphosphorylation.

aggregates with the same intensity [34]. Therefore, leptin can modulate bidirectional Aβ kinesis reducing its extracellular levels.

4.2. Leptin receptor gene polymorphism

A functional polymorphism in human leptin receptor gene (LEPR), a glutamine to an arginine substitution at codon 223 (Gln223Arg), has been associated with insulin resistance capacity and an altered leptin-binding activity [64]. Wondering if the LEPR Gln223Arg polymorphism may thus play a role in the pathogenesis of AD, Utsunomiya et al. (2010) examined the association between the LEPR Gln223Arg polymorphism and late-onset Alzheimer's disease (LOAD) in a Japanese population [64]. However, they found no significant association between the LEPR Gln223Arg polymorphism and LOAD, suggesting that this association should be evaluated in different populations.

4.3. Leptin replacement therapy and a diagnostic marker for AD

Pérez-González et al. (2014) sought to determine if leptin stimulates the proliferation of neuronal precursors in APP/Ps1 mice. They estimated the number of proliferating hippocampal cells after intracerebroventricular administration of a lentiviral vector encoding leptin. After 3 months of treatment with leptin they observed an increase of neuronal precursors. Leptin also attenuated Aβ-induced neurodegeneration [65]. Their results suggest that in APP/Ps1 mice, leptin exerts changes seeming acute neurotrophic and neuroprotective effects. These effects could serve as the basis for the design of future treatment strategies in AD. In a follow-up study, this group generated a lentivirus vector expressing leptin in a self-inactivating HIV-1 vector (HIV-leptin), and delivered this by intracerebroventricular administration to APP/PS1 transgenic mice model of AD. Three months after, brain Aβ accumulation was reduced, indicating that the gene therapy-mediated leptin administration reduced the accumulation of Aβ deposits in APP/PS1 mice. Moreover, leptin modulated neurite outgrowth in primary neuronal cultures, and rescued them from Aβ-induced toxicity. All these changes suggest that leptin may affect multiple aspects of the synaptic status, and correlate with behavioral improvements [66]. HIV-leptin treatment in APP/PS1 mice resulted in the improvement in the expression of synaptophysin in the cerebral cortex and hippocampus, and the partial restoration of learning and memory impairment [65]. Akin to this study in mice, Johnston et al. (2014) revealed individuals with early AD or MCI with low plasma leptin and remarked the benefit of a leptin replacement therapy for the patients. They concluded that development of new approaches for the delivery of neuroactive peptides is important for advancing the therapeutics of AD [67].

Johnston et al. (2014) found that plasma leptin typically reflected the levels of leptin in CSF in all groups (control/MCI/AD) in both genders. The data suggest that plasma leptin deficiency provides an indication of potential CNS leptin deficiency, further supporting the exploration of plasma leptin as a diagnostic marker for MCI or AD. If leptin deficiency plays a role in AD, individuals with early AD or MCI with low plasma leptin may benefit from leptin replacement therapy [67].

Gomes et al. (2014) using the mHypoE-N42 cell line demonstrated that oligomeric Aβ is toxic to hypothalamic cells leading to cell death. They also demonstrated that leptin and ghrelin protect these cells against Aβ-induced cell death through the activation of the leptin and

ghrelin receptors, respectively. Furthermore, ghrelin and leptin prevented superoxide production, calcium influx and mitochondrial dysfunction triggered by Aβ. They suggested that leptin and ghrelin might be considered as preventive strategies for ameliorating hypothalamic alterations in AD [59].

5. Conclusions and future directions

Although several studies support the relation between leptin and AD once it is thought that leptin may play a critical role in neuroprotection and cognitive function, there is still much to understand about its relationship with the development and progression of the AD, since some existing data in the literature are controversial. Novel researches into the mechanisms underlying the disease, new biomarkers and prevention measures have been provided. Leptin once established as a biomarker could enhance the understanding of late-onset AD risk over the life course, as well as the clinical progression of prodromal AD and manifest AD. However, it is necessary to be very careful with leptin replacement therapy and more robust studies are necessary before taking a step forward in this direction.

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8 CAPÍTULO 4

Leptin, hsCRP, TNF-α and IL6 levels from normal aging to dementia: relationship with cognitive and functional status

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ABSTRACT

Cognitive impairment, including mild cognitive impairment (MCI) and dementia, compromises the patients' cognitive abilities and, to different extents, to carry out daily activities, accompanied by personality and behavioral changes. Studies suggest that leptin, an adipokine, has a neuroprotective role against Alzheimer's disease (AD) pathology, and also that cytokines are associated with inflammatory central processes and dementia. This study aimed to evaluate serum levels of leptin, hsCRP, IL-6 and TNF-α according to Mini-Mental State Examination (MMSE) and Functional Assessment Staging (FAST) scores. Forty-three participants were included, of whom 12 with probable AD, 16 with MCI and 15 with no objective cognitive decline. We evaluated serum leptin and hsCRP levels by immunoturbidimetric method, IL-6 and TNF-α by ELISA. We observed that higher TNF-α levels were found in individuals with FAST stages 1/2 and normal scores in the MMSE. hsCRP levels were inversely correlated with FAST stages. No association with function or global cognition was observed for leptin and IL-6 levels. However, women demonstrated higher leptin serum levels than men. Lower leptin and IL-6 levels were observed in individuals aged ≥ 59 years. Our results suggest that TNF-α is involved in cognitive and functional decline and that inflammation could be a substrate of cognitive impairment at early clinical stages of dementia.

KEYWORDS: Alzheimer's disease, mild cognitive impairment, leptin, IL-6, hsCRP, TNF-α, Functional Assessment Staging, Mini-Mental State Examination.

INTRODUCTION

Dementia may be caused by a group of different disorders that usually compromise memory, besides other cognitive functions. Alzheimer's disease (AD) is the most frequent cause of dementia, causing significant and progressive cognitive and functional performance, often accompanied by personality and behavioral changes (Castellani *et al*. 2010; Heppner *et al*., 2015). AD neuropathology is characterized by altered formation of amyloid-β (Aβ) plaques and neurofibrillary tangles composed by hyperphosphorylated tau protein (Scassellati *et al*., 2004; Swardfager *et al*., 2010).

A wide degree of impairment in functional capabilities and cognition underlies the clinical profile of AD. The intermediate state between normal aging and mild cognitive impairment (MCI) is defined as subjective cognitive decline (SCD), which is characterized by a self-perceived decline, but without objective evidence of cognitive decline on standard neuropsychological tests. This condition may represent a pre-symptomatic stage of MCI, which has been reported to occur 15 years prior to MCI (Eckerström *et al*., 2017; Prichep *et al*., 2006). On the other hand, MCI refers to cognitive impairment, particularly of episodic memory regarding recent events, in subjects who maintain preserved function in daily activities and do not fulfill the diagnostic criteria for dementia (Petersen *et al*., 1999, Gauthier *et al*., 2006). MCI may follow different trajectories: it may represent a transient condition where the patient recovers the normal cognitive condition; may remain stable; or the MCI may progress to a pattern of cognitive loss resulting in a state of dementia, mainly AD (Ward *et al*., 2012). Studies indicate that 10 to 20% of individuals with 65 years or more present MCI (Hanninen *et al*., 2002; Roberts *et al*., 2008) and the risk AD development is about 10-15% per year (Petersen *et al*., 2001a).

A progressive deficit in functional activities is expected with the evolution of cognitive impairment, because it requires memory storage and executive function processes (Shankle *et al*., 2013). The Functional Assessment Staging - FAST is a measure of the degree of impairment in functional capabilities, which has been correlated with cognitive impairment (Reisberg, 1988; Shankle *et al*., 2013). Moreover, several neuropsychological batteries are useful for cognitive screening. The Mini-Mental State Examination (MMSE) is the most widely used of such scales and allows a good evaluation of global cognitive functioning.
Studies suggest that lifestyle factors, including nutritional behaviors, play a critical role in the

onset of dementia and that AD is a generalized metabolic disorder (Tezapsidis *et al*., 2009; Ríos *et al*., 2014, Whitmer *et al*., 2005; Beydoun *et al*., 2008; Parimisetty *et al*., 2016). The relationship between nutritional behavior and AD includes obesity, arterial hypertension, dyslipidemia and elevated glucose levels in the disease development or progression (Tezapsidis *et al*., 2009; Ríos *et al*., 2014). In accordance with this argument, some studies have suggested that overweight or obesity in middle age is considered an important risk factor for later development of AD (Whitmer *et al*., 2005; Beydoun *et al*., 2008; Parimisetty *et al*., 2016). Additionally, a consistent observation is that individuals with AD, despite unchanged eating habits, begin to lose weight some years before the onset of clinical symptoms, suggesting a relation between adipose tissue metabolism and AD (Buchman *et al*., 2005; Arnoldussen *et al*., 2014; Kiliaan *et al*., 2014). It has been suggested that clarifying the role of adipose tissue in health of the brain is essential to a complete understanding of degenerative dementing processes. Moreover, it has been demonstrated that higher mid-life central adiposity might increase the risk for dementia after the age of 32 years (Gustafson *et al*., 2010; 2012).

The adipose tissue is responsible for production of regulatory molecules, which may be assorted in a group - the adipokines. Adipokines release can be dysregulated in both obesity and ageing, possibly due to link between these two conditions (Buchman *et al*., 2005).

Leptin is an adipokine, first described in 1994 by Zhang *et al*., readily detectable in circulation under normal conditions, actively transported across the blood-brain barrier and acts on hypothalamic modulation of feeding behavior and energy expenditure (Campfield *et al*., 1995; Clark *et al*., 2011; Bonda *et al*., 2014). The levels of leptin in AD patients have been exploited; however, results remain inconclusive (Magalhães *et al*., 2015). Studies have shown an association of lower circulating leptin levels with AD or cognitive decline (Lieb *et al*. 2009; Bigalke *et al*., 2011; Khemka *et al*., 2014, Baranowska-Bik *et al*. 2015), other clinical studies found no differences between AD and controls (Theodoropoulou *et al*. 2012; Warren *et al*. 2012; Teunissen *et al*. 2015, Bednarska-Makaruk *et al*., 2017) and even higher circulating leptin levels in AD (Rajagopalan *et al*., 2013; Bonda *et al*., 2014). Zeki *et al*. (2013) proposed that leptin might be a better predictor of dementia/MCI than traditional anthropometric measures. A prospective study has shown that obesity may precede dementia and leptin has been associated with cognitive decline in epidemiologic studies (Barrett-Connor, 2007).

Evidence from clinical studies suggests that inflammatory mechanisms play an important role in the pathophysiological process that leads to cognitive impairment and dementia. The cytokines may participate in cognitive processes by influencing neuronal plasticity, neurogenesis, and neuromodulation (Donzis & Tronson, 2014; Marin & Kipnis, 2013), but their dual role on neurodegeneration and neuroprotection has been discussed widely. One of the major sources of cytokines is the visceral adipose tissues, also considered as adipokines. The increased adipose tissue is associated with over expression of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (de Carvalho *et al*., 2006), and the pro-inflammatory status associated with these changes provides a potential link between dementia and inflammation (Li & Yu, 2017).

A growing number of studies have searched reliable biomarkers that allow an early diagnosis of cognitive impairment, especially AD. Therefore, our study aimed to investigate the correlation between the leptin, TNF-α and IL-6 levels, global cognitive performance and functional status in a sample of elderly individuals. We also intended to explore the association of these variables with clinical parameters. We hypothesized that leptin and cytokine levels are higher in pre-symptomatic stage of cognitive decline, with a decrease in these levels with the onset of MCI and AD.

MATERIAL AND METHODS

Subjects

Subjects were recruited between June 2014 and December 2016 and 43 participants were included in this study, of whom 12 with probable AD, 16 with MCI and 15 with no objective cognitive or functional impairment. The groups were recruited among patients attending at the Geriatric and Neurology Outpatient Clinics of the Hospital das Clínicas, Federal University of Minas Gerais (UFMG), in Belo Horizonte, Brazil. The diagnosis of AD was based on the clinical criteria for probable dementia due to AD according to recommendations from the National Institute on Aging and Alzheimer's Association - NIA-AA (McKhann *et al*., 2011) and subjects with MCI were selected according to Petersen *et al*. (1999) recommendations.

Cognitive performance was evaluated according to four levels of educational attainment (illiterate; 1-3; 4-7; and >7 years of education). In the MMSE, a reference cut-off was set at

the 25th percentile, determined by the epidemiological survey of dementia in a Brazilian healthy community (Herrera *et al*., 2002) and in accordance with the baseline performance from the participants that remained free of dementia in a 3-year follow-up (Caramelli *et al*., 2007b). The MMSE values were also normalized as z scores, from the standards of performance, according to four levels of formal educational attainment obtained from the whole sample from Caramelli *et al*. (2007b) study.

Patients with no functional impairment were classified as FAST stage 1. Individuals with subjective cognitive complaints but without objective evidence of impairment were classified as FAST stage 2. FAST stage 3 included MCI patients. Patients with FAST stage 4, 5 and 6 had functional deficits that correspond to mild, moderate and severe dementia respectively (Reisberg, 1988; Shankle *et al*., 2013).

Exclusion criteria for both groups were age < 50 years, autoimmune, kidney and liver disease, cancer (past six months), acute inflammatory disease, acute myocardial infarction or stroke (past five years), other dementia diagnoses, use of anticoagulants medications, steroidal and non-steroidal anti-inflammatory medications, except acetylsalicylic acid.

This study was approved by the ethics committees of the Federal University of Minas Gerais and was conducted according to the ethical guidelines of the Declaration of Helsinki. All participants provided written informed consent prior to entering the study.

Methods

Venous blood samples were obtained after eight hours fasting using tubes anticoagulant-free (Vacuette®). The samples were centrifuged at 1,500 xg for 20 min at 4°C to obtain the serum. Aliquots were immediately processed or stored at −80 °C until the use.

The serum leptin levels were performed using an enzyme linked immunosorbent assay (ELISA) (Quantikine HS ELISA, R&D systems®, USA). The high sensitivity- C reactive protein (hsCRP) levels were determined by immunoturbidimetric method (dCRP Latex, Beckman Coulter®, USA). The serum concentrations of IL-6 and TNF-α were determined using ELISA (Quantikine HS ELISA, R&D systems®, USA). The samples were analyzed in duplicate, with an intra-assay variation < 5%. An internal quality control was used in all assays.

Body Mass Index (BMI) was measured by weight in kilograms divided by the square of the height in meters (kg/m^2) (WHO, 2000). The abdominal circumference (AC) was measured and values for men ≥ 102 cm and women ≥ 88 cm were classified as increased (WHO, 2000). The individuals were also classified according to age range: ≤ 59 years, 60-78 years, and ≥ 79 years.

A commercially kit (Biopur - Biometrix®, Brazil) was used for obtain genomic DNA from blood cells. The presence of the allele ε4 on *Apolipoprotein E* gene were evaluated by Polymerase Chain Reaction (PCR-RFLP), using the methodology described by Main *et al*. (1991).

Statistical analyses

Statistical analyses were performed using Statistical Package of the Social Sciences (SPSS) 13.0 version. The results are expressed as median (interquartile ranges) (all non-parametric). We performed the Mann-Whitney test for compare variables between two groups. A Kruskal-Wallis test, followed by Bonferroni correction, was applied to compare non-normal three groups. For categorical variables, we used the chi-square test or Fisher test when appropriated. Correlation was assessed using the Spearman rank correlation test. For all analyses, we considered $p<0.05$ statistically significant.

RESULTS

Patients presented median age= 72.0 (12.0) years, of which 15 (35%) men and 28 (65%) women. Regarding educational level, 2 patients (4.6%) were illiterate, 23 (53.5%) had 1-3, 8 (18.6%) had 4-7, and 10 (23.3%) had > 7 years of education. We observed 22 (31%) of *APOE* ε4 allele non-carriers and 13 (18.3%, 8 missing data) carriers. The frequencies of FAST stages were: 1 – 6 individuals (8.5%); 2 – 7 (9.9%); 3 – 18 (25.4%); 4 – 6 (8.5%); 5 – 4 (2.8%); and 6 – 2 individuals (2.8%). MMSE scores below the cut-offs according to educational level (altered) were observed in 17 individuals (23.9%), and the median z score was -0,102 (2.970). The median BMI and AC were 25.6 (7.0) kg/m^2 and 93.0 (21.0) cm, respectively.

Due to small number of individuals in some FAST stages, we reclassified in 1 (stages 1 and 2) and 2 (3 to 6 stages, MCI and AD), since no difference related FAST staging was observed

when considered as continuous variables (p>0.05). We observed that TNF-α levels were higher in the group 1 [2.1 (10.7) pg/mL] when compared to group 2 [1.11 (1.0) pg/mL] (p= 0.025) (Figure 1). Leptin, hsCRP and IL-6 levels were not different between these groups (p= 0.074, 0.410 and 0.608, respectively).

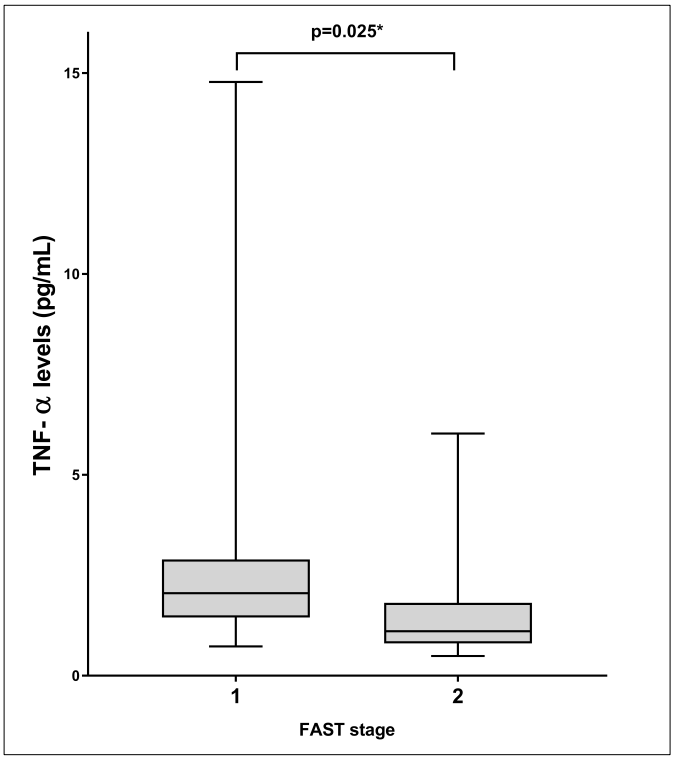

Figure 1: TNF-α serum levels on FAST stage 1 and 2 groups. FAST stage 1: subjects with no cognitive impairment and SCD – subjective cognitive decline. FAST stage 2: subjects with MCI (mild cognitive impairment) and AD (Alzheimer's disease). Significative: Mann-Whitney. *p<0,05

Similarly, MMSE was classified as normal or altered according to individual result above the cut-off for educational level, because no difference regarding MMSE was observed when considered as continuous variables (p>0.05). Individuals with altered MMSE scores presented lower TNF- α levels [1.1 (0.9) pg/mL] when compared to those with normal scores [2.1 (13.3) pg/mL] (p=0.004) (Figure 2). No differences in leptin, hsCRP and IL-6 levels were observed between these groups (p= 0.296, 0.302 and 0.104, respectively).

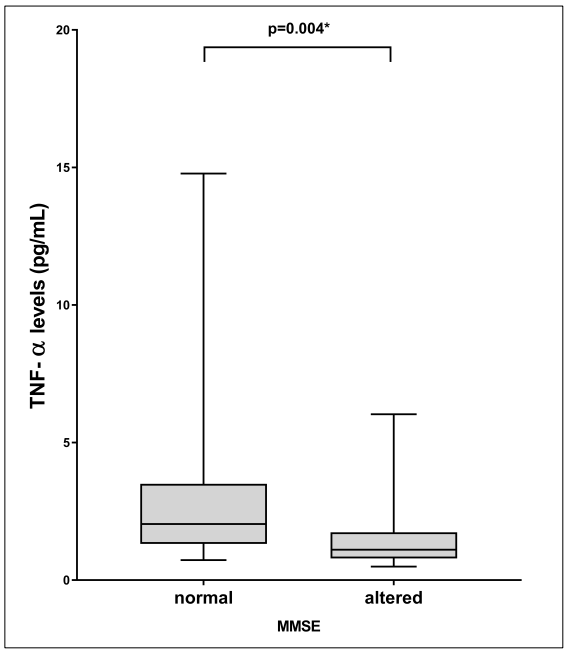

Figure 2: TNF-α serum levels on normal and altered MMSE groups. TNF-α (Tumor necrose factor-α), MMSE (Mini-mental Statement Examination). Significative: *p<0,05.

Interestingly, male presented lower leptin levels [6.2 (11.0) ng/mL] than female [24.7 (19.8) ng/mL] (p<0.001). Indeed, leptin levels changed according to age: ≤ 59 years - 1.3 (1.0) ng/mL, 60-78 years - 17.6 (19.8) ng/mL, ≥ 79 years – 29.3 (46.3) ng/mL, with individuals aged ≤ 59 years showing lower leptin levels when compared to those aged 60-78 years (p<0.001) and ≥ 79 years (p=0.011). We also observed lower IL-6 levels in the group ≤ 59 years – 0.6 (1.0), than the group 60-78 years – 2.5 (5.3), p<0.001, and ≥ 79 years – 2.5 (3.2), p= 0.018. These findings were independent of BMI or AC (p>0.05).

No association between leptin, hsCRP, TNF-α or IL-6 levels, as well as FAST stages with *APOE* ε4 allele was observed (p>0.05). However, z scores were higher in ε4 allele non-carriers [0.9 (2.50)] when compared to carriers [-1.01 (3.8)] (p=0.022). These variables did not show correlation with education (p=0.05). According to expected, FAST stages showed an inverse and significant correlation with z score (r=-0.313, p=0.041) and hsCRP levels (r=-0.570, p<0.001) (Figure 3).

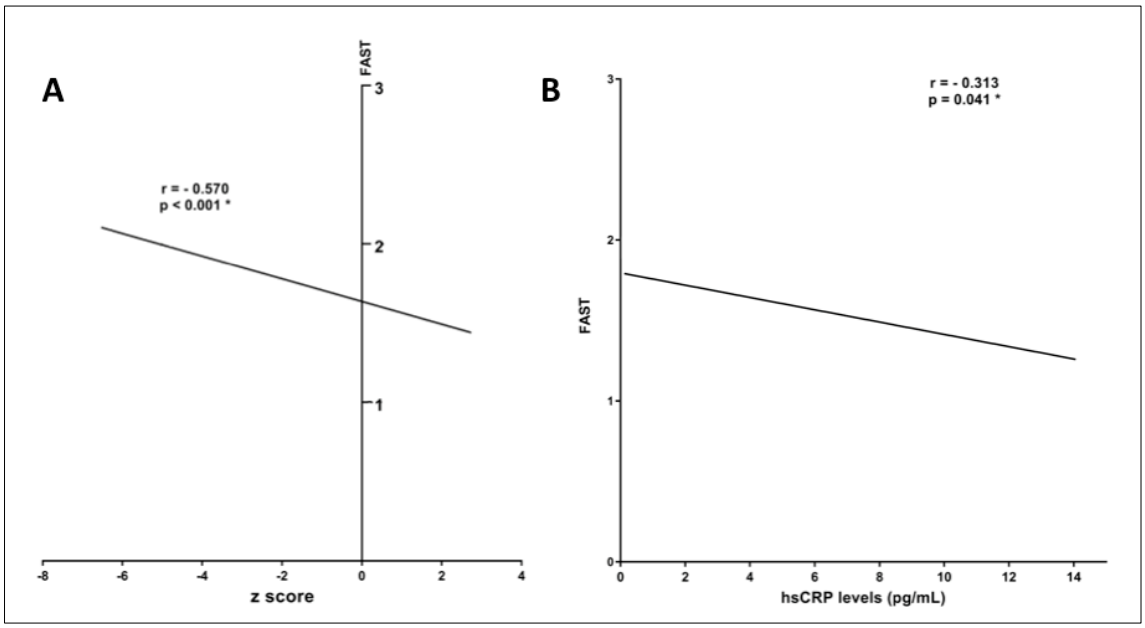

Figure 3: FAST stages correlation with z score and hsCRP levels. FAST stages, z score and high sensitivity C reactive protein (hsCRP). Significative: Mann-Whitney. *p<0,05.

DISCUSSION

Our study showed that TNF-α levels, but not leptin or IL-6, were associated with functional and cognitive performance in an elderly sample. hsCRP levels showed inverse correlation with functional impairment. Furthermore, leptin levels were increased in female and in individuals with more advanced age (i.e., ≥ 79 years).

Neuroinflammation is a common feature in neurodegenerative disorders, but it is not clear whether this process is protective or harmful, and when it starts and finishes (Calsolaro & Edison, 2016). Our results corroborate the hypothesis that inflammation is part of the pathological substrate (putatively degenerative) of cognitive impairment only at early clinical stages, since higher levels of the pro-inflammatory cytokine TNF-α were observed in individuals with FAST stages 1/2 and normal MMSE scores. Moreover, hsCRP also showed inverse correlation with FAST stages.

TNF-α is a proinflammatory cytokine implicated in the pathogenesis of systemic inflammatory and neurodegenerative diseases (Smith *et al*, 2012). Serum TNF-α levels were negatively correlated with cognitive function in patients with AD, MCI and controls by Kim *et al*. (2017), but no correlation between plasma cytokines levels, including TNF-α and cognitive scores was found by Julian *et al*. (2015) in AD. These results seem to contradict our

data. However, recently, Paouri e*t al*. (2017) observed a role of peripheral TNF-α in the modulation of the amyloid phenotype by regulating blood-derived and local brain inflammatory cell populations involved in β-amyloid clearance in mice. In this study, peripheral inhibition of TNF-α increased the amyloid deposition, without affecting brain TNF-α levels. In accordance with this study, our results suggest that the decrease of peripheral TNF-α levels along the cognitive impairment development could favor the amyloid deposition, reflected by functional and cognitive decline.

Leptin has been demonstrated to have a neuroprotective role against AD pathology (Harvey *et al*., 2006; Bednarska-Makaruk *et al*., 2017). Studies *in vitro* and *in vivo* have shown that leptin reduces tau phosphorylation and Aβ production in neuronal cells of transgenic mice models of AD (Fewlass *et al*., 2004; Greco *et al*., 2008; Greco *et al*., 2009; Greco *et al*., 2010; Greco *et al*., 2011, Folch *et al*., 2012, Guo *et al*., 2016, Malekizadeh *et al*., 2016). Moreover, Khemka *et al*. (2014) proposed that the serum levels of leptin in AD subjects correlate inversely with dementia severity. However, no association between leptin levels and FAST staging or MMSE was observed, which suggests that this peptide is not involved in the evolution of cognitive impairment. Although this is the first study comparing leptin levels with functional and cognitive scores, these findings are in agreement with previous studies that found no significant differences in leptin levels between dementia, MCI and healthy elderly controls (Theodoropoulou *et al*. 2012; Warren *et al*. 2012; Maioli *et al*., 2015; Teunissen *et al*. 2015; Bednarska-Makaruk *et al*., 2017). Moreover, Yuruyen *et al*. (2017), investigating another neuropeptide supposedly regulated by leptin (Treen & Belsham, 2013) - phoenixin, they did not find differences in the levels when comparing individuals with subjective memory complaints to patients with MCI and AD. However, different from our results, other authors observed higher leptin concentration in AD patients compared to both MCI and controls (Bonda *et al*., 2014).

The gender difference in serum leptin levels is well established (Havel *et al*., 1996). Leptin serum levels were also influenced by gender in the present sample. Women demonstrated higher leptin serum levels than men. Fulda *et al*. (2010) found that women have higher leptin levels independently from age and BMI. Saad *et al*. (1997) found that women had 40% higher leptin levels than men at any level of adiposity. According to these authors, women may be less sensitive than men to leptin lipostatic actions, leading to compensatory increase in its production and possibly its transport to the cerebrospinal fluid (Saad *et al*., 1997).

Age is associated with dysregulation of leptin signaling (Guadalupe-Grau *et al*., 2014). In our study, we found that leptin serum levels were also influenced by age, since participants with 79 years or more showed higher values. This result suggests that the loss of leptin signaling could promote a higher and compensatory leptin release in older individuals. The opposite were found by Isidori *et al*. (2000), since they found that serum leptin concentrations in humans gradually decline with aging.

In agreement with Keegan *et al*. (2017), IL-6 levels were also related to age in subjects from a community presented for a memory screening with varying degrees of memory concern. IL-6 is a pleiotropic proinflammatory cytokine, which has been considered the major cytokine in central nervous system (Erta *et al*., 2012), associated with disease progression and severity of symptoms in AD (Brosseron *et al*., 2014, Banerjee *et al*., 2017). Imbalances between IL-6 concentrations in serum and cerebrospinal fluid are observed in MCI and dementia (Schuitemaker *et al*., 2009). We showed in another study that genetic predisposition to higher production of IL-6 is an independent risk factor for cognitive decline among MCI individuals (Fraga *et al*. 2015). However, IL-6 did not showed association with functional and cognitive stages, which suggest that peripheral IL-6 levels could not reflect the effect of the central levels on brain.

The *APOE* ε4 allele is considered an independent risk factor for AD, mainly in the sporadic form of the disease (Bettens *et al*., 2010). Independently from the ethnic group, ε4 allele is more frequently found in patients with AD compared to controls and has been associated with a younger age of AD onset and more important cognitive decline (Duron *et al*., 2008; Lara *et al*., 2016). In fact, we found that ε4 allele presence correlates with lower z scores.

Abdominal obesity is associated with higher leptin levels due to central leptin resistance and higher pro-inflammatory cytokines release. However, we found no significant influence of abdominal obesity (increased AC) on leptin, hsCRP, IL-6 or TNF-α levels, with confirms the independent association of TNF-α with FAST stages and MMSE in this population.

The main limitation of our study is the small sample size. Consequently, prospective design with larger samples studies are required in order to better understand the role of leptin, hsCRP, IL-6 and TNF-α in pre-symptomatic stage of cognitive impairment.

CONCLUSIONS

Our results suggest that TNF-α levels, but not leptin and IL-6, are associated with FAST stages and MMSE scores. HsCRP also demonstrated a correlation with functional impairment. These findings suggest a role of inflammation on the dementing process that compromises functional and cognitive capabilities. Further studies are required to confirm our findings.

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CONFLICTS OF INTEREST

Conflicts of interest: none

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9 OUTRAS PUBLICAÇÕES

9.1 ARTIGO 5

ORIGINAL ARTICLE

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Cerebrospinal fluid biomarkers for the differential diagnosis of Alzheimer's disease

Aplicação de biomarcadores do fluido cerebrospinal no diagnóstico diferencial da doença de Alzheimer

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ABSTRACT

Introduction: Several studies have been conducted in order to validate cerebrospinal fluid biomarkers for the diagnosis of Alzheimer's disease (AD), aiming primarily to facilitate the early diagnosis. **Objective:** To evaluate CSF biomarkers on patients with probable AD and the applicability of the international references values in this population. **Methods:** 46 individuals were recruited and classified as probable AD (n = 19), mild cognitive impairment (MCI) (n = 5) and other dementias (n = 22). The cerebrospinal fluid (CSF) biomarkers were measured using the INNOTEST kits for enzyme-linked immunosorbent assay (ELISA). Higher Tau protein values and lower Aβ and Innotest Amyloid Tau Index (IATI) values were observed in AD group when compared with MCI; higher levels of Tau and phosphorylated Tau (P-Tau), and lower Aβ and IATI values were observed in AD group when compared to patients with other dementias. No biomarker or IATI was able to distinguish between MCI and other dementias. The kappa index between biomarkers and the clinical diagnosis was regular to Tau and IATI, and weak to Aβ and P-tau. **Conclusion:** The cut-off values for each biomarker that showed better combined sensibility and specificity differ from the reference values suggested by the manufacturer. The CSF biomarkers represent important resources that can help with the AD diagnosis, although the results interpretation must be made based on the analysis of the three analytes together. The cut-off values must be established to address the specificities and characteristics of each population.

Key words: amyloid; tau proteins; Alzheimer's disease; cerebrospinal fluid.

INTRODUCTION

Dementia is a general term representing a number of neurodegenerative impairments, whose common characteristic is limitation on autonomy and poor quality of life. Intrinsically age-related, these clinical conditions have been increasing in social and public health contexts worldwide. According to the World Health Organization (2011)[1], developing countries such as Brazil, currently have the most pronounced aging population rates that reflects a rapid growth, especially among age groups over 65 years. A systematic review of articles published in our country found a prevalence of dementia between 5.1% and 19% in the elderly aged 60 years or over[2].

Dementia includes memory impairment with loss of ability to learn new information or remember information previously learned, as well as the presence of at least one of the following alterations that may compromise the social and occupational activities of the individual: aphasia (language impairment); apraxia (impaired ability to carry out motor activities despite intact motor function); agnosia (failure to recognize or identify objects despite intact sensory function), and executive functions deficities (ie, planning, organizing, sequencing and abstraction). Among possible causes of dementia, Alzheimer's disease (DA) is the most common, accounting for 50%-70% of cases[3, 4].

In the last decade, several studies have been conducted to validate biomarkers in cerebrospinal fluid (CSF) for the diagnosis of AD, aiming primarily to facilitate the early diagnosis of this dementia syndrome. The studies are dedicated primarily for measuring, in the cerebrospinal fluid, the two key proteins involved in the disease: a) beta-amyloid protein ($A\beta_{1-42}$ or simply Aβ), the main component

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of amyloid plaques, which is decreased in the CSF of patients with AD due to its deposition in brain parenchyma, and b) tau protein and phosphorylated Tau (P-tau), which are increased in the CSF of patients due to neuronal degeneration associated with the intracellular accumulation of neurofibrillary tangles[5-10].

The examination of these biomarkers in the CSF may increase the accuracy of diagnosis in clinical practice, both in dementia phase as in mild cognitive impairment (MCI), which is described as the intermediate state between normal cognitive aging and dementia, or more specifically, AD[11]. Moreover, these biomarkers have been correlated with the intensity of neuropathological lesions and have demonstrated sensitivity and specificity of approximately 85%-90% for AD diagnosis[6, 12]. CSF biomarker analysis may be applied for identifying asymptomatic individuals at risk of developing and preclinical stages of AD, elucidation of atypical AD cases, monitoring disease progression, and evaluation of response to treatment[13].

Studies show that the results of biomarkers should always be evaluated as a whole to characterize the pathological signature in AD[5-10], which gives strength to the diagnostic laboratory finding. Therefore, Innotest Amyloid Tau Index (IATI) obtained by the relation between Aβ and Tau values: $(A\beta_{1\text{-}42}/(240 + 1.18 \times \text{Tau})$ shows better performance diagnosis for AD than the isolated result of each biomarker[14, 15].

Despite the advantages of using biomarkers in CSF to aid in the diagnosis of AD, there some are still challenges in the implementation of these tools in the laboratory routine, especially regarding the reproducibility of results and the establishment of optimal cut-off values. This difficulty is exemplified by the fact that in multicenter studies, including different laboratories using the same biochemical assay, a large variability of these biomarkers levels have been found[12]. A number of reasons may explain the interlaboratory variations of these measurements; among them: demographic characteristics of patients, source of recruitment, severity of disease, and selection and diagnosis criteria. Other factors may also be involved, such as the process of obtaining the CSF, collection and sample transportation, besides the storage conditions. Other interfering include variations of the kits used in the assay, laboratory equipment and variations in test procedure[12]. Therefore, it is essential that each laboratory establish its own reference values or a reproducible methodology used in other laboratories[14].

OBJECTIVE

This study aimed at assessing biomarkers from the cerebrospinal fluid, Aβ, Tau and P-tau proteins, in a sample of the Brazilian population, since there are no validation studies of these biomarkers in this population. Thus, our results may contribute to the suitability of using these CSF biomarkers and determining reference values in clinical laboratories in Brazil.

METHODS

Participants

We recruited 46 patients seen in the Neurology clinic at the Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte (MG), from November 2012 to March 2014. The average age was 65.89 ± 9.65, of which 29 female patients and 17 male.

The participants underwent clinical and neurological exams, including cognitive and functional assessment. Diagnoses were based on the criteria of the National Institute on Aging and the Alzheimer's Association workgroup[16] and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[17]. Based on the clinical diagnosis, the participants were divided into the following groups: probable AD ($n = 19$), MCI ($n = 5$), other dementias/clinical conditions: behavioral variant frontotemporal dementia (bvFTD) ($n = 19$), primary progressive aphasia (PPA) ($n = 2$), and antibodies anti-N-methyl-D-aspartate (NMDA) receptor encephalitis ($n = 1$), these conditions' symptoms overlap with MCI or AD.

This study was approved by the Research Ethics Committee of the UFMG, and all participants or their caregivers signed a consent form prior to collection.

CSF analysis

Lumbar puncture was performed to collect the cerebrospinal fluid with participants fasted for eight hours. The samples were centrifuged at 3,000 revolutions per minute (rpm) for 10 minutes, at 4ºC, maximum 4 hors after collection. Then, they were frozen in polypropylene tubes at -80ºC until the time of analysis. The biomarkers were measured using the INNOTEST hTAU Ag, INNOTEST PHOSPHO-TAU (181P), and INNOTEST β-Amyloid (1-42) (Innogenetics, Bélgica) kits by enzyme-linked immunosorbent assay (ELISA) technique, the manufacturer's instructions were strictly followed. The reference values indicated by the manufacturer are: IATI < 0.8 pg/ml – indicative of AD; > 1.2 pg/ml – normal; Aβ < 500 pg/ml; Tau total > 375 pg/ml and P-tau > 60 pg/ml. The samples were analyzed simultaneously

for the three biomarkers using kits of the same lot, and intra-assay variations based on replicates was < 5%. In all assays was used an internal quality control.

Statistical analysis

Statistical analyzes were performed using SPSS v. 13.0 software. Shapiro-Wilk test was used to evaluate the normality of the variables. The comparison for two groups was performed by the Mann-Whitney or *t*-test for non-parametric and parametric variables, respectively. The comparison between the three groups was performed by the Analysis of Variance (ANOVA) post-hoc least-significance difference (LSD) or Kruskall-Wallis, followed by Mann-Whitney test with Bonferroni correction, for parametric and nonparametric variables, respectively. Correlations were evaluated by Pearson, for parametric variables, or Spearman, for nonparametric. The agreement between the variables was evaluated by the kappa index. Receiver operating characteristic (ROC) curves were used to represent the sensitivity and specificity to AD diagnose in different cut-off values. The best cut-off for each marker has been established by the highest value of the Youden index based on ROC curve. We considered $p < 0.05$ value significant.

TABLE 1 – Comparison of Tau, P-tau and Aβ values and IATI in groups with AD, MCI and other dementia

Biomarkers	AD ($n = 19$)	MCI ($n = 5$)	Other dementia ($n = 22$)	*p* value
Tau (pg/ml)	541 (502)	197 (208)	183 (119)	< 0.001*[a, b]
P-tau (pg/ml)	65 (32)	51 (22)	40 (16)	< 0.001*[b]
Aβ (pg/ml)	624 ± 212	959 ± 250	852 ± 293	< 0.001*[c, d]
IATI	0.5 (0.4)	2.1 (1.3)	2.1 (1.2)	< 0.001*[a, b]

Variable expressed as median (interquartile range) or mean ± standard deviation.

P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index; AD: Alzheimer's disease; MCI: mild cognitive impairment; a: p = *0.007 between AD × MCI to Tau and IATI; b:* p < *0.001 between AD × other dementia to Tau, P-tau and IATI; c:* p = *0.005 between AD × MCI to Aβ, d:* p = *0.002 between AD × other dementia to Aβ; * significant:* p < *0.05.*

TABLE 2 – Comparison of Tau, P-tau and Aβ values and IATI in groups with AD/MCI e other dementia

Biomarkers	AD/MCI ($n = 24$)	Other dementia ($n = 22$)	*p* value
Tau (pg/ml)	488 (603)	183 (119)	< 0.001*
P-tau (pg/ml)	60 (26)	40 (16)	< 0.001*
Aβ (pg/ml)	685 (252)	852 (293)	0.019*
IATI	0.6 (0.9)	2.1 (1.2)	< 0.001*

Variable expressed as median (interquartile range) or mean ± standard deviation.

*P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index; AD: Alzheimer's disease; MCI: mild cognitive impairment; * significant:* p < *0.05.*

RESULTS

The median values and average concentration of each biomarker measured in the CSF and the IATI calculated are shown in **Tables 1** and **2**. In Table 1, individuals with probable AD, MCI and other dementia/other medical conditions; in Table 2, individuals with AD or MCI in relation to other dementias.

We observed higher values of Tau protein and lower values of Aβ and IATI in the AD group when compared with MCI group. We was also observed increased levels of Tau e P-tau, as well as lower levels of Aβ and IATI in AD group when compared with patients with other dementias. However, no biomarker or IATI was able to distinguish between MCI and other dementias (Table 1). In addition, significant differences were found between AD/MCI groups and other dementias with higher levels of Tau, P-tau and lower levels of Aβ and IATI in the first group.

Considering the diagnosis of AD based on IATI reference values provided by the manufacturer (< 0.8) compared with the clinical diagnosis (in this case considered the gold standard), sensitivity 63.6%, specificity 89.6%, positive predictive value (PPV) 87.5%, negative predictive value (NPV) 68.4%, and accuracy 75.8% was obtained for the diagnosis of AD. The result of the kappa index among the variables and the clinical diagnosis was regular for Tau and IATI (0.568 for both), and weak to Aβ (0.111) and P-tau (0.371).

The ROC curve was determined for the variables Tau, P-tau, Aβ and IATI, considering the AD classification according to the clinical diagnosis (**Figure**). It was observed an area under the curve 0.821 for Tau and 0.817 for p-tau, and good the relationship between these biomarkers and clinical diagnosis is considered[18]. As for Aβ and IATI, the area under the curve was 0.701, and 0.777, this relationship is considered poor and regular, respectively[18].

Analyzing the same ROC curves, we evaluated the sensitivity and specificity criteria based on different concentrations of the markers, which were simulated as possible reference value or cut-off (**Table 3**). Only the concentrations which resulted in sensitivity and specificity values > 60% were considered. For Tau variable, concentrations from 244.5 to 327.5 pg/ml showed sensitivity values around 70%-80% with specificity between 84%-70%, respectively, for the diagnosis of AD, and the cut-off 265.7 pg/ml, the value that best showed balance between these parameters. For P-tau protein, the cut-off 47.7 pg/ml showed both variables around 80% and 75%, and the cut-off 680 pg/ml value for Aβ the one that presented

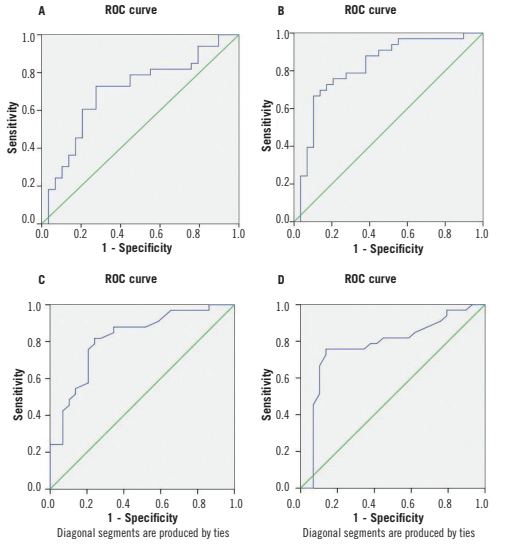

FIGURE – *ROC curve for Aβ (A), Tau (B), P-tau (C) and IATI (D) biomarkers considering the clinical diagnosis as reference*

ROC: receiver operating characteristic; Aβ: amyloid beta-protein; P-tau: phosphorylated Tau protein; IATI: Innotest Amyloid Tau Index.

better sensitivity and specificity combined. For IATI the 1.05 cut-off showed best combined values of sensitivity and specificity, around 75% and 84%, respectively.

When Tau, P-tau and Aβ variables were correlated, there was a strong positive correlation between Tau and P-tau levels (r = 0.917, $p < 0.001$) and strong negative correlation between Aβ and Tau levels (r = -0.659, $p < 0.001$), and Aβ and P-tau (r = -0.612, $p < 0.001$).

DISCUSSION

To our knowledge, this is the first study to assess the concentration of CSF markers for the diagnosis of AD in Brazil. Although this is a preliminary study that requires a larger sample size to validate the findings, the obtained data demonstrate the need for the laboratory to establish the reference values, according to the characteristics of each population, for the diagnosis of the disease.

Currently, the majority of AD patients are diagnosed based on clinical criteria and exclusion of other diseases that cause dementia. While these criteria are widely used, their sensitivity varies around 70.9%-87.3% and specificity between 44.3%-70.8%[19]. Thus, a large percentage of patients are misclassified, particularly the cases of early AD, atypical AD or with multiple etiologies[20]. CSF

TABLE 3 – Coordinates of each ROC curves in the diagnosis of probable AD for Tau, P-tau and Aβ variables and IATI in the group with clinical AD

Variable	Positive if greater or less than	Sensitivity	1-Specificity
Tau	190	0.879	0.379
	194.5	0.848	0.379
	197.5	0.818	0.379
	206.5	0.788	0.379
	227	0.788	0.345
	241.5	0.788	0.310
	244.5	0.788	0.276
	251	0.758	0.276
	257.7	0.758	0.241
	265.7	0.758	0.207
	277.5	0.727	0.207
	292.5	0.727	0.172
	304	0.697	0.172
	327.5	0.697	0.138
	353.5	0.667	0.138
	384	0.667	0.103
	418.5	0.636	0.103
	435.5	0.606	0.103
P-tau	42.2	0.879	0.379
	44	0.879	0.345
	45.5	0.848	0.345
	46.7	0.818	0.276
	47.7	0.818	0.241
	50	0.788	0.241
	53	0.758	0.207
	54.5	0.606	0.207
Aβ	648.5	0.606	0.207
	656.5	0.606	0.241
	661.5	0.606	0.276
	663	0.636	0.276
	664.5	0.667	0.276
	665.5	0.697	0.276
	680	0.727	0.276
	706	0.727	0.310
	728	0.727	0.345
	745	0.727	0.379
IATI	0.75	0.606	0.103
	0.85	0.667	0.103
	0.95	0.727	0.138
	1.05	0.758	0.138
	1.15	0.758	0.207
	1.30	0.758	0.241
	1.55	0.758	0.345
	1.75	0.788	0.379

ROC: receiver operating characteristic; AD: Alzheimer's disease; P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index.

biomarkers are important to optimize the diagnosis, especially in differentiating AD from other neurodegenerative disorders such as frontotemporal, vascular, psychiatric dementia or neuroinfectious diseases.

According to members of Alzheimer's Biomarkers Standardization Initiative (ABSI)[21], the measurement of CSF biomarkers should be considered for patients with early AD, MCI or with atypical presentations of dementia with complex differential diagnosis. Furthermore, they must be evaluated in together with Tau, P-tau and Aβ values for greater accuracy in the AD diagnosis, which can be interpreted using in the IATI calculation.

In this study, the use of Tau, Aβ and IATI markers calculated was useful for the differential diagnosis of MCI or other dementia and AD, while the p-tau values differentiated only AD and other dementia groups, which confirms the usefulness of these markers in the differential diagnosis and the need for the analysis to be performed together. Furthermore, data suggest the use of markers for checking the conversion of prodromal MCI stage to AD. However, it should be noted that the CSF markers were unable to differentiate the individual with MCI from those with other types of dementia, and, therefore, it is not suitable for cases where the AD is unlikely.

When the AD and MCI groups were collected and the values of variables compared to the group with other dementia, all biomarkers and IATI showed differences between groups, suggesting its usefulness in the diagnosis of pre-clinical and clinical stages of AD compared with other dementias.

Although INNOTEST® kit is not the only kit commercially available for the quantification of these markers, it is known that its use is widespread, and in Brazil it is already being applied in the routine laboratory. Considering the clinical diagnosis of AD and diagnosis performed in our patients based on IATI provided by the manufacturer, were observed low sensitivity and NPV, as well as regular to weak relationship with the clinical diagnosis measured by the kappa index, which suggests that the cut-off value of IATI suggested by the manufacturer is not suitable for the population studied.

In fact, the variability of the cut-off between different laboratories, using the same, can reach 20%-30%[14, 22]. Since these values are calculated using the results from patients with clinical diagnosis of AD, patients with other types of dementia and healthy individuals may exhibit altered biomarker values without necessarily demonstrate the clinical features of the disease, since AD may be present up to 20 years before the onset of symptoms[23].

There was variation in sensitivity and specificity according to different cut-off values. The values that showed better balance between the two criteria were suggested (**Table 4**), which differed from the values suggested by the kit manufacturer. However, choosing the best cut-off should be discussed with clinicians, since the impact of false positive or false negative results should be evaluated selectively. According to ABSI, because there is no yet an effective therapy for AD, it is better to incorrectly diagnose some patients who actually have the disease than patients classified as healthy individuals[24]. It will also be necessary to establish, based on Brazilian large cohort studies, the "gray zone" or each biomarker and IATI value. Although commonly it has been used values 10% higher or lower than the reference value for determining test positivity or negativity[21], this value is arbitrary and its interpretation must be done with caution – an um "unlikely AD" does not mean an "excluded AD"[24].

It should also be considered that the AD develops a dynamically and progressively, which means that the cut-off values can be modified depending on the course of the disease (preclinical and clinical phase) and age group. The final diagnosis should be complemented, whenever possible, with imaging tests such as magnetic resonance imaging (MRI) and positron emission tomography (PET)[25-27].

It is noteworthy that no biomarker provided sufficient sensitivity and specificity for the diagnosis of AD when assessed alone, which justifies the recommendation of a joint assessment of the three biomarkers. The mathematical calculation of the IATI, based on these markers, that although has demonstrated a strong correlation, meaning that they have the same tendency for the diagnosis, did not show sufficient accuracy to be used alone for the diagnosis of AD. This fact confirms the need to include other parameters along with the cognitive tests and clinical features for the diagnosis to be completed.

TABLE 4 – Comparative description of cut-off values for diagnosis of AD according to the kit used and the clinical diagnosis

Biomarkers	INNOTEST®	Clinical diagnosis
Aβ	< 500 pg/ml	< 680 pg/ml
Tau	> 375 pg/ml	> 268 pg/ml
P-tau	> 60 pg/ml	> 48 pg/ml
IATI	< 0.8	< 1.05

AD: Alzheimer's disease; Aβ: amyloid beta-protein; P-tau: phosphorylated Tau protein; IATI: Innotest Amyloid Tau Index.

CONCLUSION

Although insufficient to complete the diagnosis of AD alone, CSF biomarkers represent important resources that can

assist the medical team. It is true that the search for predictive biomarkers for AD is a high priority on research related to neurodegenerative diseases and, in the near future, it is expected to establish cut-off values that meet specificities of each population, enabling the use of these tools in the search of early diagnosis of AD and modifying perspective of the course of the disease.

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RESUMO

Introdução: *Estudos têm sido conduzidos no sentido de validar biomarcadores no liquor para o diagnóstico da doença de Alzheimer (DA), objetivando, sobretudo, facilitar o diagnóstico precoce.* ***Objetivo:*** *Avaliar os biomarcadores do liquor em indivíduos com provável DA, bem como a aplicabilidade dos valores de referência internacionais nesta população.* ***Métodos:*** *Foram recrutados 46 indivíduos, sendo classificados como provável DA (n = 19), comprometimento cognitivo leve (CCL) (n = 5) e outras demências (n = 22). Os biomarcadores foram dosados no liquor utilizando-se os* kits *INNOTEST' por ensaio imunossorvente ligado à enzima (ELISA). Maiores valores de proteína Tau e menores valores de Aβ e índice Innotest Amiloide Tau Index (IATI) foram observados no grupo de DA quando comparados com o de CCL; maiores níveis de Tau e Tau fosforilada (Tau-P) e menores valores de Aβ e IATI foram observados no grupo de DA quando comparados com os pacientes que apresentavam outras demências. Nenhum biomarcador ou o IATI foi capaz de discernir entre CCL e outras demências. O índice* kappa *entre os biomarcadores e o diagnóstico clínico foi regular para a Tau e IATI, e fraco para Aβ e Tau-P.* ***Conclusão:*** *Os valores de* cut-off *para cada biomarcador que apresentou melhor sensibilidade e especificidade conjugadas diferiram dos valores de referência sugeridos pelo fabricante. Os biomarcadores do liquor representam importantes recursos que podem auxiliar no diagnóstico da DA, mas a interpretação dos resultados deve ser feita com base na análise dos três analitos em conjunto. Os valores de* cut-off *devem ser estabelecidos de modo a atender as especificidades e as características de cada população.*

Unitermos: *amiloide; proteínas tau; doença de Alzheimer; líquido cefalorraquidiano.*

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9.2 ARTIGO 6

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VIEW AND REVIEW

Alzheimer's disease and cytokine IL-10 gene polymorphisms: is there an association?

A doença de Alzheimer e os polimorfismos no gene da citocina IL-10: há alguma associação?

Carolina Antunes Magalhães[1], Maria das Graças Carvalho[1], Lirlândia Pires de Sousa[1], Paulo Caramelli[2], Karina Braga Gomes[1]

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia. In the last 15 years, a new theory has proposed the autoimmune mechanism as a trigger for AD. Studies on the association between AD and inflammatory biomarkers have yielded controversial results. Interleukin-10 (IL-10), an anti-inflammatory mediator, has been pointed out as one of the main cytokines associated with the occurrence of AD. Moreover, treatment that increases IL-10 levels could be a potential therapy for AD, since this cytokine acts on amyloid and pro-inflammatory molecule reduction. Based on the current literature, this study reviews evidence regarding the role of *IL-10* polymorphisms in the context of AD, which has been shown to be of paramount importance for attenuating neuroinflammation, cognitive dysfunction and neurodegeneration.

Keywords: Alzheimer disease; inflammation; Interleukin-10.

RESUMO

A doença de Alzheimer (DA) é a forma mais comum de demência. Nos últimos 15 anos, uma nova teoria propõe um mecanismo autoimmune como o gatilho para a DA. Associações entre DA e biomarcadores inflamatórios têm sido registradas, contudo com resultados controversos. A interleucina-10 (IL-10), um mediador anti-inflamatório, tem sido apontada como uma das principais citocinas associadas com a ocorrência de DA. Além disso, os tratamentos que aumentam os níveis de IL-10 podem ser uma terapia potencial para DA, uma vez que esta citocina atua sobre a redução de substância amiloide e de moléculas pró-inflamatórias. Baseando-se em literaturas atuais, este estudo revisa evidências relacionadas com o papel da IL-10 e seus polimorfismos no contexto da DA, o qual se mostrou ser de fundamental importância para atenuar a neuroinflamação, a disfunção cognitiva e a neurodegeneração.

Palavras-chave: doença de Alzheimer; inflamação; interleucina-10.

Alzheimer's disease (AD), the most common form of dementia, is a global public health problem challenging the older generation[1]. Alzheimer's disease is a neurodegenerative disorder characterized by injury to brain regions responsible for controlling memory and other cognitive functions. In this way, this disease compromises the ability to learn, reason, communicate, and carry out daily activities, and is accompanied by personality and behavioral changes[2].

According to the Alzheimer's Association, in the United States, one person develops AD every 67 seconds. By 2050, one case every 33 seconds is predicted, resulting in one million new cases per year[1]. Nitrini *et al.* found a prevalence for dementia in seven percent of the elderly, aged 65 or older, in Latin America[3].

Neuropathology in AD is characterized by altered formation of amyloid-β (Aβ) plaques and hyperphosphorylation of the tau protein associated with neurofibrillary tangles[4,5]. According to Rosenberg et al.[6], genotype-phenotype correlations of AD provide a comprehensive appreciation of the spectrum of disease causation. The inflammatory process is another main pathophysiological factor associated with AD[7,8].

In the last 15 years, a new theory has proposed the autoimmune mechanism as a trigger for AD. This theory involves a dysregulation of the blood-brain barrier, neurons, microglia, astrocytes and multiple cytokines[9,10,11]. As reviewed by Naert and Rivest, activated microglia and astrocytes secrete inflammatory cytokines and chemokines, while age-related inflammation and chronic infection with herpes viruses might contribute to the systemic inflammation[12].

Microglia activation is supposed initially to be a result of tissue injury and amyloid plaque deposition due to a cytotoxic

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response in the brain[13,14]. Activated microglia and astrocyte clusters at sites of neuritic plaques release a variety of inflammatory mediators[15], including pro- and anti-inflammatory cytokines that play critical roles in the development and progression of AD[16,17,18,19].

The associations between AD and inflammatory biomarkers, including the interleukins IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IL-18, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and the C-reactive protein have been registered with controversial results[20]. However, interleukin-10 (IL-10), an anti-inflammatory mediator, has been pointed out as one of the main cytokines associated with the occurrence of AD[5,9,10]. Therefore, this study reviewed evidence of the role of IL-10 and its genetic polymorphisms in the context of AD. Our hypothesis is that decreased levels of IL-10, an anti-inflammatory cytokine, and its polymorphisms contribute to an increase in the inflammatory process, which should favour the development of AD, reinforcing the link between inflammation and cognitive decline in elderly people.

METHODS

Our search was conducted in Pubmed, the Cochrane Library, Science Direct, Scopus and Web of Science databases with the terms "Alzheimer's disease", "inflammation", "interleukin-10", and studies reporting on "associations between Alzheimer's disease and interleukin-10", with no date restrictions. In this search, 60 works published between 1989 and 2016, in the English language, were identified and included in the present review.

IL-10 and immunomodulation

Cytokines are small proteins secreted by activated cells. They can affect other target cells or even the same cell that secreted these cytokines. Cytokines are responsible for the communication between cells and play an important role in the physiological and pathological inflammation processes[21]. Interleukins, one of the main types of cytokines, are small glycoproteins, secreted by activated macrophages and leukocytes. Interleukins are involved in T lymphocyte activation, proliferation and toxicity. Defects of interleukin production may be associated with several disorders. There are more than 36 types of interleukins chronologically numbered in the order of their discovery, one of them being IL-10[22].

Interleukin-10 is a 36-KDa homodimeric cytokine described by Fiorentino *et al.* as a "cytokine synthesis inhibitory factor", because of its ability to suppress cytokine production from all T cell types[23]. The main IL-10 sources *in vivo* are monocytes, macrophages, dendritic, B, NK and mast cells, T-cells, as well as neutrophils and eosinophils. In monocytic cells, IL-10 influences antigen presentation, release of immune mediators and phagocytosis[21].

Interleukin-10 opposes the actions of the pro-inflammatory cytokines and appears to be a suppressor of both immunoproliferative and inflammatory responses in the brain, reducing synthesis of pro-inflammatory cytokines, suppressing cytokine receptor expression, and inhibiting receptor activation[21,24]. Expression of the pro-inflammatory cytokines with a central role in inflammation and cell death, such as IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α, and IFN-γ, are negatively controlled by the immunomodulatory action of IL-10[23].

Alzheimer's disease and IL-10 effects

It is known that a chronic inflammatory process accompanies AD. However, it remains unclear whether inflammation is a reaction to the pathology of AD or a contribution to the onset, or progression, of the disease[25]. The observation of the reactive astrocytes and activated microglia cells, associated with senile plaques in AD, reinforces the inflammatory mechanisms in the pathogenesis of this disease. This mechanism is also supported by the observation of a decreased incidence of AD in patients who receive long-term nonsteroidal anti-inflammatory drugs[26].

According to Combarros et al.[25], certain combinations of genetic variants in the regulatory regions of the two genes, i.e. *IL-6*-174G/C (rs1800795) and *IL-10*-1082A/G (rs1800896) contribute to chronic inflammation in elderly people, increasing the risk of AD. An imbalance between pro-inflammatory and anti-inflammatory cytokines may, therefore, be an important phenomenon in AD. This hypothesis is supported by studies, which described an increase of seven- to ten-fold in the production of IL-1β over IL-10 levels in AD patients when compared with control subjects[26]. Indeed, Rota *et al.* did not detect abnormal levels of IL-10 in either cerebrospinal fluid or serum of AD patients[27].

Richwine *et al.* observed that a peripheral injection with lipopolysaccharide, in IL-10-deficient mice, causes a prominent cognitive deficit when compared with wild-type mice[28]. Kiyota et al.[10] demonstrated that IL-10 significantly reduced neuroinflammation, enhanced neurogenesis and improved spatial cognitive dysfunction in transgenic AD mouse models. They showed that treatment with IL-10-adeno-associated virus of double-transgenic mice expressing familial AD mutants of amyloid precursor protein+presenilin-1 (APP+PS1 Tg), could suppress astro/microgliosis. According to the authors, these findings support the concept that IL-10 may ameliorate neuroinflammation, cognitive dysfunction and neurodegeneration.

Corroborating these findings, Henderson[29] suggested that post-menopausal administration of estrogens may delay the onset, or contribute to the prevention of AD[29] by increasing the secretion of IL-10 from microglial cells[30,31]. Moreover, resveratrol, a natural polyphenol reported to have anti-inflammatory

effects, is able to up-regulate both *IL-10* gene expression and IL-10 levels, which could explain its neuroprotective properties[32]. Bagyinszky et al.[22] disclosed that IL-10 treatment could be a potential therapy for AD since this cytokine could act on amyloid reduction by inducing the production of anti-inflammatory molecules, and inhibition of pro-inflammatory cytokines, probably by down-regulating their expression.

However, Guillot-Sestier et al.[33] found that crossing the (APP+PS1 Tg) mouse model of cerebral amyloidosis with animals deficient in IL-10 demonstrated that genetic blockade of IL-10 mitigates cerebral amyloidosis in APP/PS1 mice. In line with these results, Chakrabarty *et al.* reported that enhanced IL-10 expression in brains of APP transgenic mice leads to increased Aβ accumulation and worsening of behavioral deficits[34]. These results suggest that rebalancing cerebral innate immunity and promoting beneficial neuroinflammation may be more efficacious than generalized anti-inflammatory therapy for AD. Indeed, according to Bryson and Lynch[35], anti-inflammatory therapy has not been proven to be of value in the treatment of AD and inflammatory changes, once a certain stage of inflammation is reached.

Zheng et al.[36] reviewed studies in order to understand the importance of the role of cytokines or neuroinflammation in AD etiology and pathogenesis, suggesting the imbalance of pro- and anti-inflammatory activity in AD. According to them, inconsistent outcomes involve IL-10: this cytokine drives macrophage polarization – M1 to M2, which is associated with deactivation of microglia; overexpressing tau increases secretion of IL-10 in rat microglia, which show greater phagocytosis of microspheres; knockout mice show the benefit of IL-10 removal[32]. However, some meta-analysis studies have not found significant differences in IL-10 levels between subjects with mild cognitive impairment (MCI) and healthy controls. Moreover, IL-10 overexpressing in AD animal models weakened the phagocytosis of soluble Aβ by microglia and exacerbated Aβ deposits in cognitive impairment. They highlighted that the association of IL-10 with AD requires further study based on genetic polymorphisms as well as the changing levels of this cytokine in AD patients[36].

Alzheimer disease and IL10 gene polymorphisms

The pro- and anti-inflammatory cytokine genes have been studied as potential candidates for the individual's susceptibility to AD; however, no preferential role has been clearly identified[37], even with opposing results[38] (Table).

Interleukin -10 is encoded by a gene located on the long arm of chromosome 1 between positions 31 and 32[39]. The regulatory regions of the *IL-10* gene have been associated with chronic inflammatory diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, as well as the development of dementia[9,40]. Some polymorphisms have been associated with *IL-10* gene expression (Figure).

Several single nucleotide polymorphisms (SNPs) in the regulatory region of *IL-10* were reported to be associated with modulation of IL-10 production. This may result in an imbalance of the regulatory effect of IL-10 on pro-inflammatory cytokines with a subsequent imbalance of the immune response[41,42]. Lio et al.[41] evaluated the role of *IL-10* polymorphisms and AD development in a group of patients from northern Italy. In their 132 AD patients and 213 healthy controls, they investigated the prevalence of SNPs -1082A/G, -819C/T (rs1800871) and -592C/A (rs1800872) in the *IL-10* promoter region. The frequency of -1082A carriers, which are associated with a low production of IL-10, was significantly increased among AD patients. Thus, these authors concluded that the presence of the -1082A allele, associated with a low production of IL-10, may be considered as an additive and independent genetic risk factor for AD[41]. In the same year, Depboylu et al.[40] investigated the polymorphisms -1087A/G (rs1800896), -824C/T (rs1800871) and -597C/A (rs1800872) in 406 AD patients and 251 unrelated healthy controls from Germany. They found no significant differences in the allelic distribution of these polymorphisms between AD patients and controls.

Arosio et al.[43] investigated the prevalence of -1082A/G, -819C/T and -592C/A polymorphisms and IL-10 production by peripheral blood mononuclear cells in 65 AD patients and 65 controls, selected from an Italian population. In the AD patients, an increase of the -1082A allele and a decrease of -1082GG genotype frequencies were observed. They found that the homozygosity for the A allele was associated with a higher risk of AD. The same authors, six years later, analyzed the genotype and allele frequencies of *IL-10* -1082A/G polymorphism in 138 patients with MCI diagnosed, respectively, as amnestic (a-MCI) and with multiple impaired cognitive domains (mcd-MCI) in Caucasians from northern Italy[44]. The allele frequencies of this SNP in a-MCI patients were similar to those of AD patients, whereas those of mcd-MCI patients were comparable to controls. According to the authors, IL-10 may partly explain the conversion of a-MCI to AD, or be a genetic marker of susceptibility[43,44].

In addition, studying the Italian population, Scassellati et al. analyzed 215 AD patients and 153 controls. They observed that three SNPs (-1082A/G, -819C/T and -592C/A) have linkage disequilibrium, resulting in three haplotypes GCC, ACC and ATA. The haplotype GCC/ACC was more frequent in AD patients[4]. Some years later, another study involving an Italian population investigated allele frequency and distribution of the -1082A/G and -819C/T polymorphisms in 222 sporadic AD patients and 179 normal controls. They found that haplotype -1082A/-819T was significantly associated with an increased risk of developing AD[45].

Ma et al.[46] investigated three SNPs (-1082A/G, -819C/T and -592C/A) in 95 AD patients and 117 age-matched healthy Chinese subjects. They found a strong association between AD and two *IL-10* polymorphisms. The reduced expression of *IL-10* was associated with the -819C and -592C alleles, and the authors concluded that the functional polymorphisms of the *IL-10* gene act as a risk factor for AD.

Table. Association between IL-10 polymorphisms and Alzheimer's disease (AD) in different studies.

Authors	Location	Groups	IL-10 polymorphism(s)	Conclusion
Depboylu et al.,[40] 2003	Germany	AD: 406 Control: 251	-1087A/G -824C/T -597C/A	No significant differences have been found between AD patients and controls.
Lio et al.,[41] 2003	Italy	AD: 132 Control: 213	-1082A/G -819C/T -592C/A	The presence of -1082A allele associated with a low production of IL-10, may be considered as an additive and independent genetic risk factor for AD.
Scassellati et al.,[4] 2004	Italy	AD: 215 Control: 153	-1082G/A -819C/T -592C/A	Haplotype frequencies did not reveal differences. However, the genotype GCC/ACC was more frequent in AD.
Arosio et al.,[43] 2004	Italy	AD: 65 Control: 65	-1082A/G -819C/T -592C/A	In AD there was a significant increase of the -1082A allele and a decrease of -1082GG genotype frequencies.
Ma et al.,[46] 2005	China	AD: 95 Control: 117	-1082A/G -819C/T -592C/A	The reduced expression of IL-10 was associated with the -819C and -592C alleles.
Culpan et al.,[39] 2006	England	AD: 160 Control: 92	-3538T/A/-1354G/A -1082A/G/-819C/T -592C/A	None of the SNPs found to be associated with AD.
Ramos et al.,[48] 2006	America	AD: 265 Control: 347	-1082A/G -592C/A	No difference was observed between AD patients and controls.
Bagnoli et al.,[45] 2007	Italy	AD: 222 Control: 179	-1082A/G -819C/T	Haplotype -1082A/-819T was associated with an increase in the risk of developing AD.
Vural et al.,[49] 2009	Turkey	AD: 101 Control: 138	-1082A/G	Heterozygous (AG) or A allele carriers (AG+AA genotype) were associated with approximately two-fold increase in the risk of AD.
Combarros et al.,[25] 2009	England, Spain, Netherland and Germany	AD: 1. 757 Control: 6. 295	-1082A/G	Dysregulation of the *IL-10* gene contributes to chronic low-grade inflammation in some elderly people and increases the risk of AD.
Arosio et al.,[44] 2010	Italy	MCI: 138 AD and Control: Arosio et al., 2004	-1082A/G	The allele frequencies of this SNP in a-MCI subjects were similar to those of AD patients, whereas those of mcd-MCI were comparable to controls.
Ribizzi et al.,[50] 2010	Caucasian population	AD: 19 Control: 20	-1082A/G -819C/T	The -819C allele was raised in AD group and associated with low producers of IL-10.
Moraes et al.,[42] 2013	Brazil	AD: 120 Control: 412	-1082A/G	The SNP -1082A/G exhibited an effect in predisposition to the onset of AD. Almost 40% lower chance of AD among homozygotes of the *IL10* -1082A allele.
Kang et al.,[53] 2015	Korea	AD: 86 No AD: 625	-1082A/G	No significant association between AD patients and No AD patients.
Vargas-Alarcón et al.,[54] 2016	Mexico	AD: 122 Vascular dementia: 67 Mixed dementia: 32 Control: 986	-1082A/G -819C/T	Identified two risk haplotypes (ATA and CTA) and four protection haplotypes (ATG, CTG, ACG and CCG).
Fraga et al.,[56] 2016	Brazil	Cognitive impairment: 135 Control: 124	-1082A/G -819C/T -592C/A	Haplotype -1082/-819/-592, associated with lower expression of IL-10 were more frequent in patient group.

AD: Alzheimer's disease.

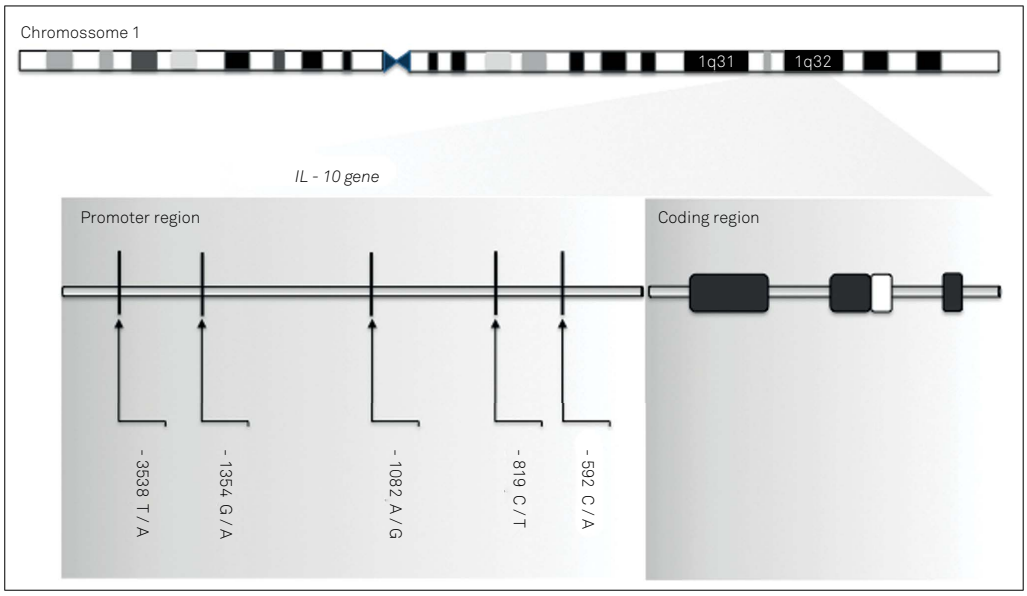

Figure. Chromosome 1, *IL-10* gene and SNPs in promotor region.

Culpan et al.[47], in a retrospective case-control study, examined the brain tissue from 160 patients with neuropathologically confirmed AD and 92 neuropathologically normal non-demented elderly controls, from a population in England. They evaluated five SNPs -3538T/A (rs1800890), -1354G/A (rs1800893), -1082A/G, -819C/T, -592C/A, and two microsatellites (IL-10-G, IL-10-R) in the promoter region of the *IL-10* gene. None of the SNPs or microsatellites was found to be associated with AD. Levels of IL-10 protein and gene expression also did not appear to be related to AD. These results are consistent with those reported in Italian and German patients[4,40], but differed from those in others studies in Italian and Chinese populations[41,43 45,46].

In the same year, Ramos et al.[48] evaluated the influence of promoter region polymorphisms in the *IL-10* gene and the risk of late-onset AD in 265 older white patients and 347 white control subjects in the American population. No difference was observed for *IL-10*-1082A/G and -592C/A allelic and genotypic frequencies between the groups.

Vural et al.[49] investigated the SNP-1082A/G as a susceptibility factor for AD in 101 sporadic AD patients and 138 healthy controls in the Turkish population. Heterozygous (AG) or A allele carriers (AG+AA genotype) for this polymorphism were associated with approximately a two-fold increase in the risk of AD.

Ribizzi *et al.*, also analyzing Caucasian individuals, evaluated the genotypic and allelic polymorphisms of 13 cytokine genes (*IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-G, TGF*-β, *TNF*-α), and of cytokine receptors (*IL-1R, IL-1RA, IL-4RA*) in 19 AD patients and 20 controls affected by non-inflammatory neuropsychiatric disease. They suggested the presence of a pro-inflammatory environment in AD patients, corroborated by the low expression of *IL-10* when the -819C allele was present[50].

Zhang et al.[50] performed a meta-analysis on the association between *IL-10* -1082A/G polymorphism and AD risk (2,158 patients and 2,088 controls in 12 case-control studies)[51]. The results indicated that A allele carriers (AA + AG) had a 27% increased risk of AD, when compared with the homozygote GG. In the analysis of the ethnic subgroup, significant elevated risk was associated with A allele carriers in Europeans but not in Asians, suggesting genetic diversity among ethnicities. However, because there was only one study performed in Asians, these results may not be valid for this population, according to the authors[47]. Di Bona et al.[52] also investigated, by meta-analysis, the association of the common IL-10 polymorphisms with AD risk. Fifteen studies investigating the association between the polymorphisms -1082A/G, -819C/T and -592C/A and AD were analyzed. The data suggested an association between the -1082A allele and risk of AD. They did not find an association with AD for the -819C/T and -592C/A polymorphisms.

Moraes et al.[42] compared the polymorphic genotype distribution across outpatients with late-onset AD and non-cognitively impaired subjects (120 AD patients and 412 healthy controls) from Brasília, midwest Brazil. They evaluated polymorphisms in *IL-1*α, *IL-1*β, *IL-6, IL-8, IL-10, IL-12*β,

IL-18, TGF-β1, TLR-4 and *TNF-α* genes. Only *IL-10* (-1082A/G) and *IL6* (-174C, rs1800795) genes, in recessive and dominant models, respectively, exhibited an effect on the predisposition to AD. Their findings showed an almost 40% lower chance of AD among homozygotes of the *IL-10* -1082A allele[42].

Kang et al.[53] investigated the involvement of alleles associated with higher production of proinflammatory and lower production of anti-inflammatory cytokines in 732 elderly Korean individuals with AD or depression. Genotyping was performed for six pro-inflammatory (*IL-1β, IL-6, IL-8, TNF-α*) and two anti- inflammatory (*IL-4* and *IL-10*-1082A/G) cytokines genes. *TNF-α* and *IL-8* were significantly associated with AD, and IL-1β with late-life depression. They found no significant association between anti-inflammatory cytokine gene polymorphisms and AD or late-life depression[53].

Vargas-Alarcón et al.[54] conducted the first study in a Mexican population that considered the analysis of *IL-10* SNPs in patients with AD, vascular dementia and mixed dementia (AD/vascular dementia). They analyzed genotypes, allele distributions and haplotypes of *IL-10* promoter polymorphisms -592 C/A, -819 C/T and -1082 A/G, in 986 healthy controls and 221 patients, with 122 patients with AD, 67 with vascular dementia and 32 with mixed dementia. They observed associations between the *IL-10* SNPs with mixed dementia when compared with controls, with dominant, and overdominant inheritance models[54]. Moreover, these polymorphisms were associated with a lower risk of developing AD and vascular dementia when compared with controls. Patients with dementia also showed increased frequency of ATA, CTG, and CTA haplotypes when compared with controls. They identified two risk haplotypes: ATA and CTA and four protection haplotypes: ATG, CTG, ACG and CCG[54].

Mun et al.[55], in a meta-analysis, re-evaluated and updated the associations between IL gene polymorphisms [-889C > T (rs1800587) in *IL-1α*, -511C > T (rs16944) in *IL-1β*, -174C > G (rs1800795) in *IL-6* and -1082G > A in *IL-10*] and the risk of AD. Their results suggested that the -889C > T polymorphism may be a potential risk factor in AD. However, the other three polymorphisms, including the -1082G > A polymorphism of *IL-10*, may not be a risk factor for AD[55].

Our group investigated the frequency of *IL-10* -1082A > G, -819C > T and -592C > A SNPs in a sample of healthy and cognitively impaired elderly, to verify the association between these SNPs and the cognitive and functional performance of individuals aged 75 years and above. In this study, 259 Brazilian participants were included, 135 with cognitive impairment (81 with cognitive impairment with no dementia, and 54 demented seniors) and 124 age-matched and gender-matched cognitively healthy controls. The results showed that the haplotypes associated with lower gene expression were more frequent among individuals with cognitive impairment. Moreover, carriers of the -1082G allele also had better performances in brief cognitive screening tests. Carriers of -819T and -592A alleles showed worse performance than non-carriers in the same tests. In relation to the *IL-10* haplotypes, individuals with higher or intermediate expression of IL-10 had better performances in the screening tests. The results suggest a potential role for these SNPs in the development of cognitive impairment with no dementia, and dementia, which may influence the cognitive performance of these patients[56].

The discrepancies between the studies may be explained by uncontrolled confounding factors, gene-gene interaction or by the fact that some polymorphisms present with different allelic frequencies in certain populations, since genotype distribution of *IL-10* polymorphisms has been found to be different in Caucasian and Asian populations[36].

None the studies discussed found differences in either IL-10 haplotype or genotype distributions among AD patients who did, or did not, carry the allele 4 (e4) of the ApoE gene, concluding that the *IL-10* polymorphisms are an additive and independent risk factor for AD.

CONCLUSION

The studies showed that immunity has an important role in AD onset/progress. Although *IL-10* SNP frequency has shown heterogeneity in different populations, several studies, including our investigation in a Brazilian cohort, suggest that these polymorphisms, particularly -1082A/G, are an important risk factor for AD. However, the mechanism in which IL-10 may ameliorate neuroinflammation, cognitive dysfunction or neurodegeneration is not completely clear. Therefore, other molecular studies, to clarify the AD etiology, are necessary for solution management and prevention of this complex disease.

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10 DISCUSSÃO

No capítulo 1 do nosso trabalho, avaliamos a relação de marcadores inflamatórios e das MPs na DA. Dentre as citocinas avaliadas, IL-6, IL-1β e TNF-α, bem como a PCR-us, apresentaram níveis séricos significativamente reduzidos em pacientes com DA quando comparados com controles cognitivamente saudáveis. Já a IL-10 não apresentou diferença entre os grupos. Embora seja conhecida a influência do IMC nos níveis das citocinas (Larsson *et al,* 2015), em nosso estudo o IMC e a CA não diferiram entre os grupos e não influenciaram os níveis destes marcadores.

A neuroinflamação é um evento comum na DA, onde as células da glia estão mais ativadas, resultando em um aumento da produção de citocinas pró-inflamatórias (Martorana *et al*., 2012; Rubio-Perez e Morrilas-Ruiz, 2012; Heneka *et al*., 2015, Su *et al*., 2016; Calsolaro and Edison*;* 2016; White *et al*., 2017). Calsolaro e Edison (2016) sugeriram que alguns marcadores inflamatórios podem aumentar temporariamente no estágio pré-clínico ou no estágio clínico inicial da doença e também aumentar rapidamente durante a progressão da doença. Estudos com diferentes delineamentos e variadas amostras levam a resultados conflitantes que dificultam o uso das citocinas como biomarcadores para a DA (Hesse *et al*., 2016). Hesse *et al*. (2016) analisaram IL-1β, IL-8 e TNF-α em amostras de líquor e soro de pacientes com DA e encontraram níveis reduzidos de IL-8 nessas amostras, além de uma correlação negativa entre MEEM e IL-1β. Savas *et al*. (2016) encontraram altos níveis sanguíneos de IL-6, mas não de PCR-us e TNF-α, em pacientes com DA comparando-os com controles.

Popp *et al*. (2017) avaliaram biomarcadores inflamatórios associados com a DA e a associação deles com biomarcadores liquóricos (proteína amiloide e proteína Tau). As citocinas IL-1β, IL-6, TNF-α e IL-10 não demonstraram correlação com os biomarcadores liquóricos para a DA. Eles sugeriram que a inflamação é parte da patologia da DA somente nos estágios clínicos iniciais. Nossos resultados sugerem que há uma diminuição nos níveis de citocinas com o curso clínico da doença.

As MPs estão envolvidas no desenvolvimento neuronal, na atividade sináptica, na regeneração neural e em mecanismos protetores (Verderio *et al*., 2012; Lai e Breakefield, 2012). Elas também são capazes de transferir proteínas entre células e estão implicadas em desordens neurodegenerativas como a DA (Matsubara *et al*., 2002). Níveis elevados de MPs foram detectados no líquor e plasma de indivíduos com doenças ligadas ao sistema nervoso

central (Verderio *et al*., 2012; Minagar *et al*., 2001; Combes *et al*., 2005). Nós sugerimos que níveis aumentados de tipos específicos de MPs no plasma podem representar marcadores biológicos confiáveis para a DA.

É importante destacar que a DA está associada a condições inflamatórias, a formação de novelos neurofibrilares, ao desenvolvimento de placas amiloides, a degeneração neuronal progressiva, a injúria microvascular, ao bloqueio capilar e a desregulação da barreira hematoencefálica (Yun *et al*., 2016). Consequentemente, caracterizamos as MPs de acordo com a sua célula de origem e associadas a esses mecanismos. Nós observamos que o número de MPs total estava aumentado nos pacientes com DA quando comparados com o grupo controle, especificamente os níveis de MPs que expressam fator tecidual (TFMPs), MPs derivadas de leucócito (LMPs) e de neurônio (NMPs), o que sugere o potencial desses biomarcadores na doença. Além disso, foi encontrada uma associação independente do TNF-α e das NMPs com a DA. Segundo nosso conhecimento, este é o primeiro estudo que investiga os níveis de NMPs em pacientes com DA comparados com indivíduos cognitivamente saudáveis e que estabelece um algoritmo de diagnóstico da DA utilizando mardores inflamatórios e MPs. Destacamos a observação de correlações inversas das LMPs, PMPs e TFMPs com a proteína βA, assim como das NMPs com o índice IATI. Estes resultados reforçam o potencial das MPs como marcadores no diagnóstico da DA.

No capítulo 2, avaliamos os níveis de MPs em indivíduos com e sem comprometimento cognitivo em uma população de idosos. O DCS é uma definição recente que criou uma eminente necessidade de biomarcadores que permitam identificar quais indivíduos com DCS evoluirão para CCL/demência e quais não. Considerando que as MPs estão envolvidas na neuroinflamação e na hemostasia (Zamolodchikov e Strichland, 2016; Kumar *et al*., 2017; Bagyinszky *et al*., 2017), investigamos a correlação entre os níveis de MPs e a performance global cognitiva e o estado funcional desses indivíduos.

Encontramos que níveis elevados de PMPs, LMPs e TFMPs estão associados com o declínio cognitivo. Além disso, observamos que níveis elevados de LMPs e NMPs se correlacionam com o comprometimento funtional nessa população. Ainda segundo nosso conhecimento, este é o primeiro estudo que investiga os níveis de MPs de acordo com a performance funcional e cognitiva no idoso cognitivamente saudável até o idoso em estado demencial.

No capítulo 3, avaliamos os níveis de leptina, TNF-α, IL-6 e PCR-us em pacientes com declínio cognitivo, entre estes, indivíduos com CCL e pacientes com DA e os comparamos com indivíduos sem comprometimento cognitivo e indivíduos com declínio

cognitivo subjetivo (DCS). A leptina, com seu papel neuroprotetor contra a patologia da DA, poderia ser um potencial biomarcador para a diferenciação entre o envelhecimento normal e o estado de demência. Entretanto, não foi encontrada diferença significativa nos níveis de leptina entre os grupos estudados. Já foi demonstrado que a obesidade abdominal está associada com níveis elevados de leptina devido a uma resistência central a este peptídeo (Tezapsidis *et al*., 2009, Hazzouri *et al*., 2013), no entanto, nós não encontramos influência da obesidade abdominal (aumento da circunferência abdominal) assim como do índice de massa corpórea (IMC) nos níveis de leptina entre os participantes.

Nosso resultado sugere que a leptina não está envolvida na evolução do declínio cognitivo. Este é o primeiro estudo a avaliar níveis de leptina em um grupo com DCS e; embora outros autores tenham encontraram níveis elevados de leptina em pacientes com DA comparados com grupos CCL e controle (Bonda *et al*., 2014), nossos achados estão em concordância com estudos prévios que também não encontraram diferença nos níveis de leptina entre idosos com demência, CCL e grupo controle (Holden *et al*., 2009; Lieb *et al*., 2009; Bigalke *et al*., 2011; Theodoropoulou *et al*. 2012; Warren *et al*. 2012; Khemka *et al*., 2014, Baranowska-Bik e*t al*., 2015; Maioli *et al*., 2015; Teunissen *et al*., 2015; Bednarska-Makaruk *et al*., 2017).

Os participantes com declínio cognitivo (CCL e DA) apresentaram níveis reduzidos de TNF-α quando comparados com o grupo que inclui os indivíduos sem comprometimento cognitvo e com DCS. Assim como os indivíduos com MEEM alterado apresentaram níveis reduzidos de TNF-α quando comparados com indivíduos com resultados normais de MEEM. Recentemente, Paouri e*t al*. (2017) em um estudo com ratos, observaram que a inibição periférica de TNF-α aumentou a deposição de proteína β-amiloide, corroborando com os nossos resultados. Não foi encontrada diferença nos níveis das outras proteínas (IL-6 e PCR-us). Embora a PCR-us tenha mostrado uma significativa correlação inversa com o comprometimento funcional.

A principal limitação do nosso trabalho é o pequeno tamanho amostral, o que pode ser justificado pelos rígidos critérios de inclusão estabelecidos com o intuito de tornar o mais confiável possível o diagnóstico pré-estabelecido. Dessa forma, tornam-se necessários outros estudos propectivos com maior tamanho amostral para que nossos resultados sejam validados em outras populações.

11 CONCLUSÃO

Os resultados do presente trabalho sugerem que a inflamação, bem como parâmetros hemostáticos, estão envolvidos na fisiopatologia das condições clínicas que cursam com o declínio cognitivo, uma vez que níveis de micropartículas derivadas de leucócitos, neurônio e micropartículas que carreiam fator tissular, bem como IL-6, IL-1β e TNF-α, foram diferenciados entre pacientes com Alzheimer e indivíduos cognitivamente saudáveis. Além disso, embora os níveis de leptina não tenham mostrado associação com a perda cognitiva, os níveis de TNF-a, bem como de micropartículas, estão associados ao declínio cognitivo e funcional, sugerindo serem potenciais biomarcadores para o diagnóstico de estágios iniciais da demência.

12 PERSPECTIVAS

- Separação das MPs por tipo celular e identificação de microRNAs carreados por elas;
- Realização de um estudo prospectivo de acompanhamento dos pacientes com CCL e com DCS, com dosagens seriadas dos marcadores aqui avaliados.

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14 ANEXOS

14.1 Carta de aprovação do COEP

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Projeto: CAAE –09638212.8.0000.5149

Interessado(a): Prof. Paulo Caramelli
Departamento de Clínica Médica
Faculdade de Medicina - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 29 de janeiro de 2013, o projeto de pesquisa intitulado **"Investigação de biomarcadores diagnósticos em pacientes com comprometimento cognitivo leve e doença de Alzheimer"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

14.2 Ficha Clínica

Pesquisa: Avaliação dos parâmetros hemostáticos e inflamatórios em indivíduos com Comprometimento Cognitivo Leve e Doença de Alzheimer

FICHA DE AVALIAÇÃO CLÍNICA

Data: _____

Nome: _____

Sexo: _____ **Idade:** _____(anos) **Número do Prontuário:**_____

- ✓ **Critérios de inclusão:**

1. Pacientes com Comprometimento Cognitivo Leve e provável Doença de Alzheimer entre 50 a 90 anos – diagnóstico pelos médicos do ambulatório de acordo com critérios estabelecidos (NIA/AA (2011) para DA e IWG on MCI (2004) para CCL).
2. Indivíduos do grupo controle – indivíduos sem comprometimento cognitivo (avaliação pelos médicos do ambulatório), pareados segundo sexo e idade com o grupo caso.

- ✓ **Critérios de exclusão:**

1. Insuficiência renal crônica
2. Câncer ou doença autoimune
3. Doenças hepáticas
4. Processo infeccioso/inflamatório atual ou recente (nas 4 últimas semanas)
5. Histórico de infarto agudo do miocárdio (últimos 6 meses)
6. Uso atual de anti-inflamatórios (exceto AAS)
7. Uso atual de anticoagulantes
8. Outras demências que não a DA

✓ **Escolaridade:**

Anos de estudo:_____

( ) 1 º grau completo ( ) 2º grau incompleto ( ) 2º grau completo ( ) Superior Incompleto ( ) Superior Completo ( ) Pós-graduação/Especialização

✓ **Estado civil:**

( ) Casado(a) ( ) Relação estável ( ) Solteiro(a) ( ) Divorciado(a) ( )Viúvo(a)

✓ **Renda:**

No **MÊS PASSADO**, qual foi aproximadamente sua renda familiar **LÍQUIDA**, isto é, a soma de rendimentos, já com descontos, de todas as pessoas que contribuem regularmente para as despesas de sua casa?
( ) Menos de 724 reais (<1 salário)
( ) Entre 724 e 1448 reais (1 <salário<2)
( ) Entre 1448 e 2172 reais (2 <salário<3)
( ) Entre 2172 e 2898 reais (3 <salário<4)
( ) Entre 2898 e 3620 reais (4 <salário<5)
( ) Entre 3620 e 4344 reais (5 <salário<6)
( ) Entre 4344 e 5068 reais (6 <salário<7)
( ) Entre 5068 e 5792 reais (7 <salário<8)
( ) Entre 5792 e 6516reais (8 <salário<9)
( ) 6516 reais ou mais (salário>9)
( ) NÃO SABE/NÃO QUER RESPONDER

Quantas pessoas (adultos e crianças), **INCLUINDO O(A) SR(A),** dependem dessa renda para viver? Se for o caso, inclua dependentes que recebem pensão alimentícia, mas **NÃO INCLUA** empregados domésticos para os quais o(a) Sr(a) paga salário.
|___|___| pessoas
( ) NÃO SABE/NÃO QUER RESPONDER

✓ **Exame Físico:**

FC: _____	PAS: _____	PAD: _____
Peso: _____	Altura: _____	CA: _____
Cálculo do IMC: _____	Circunferência quadril: _____	

Exame Cardiovascular: _____

_____

✓ **Exames Complementares:**

Data:

Hg/HT	Glicemia	CT	HDL	LDL	TG	Outro	Outro	Outro

Alguma vez um médico lhe informou que o (a) Sr(a)/você teve ou tem alguma das doenças abaixo? Quando foi informado (época do diagnóstico)?

( ) Doença de Alzheimer
( ) Pressão alta ou hipertensão arterial
( ) Diabetes (açúcar alto no sangue ou na urina)
( ) Insuficiência renal
( ) Anemia
( ) Insuficiência cardíaca (coração grande ou dilatado)

( ) Colesterol alto
( ) Dores nas costas ou problemas de coluna
( ) Dores nas juntas (artrose ou artrite, reumatismo)
( ) Asma/bronquite
( ) Cirrose
( ) Câncer
( ) Outros:_____

Outras comorbidades relatadas pelo clínico ou constantes do prontuário:

_____

_____

- ✓ **Quantos fatores de risco? _____**

Idade (homem > 55 e mulheres > 65 anos)
Tabagismo
Dislipidemia: triglicérides ≥ 150 mg/dL; LDL ≥ 160 mg/dL; HDL < 40 mg/dL(homens) e HDL < 50 mg/dL(mulheres) – **Quinta Diretriz**
Diabetes Mellitus
Obesidade
História familiar prematura de doença cardiovascular: (homem < 55 e mulheres < 65 anos)
Hipertensão

- ✓ **Condições clínicas associadas: _____**

Doença cerebrovascular (AVE, AVEI, AVEH)
Doença cardíaca (infarto, angina, revascularização coronária, insuficiência cardíaca)
Doença renal (nefropatia diabetic, clearance < 60 mL/mim)
Doença arterial periférica
Retinopatia avançada
Menopausa
Histórico familiar de Doença de Alzheimer

✓ **Medicação** (considere também como remédio qualquer vitamina ou remédio natural)**:**
Tomou alguma medicação de uso regular ou por qualquer outro motivo nos últimos 7 dias? Sim ( ) Não ( )

Medicamento	Apresentação	dose	Número x ao dia	Tempo de uso (meses)	Prescrito por médico ?

Informações provenientes de: ( ) receita/prontuário ( ) verbal do paciente

✓ **Cigarro:**

01. É ou já foi fumante, ou seja, já fumou ao longo da sua vida?	
( ) Não	
( ) Sim	
02. Com que idade começou a fumar?	
|_|_| anos de idade ( ) NÃO SABE/NÃO QUER RESPONDER	
03. Fuma cigarros atualmente?	
( ) Não ----------à	04. Com que idade o(a) senhor(a) parou de fumar pela última vez? |_|_| anos ( ) NÃO SABE/NÃO QUER RESPONDER

( ) Sim
04. Em geral, quantos cigarros por dia fuma (ou fumava)?
|_|_| cigarros
05. Ao todo, durante quantos anos fumou ou fuma? Desconte os períodos em que deixou de fumar.
|_|_| anos ( ) NÃO SABE/NÃO QUER RESPONDER

06. Convive com pessoas que fumam no mesmo ambiente (sala de trabalho, em casa, no automóvel)?	
( ) Sim ----------à ( ) Não	Onde? . Em casa? ( ) Não ( ) Sim . No trabalho? ( ) Não ( ) Sim . No automóvel? ( ) Não ( ) Sim

Carga tabágica: (nº. cigarros por dia/20) * nº anos que fumou.

- ✓ **Consumo de álcool:**

1. Já consumiu bebida alcoólica? ( ) SIM ( ) NÃO

2. Que idade tinha quando começou a consumir bebida alcoólica?_____(anos)

3. Atualmente, consome bebidas alcoólicas? ( ) SIM ( ) NÃO

4. Por quantos anos consumiu/ingeriu bebidas alcoólicas, antes de parar de beber?_____ (anos)

5. No passado, quais os tipos de bebida alcoólica consumia (fazia uso)?
_____
_____
_____

6. Qual era o número usual de drinques por semana, antes de parar de beber?_____

7. SE BEBE ATUALMENTE. Há quantos anos bebe? (Não conte os anos em que não bebeu) _____ (anos)

8. Qual é o número usual de drinques por semana? _____

9. Durante as últimas 24 horas, quantos drinques bebeu? _____

10. No último mês, qual foi o maior número de drinques tomou em um único dia? _____

- ✓ **Eventos:**

1. Nos ÚLTIMOS 12 MESES, esteve hospitalizado(a) por uma noite ou mais, em razão de doença ou acidente?	
( ) [ ] Não	
( ) NÃO QUER RESPONDER	
( ) Sim, uma vez ----------→	. Qual o motivo dessa internação?
	_____ _____ _____ ( ) NÃO SABE/NÃO QUER RESPONDER
	.Quando foi que isso aconteceu? (LEIA AS ALTERNATIVAS)
	( ) Há menos de 1 mês ( ) Entre 1 e 6 meses atrás ( ) Entre 7 e 12 meses atrás ( ) NÃO SABE/NÃO QUER RESPONDER
( ) Sim, mais de uma vez -------→	. Quais os motivos dessas internações?
	_____ _____ _____ _____ _____ _____ ( ) NÃO SABE/NÃO QUER RESPONDER
	. Quando foi a ÚLTIMA VEZ que isso aconteceu?

	(LEIA AS ALTERNATIVAS)
	( ) Há menos de 1 mês ( ) Entre 1 e 6 meses atrás ( ) Entre 7 e 12 meses atrás ( ) NÃO SABE/NÃO QUER RESPONDER
2. Nos ÚLTIMOS 12 MESES, faleceu algum parente próximo (pai, mãe, cônjuge, companheiro(a), filho ou irmão)?	
( ) Não	
( ) NÃO QUER RESPONDER	
( ) Sim, um parente --------→	. Quando foi que isso aconteceu? (LE
	( ) Há menos de 1 mês ( ) Entre 1 e 6 meses atrás ( ) NÃO SABE/NÃO QUER RESPONDER
( ) Sim, mais de um parente ----→	. Quando foi a última vez que isso aconteceu? (LEIA AS ALTERNATIVAS)
	( ) Há menos de 1 mês ( ) Entre 1 e 6 meses atrás ( ) Entre 7 e 12 meses atrás ( ) NÃO SABE/NÃO QUER RESPONDER

- ✓ **Atividade física:**

01. Quantos dias por semana o(a) Sr(a) faz atividade física?	
[ ] nenhum	
|___|___| dias por semana Qual?	02. Nos dias em que o(a) Sr(a) faz essa atvidade, quanto tempo no total elas duram por dia?
	|___|___|___| minutos/dia
02. Se incapacitado, costumava realizar atividades físicas?	

|___|___| dias por semana Qual?	02. Quanto tempo no total elas duravam por dia? |___|___|___| minutos/dia

- ✓ **Estado emocional:**

Escala de Depressão Geriátrica – GDS15	Valor = 1	Valor = 0
1- Está satisfeito (a) com sua vida?	Não	Sim
2- Diminuiu a maior parte de suas atividades e interesses?	Sim	Não
3- Sente que a vida está vazia?	Sim	Não
4- Aborrece-se com freqüência?	Sim	Não
5- Sente-se de bem com a vida na maior parte do tempo?	Não	Sim
6- Teme que algo ruim possa lhe acontecer?	Sim	Não
7- Sente-se feliz a maior parte do tempo?	Não	Sim
8- Sente-se freqüentemente desamparado (a)?	Sim	Não
9- Prefere ficar em casa a sair e fazer coisas novas?	Sim	Não
10- Acha que tem mais problemas de memória que a maioria?	Sim	Não
11- Acha que é maravilhoso estar vivo agora?	Não	Sim
12- Vale a pena viver como vive agora?	Não	Sim
13- Sente-se cheio(a) de energia?	Não	Sim
14- Acha que sua situação tem solução?	Não	Sim
15- Acha que tem muita gente em situação melhor?	Sim	Não
Valor Total (Total > 5 = suspeita de depressão)	_____	

Escala de Cornell de Depressão na Demência – CSDD	2 pontos	1 ponto	0 ponto
A. Sintomas Relativos ao Humor	**Severo**	**Moderado**	**Outros**
1- Ansiedade, expressão ansiosa, ruminações, preocupações			
2- Tristeza, expressão triste, voz triste, choro			
3- Ausência de reação aos eventos agradáveis			
4- Irritabilidade, facilidade em ficar contrariado, humor lábil			
B. Distúrbios do Comportamento	**Severo**	**Moderado**	**Outros**
5- Agitação, não consegue ficar no lugar, se contorce, puxa os cabelos			
6- Lentidão psicomotora: dos movimentos, da fala, das reações			
7- Numerosas queixas somáticas (anotar ausente se apenas sintomas gastrintestinais)			
8- Perda de interesse, menor implicação nas atividades habituais (anotar apenas se a mudança ocorreu de forma rápida, em menos de 1 mês)			
C. Sintomas Somáticos	**Severo**	**Moderado**	**Outros**
9- Perda de apetite, come menos do que usualmente			
10- Perda de peso (anotar severa se superior à 2,5 kg em 1 mês)			
11- Falta de energia, se cansa facilmente, incapaz de sustentar uma atividade (anotar apenas se a mudança ocorreu de forma rápida, em menos de 1 mês)			
D. Funções Cíclicas	**Severo**	**Moderado**	**Outros**
12- Variações de humor durante o dia, sintomas mais acentuados pela manhã			
13- Dificuldades para dormir, dorme mais tarde do que usualmente			
14- Despertar noturno frequente			
15- Despertar matinal precoce, mais cedo do que usualmente			
E. Distúrbios Ideatórios	**Severo**	**Moderado**	**Outros**
16- Idéias de suicídio, pensa que a vida não vale a pena de ser vivida, deseja morrer			
17- Auto-depreciação, se queixa dele próprio, pouca estima de si, sentimento de fracasso			
18- Pessimismo, antecipação do pior			
19- Idéias delirantes congruentes ao humor, idéias delirantes de pobreza, de doença ou de perda			

Valor Total (> 12, provável depressão)	_____/ 38 pontos

PONTUAÇÃO

CUMULATIVE ILLNESS RATING SCALE FOR GERIATRICS (CIRS-G)

Miller, Paradis, and Reynolds 1991

Paciente: _____Idade: _____

Avaliador: _____Data: _____

Instruções: escreva breves descrições do(s) problema(s) médico(s) que justifiquem a pontuação, na linha em frente a cada item. Use como referência o Manual CIRS-G. (Se precisar de mais espaço para escrever, utilize o verso da folha).

Estratégia de avaliação:

0 - Sem problema
1 - Problema leve atual ou problema significante no passado
2 - Invalidez ou morbidade moderada/requer terapia de "primeira linha"
3 - Invalidez significante severa ou constante/problemas crônicos incontroláveis
4 - Extremamente severo/requer tratamento imediato/fase final da insuficiência do órgão /severa dificuldade na função

Sistemas	Descrição	Pontuação
Coração		
Vascular		
Hematopoiético		
Respiratório		
Olhos, orelhas, nariz, garganta e laringe		
Trato gastrointestinal superior		
Trato gastrointestinal inferior		
Fígado		
Renal		
Genitourinário		
Músculoesquelético/tegumentar		
Neurológico		
Endócrino/metabólico e peito		
Doença psiquiatra		

TOTAL DE CATEGORIAS AVALIADAS: _____ PONTUAÇÃO TOTAL: _____
Index de severidade (pont. total/núm. de categorias avaliadas): _____
Número de categorias no nível de severidade 3: _____

Número de categorias no nível de severidade 4: _____

Mini – Mental

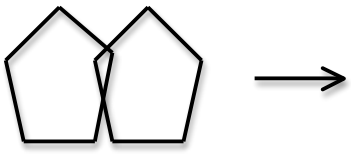
1. Orientação temporal: /10
   - 1.1. Dia do mês:
   - 1.2. Mês:
   - 1.3. Ano:
   - 1.4. Dia da semana:
   - 1.5. Hora aproximada:
   - 1.6. Local específico:
   - 1.7. Local geral:
   - 1.8. Bairro ou rua próxima:
   - 1.9. Cidade:
   - 1.10. Estado:
2. Registro: /3
   - Repetir:
3. Atenção e cálculo: /5
   - 100 – 7 = 93 – 7 = 86 – 7 = 79 – 7 = 72 – 7 = 65
4. Evocação: /3
   - Quais as três palavras perguntadas anteriormente?
5. Linguagem: /8
   - 5.1. Nomear 2 objetos: relógio e caneta /2
   - 5.2. Repetir: "Nem aqui, nem ali, nem lá" /1
   - 5.3. Comando de 3 estágios: /3

   "apanhe esta folha de papel com a mão direita, dobre-a ao meio e coloque-a no chão".
   - 5.4. Escrever uma frase completa: /1

   - 5.5. Ler e executar: /1

FECHE OS OLHOS

6. Praxias: /1
   - Copiar o diagrama em anexo:



Total: /30

14.3 Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

Título da Pesquisa: **AVALIAÇÃO DOS PARÂMETROS HEMOSTÁTICOS E INFLAMATÓRIOS EM INDIVÍDUOS COM COMPROMETIMENTO COGNITIVO LEVE E DOENÇA DE ALZHEIMER**

Unidade: Faculdade de Farmácia da Universidade Federal de Minas Gerais

Pesquisadores responsáveis: Prof. Dr. Paulo Caramelli
Prof. Dra. Karina Braga Gomes Borges
Prof. Dra. Lirlândia Pires de Sousa
Doutoranda Carolina Antunes Magalhães

Endereço: Departamento de Análises Clínicas e Toxicológicas
Faculdade de Farmácia da UFMG. Telefone: (31) 3409-6895
Av. Antônio Carlos, 6627, Pampulha, Belo Horizonte, MG. CEP: 30.270 - 901

LEIA CUIDADOSAMENTE AS INFORMAÇÕES ABAIXO:

Esse documento tem como finalidade convidá-lo a participar no projeto de pesquisa "**INVESTIGAÇÃO AVALIAÇÃO DOS PARÂMETROS HEMOSTÁTICOS E INFLAMATÓRIOS EM INDIVÍDUOS COM COMPROMETIMENTO COGNITIVO LEVE E DOENÇA DE ALZHEIMER**".

Esta pesquisa tem como objetivo estudar algumas substâncias que podem estar presentes no sangue e no líquor ("líquido da espinha") de pacientes com comprometimento de memória ou de outras funções cerebrais relacionadas, tal qual ocorre nas chamadas demências, como a doença de Alzheimer. Estas substâncias podem vir a ser, no futuro, importantes para diagnosticar precocemente estas doenças. Você poderá participar como membro do grupo de pacientes que apresentam comprometimento de memória ou do grupo de indivíduos saudáveis sem comprometimento de memória para comparação com o primeiro.

Em pacientes com doença de Alzheimer ou com outras demências o exame do líquor ("líquido da espinha") é um método que ajuda no diagnóstico, porque algumas substâncias que se acumulam no cérebro podem ser vistas neste fluido. Assim, não é obrigatório que este exame seja feito, mas a sua realização pode ser importante para a confirmação do diagnóstico e para afastar algumas doenças inflamatórias ou infecciosas que causam demência. Caso o(a) senhor(a) concorde com a realização deste exame, precisamos de sua autorização para que um pequeno volume do líquor seja colhido. O líquor ("líquido da espinha") será coletado por um médico neurologista com experiência na realização do exame, em uma sala apropriada do ambulatório de Neurologia do Hospital das Clínicas da UFMG. O procedimento não requer anestesia ou o uso de qualquer medicamento antes ou depois. O(a) senhor(a) ficará consciente e sentirá um desconforto semelhante a uma "picada de agulha". O(a) senhor(a) deverá ficar deitado durante o procedimento e durante o período de observação que será de cerca de uma hora, para evitar o surgimento de dor de cabeça ou tonturas que por vezes podem aparecer após este exame. Se estes sintomas surgirem será garantido atendimento no ambulatório de Neurologia do Hospital das Clínicas da UFMG por um dos médicos pesquisadores responsáveis ou pelo médico neurologista de plantão no Pronto Atendimento do hospital.

O exame das substâncias presentes no sangue, que faz parte desta pesquisa, consiste em uma coleta de amostra de sangue, para a identificação de algumas proteínas que podem estar presentes na doença de Alzheimer ou em outras demências. Caso o(a) senhor(a) concorde com a realização deste exame, precisamos também de sua autorização para que um pequeno volume do sangue seja colhido. A coleta do sangue será realizada por um médico ou enfermeiro com experiência, em uma sala apropriada do ambulatório de Neurologia do Hospital das Clínicas da UFMG. No momento da coleta o(a) senhor(a) poderá sentir um leve desconforto pela picada da agulha. Apesar de raras, podem surgir algumas complicações como, hematomas, sangramentos ou infecção cutânea.

Cabe ressaltar que caso haja necessidade de nova coleta de qualquer uma das amostras e se esta coleta ocorrer em outro dia que não o da consulta de acompanhamento, garantimos os custos com o deslocamento. Informamos ainda que seu prontuário poderá ser consultado para obtenção de dados clínicos a seu respeito, no decorrer da pesquisa.

Os pesquisadores responsáveis assumem a responsabilidade de assistência integral às complicações ou danos decorrentes dos riscos previstos, bem como de informar aos pacientes caso esta pesquisa demonstre algum avanço que seja importante para o tratamento da doença. Estes exames não são obrigatórios, mas a sua realização pode ser importante para a confirmação do diagnóstico. Se o(a) senhor(a) não quiser participar seu atendimento médico no hospital não será prejudicado e suas consultas continuarão a ser realizadas do mesmo modo. O(a) senhor(a) poderá desistir ou retirar seu consentimento a qualquer momento sem nenhum prejuízo para seu atendimento no ambulatório.

BENEFÍCIOS

O(a) senhor(a) não receberá dinheiro ou outro bem material para participar deste estudo.

DIREITOS LEGAIS

A condição acima “Benefícios” não limita os seus direitos legais.

CONFIDENCIALIDADE DOS DADOS

Conforme a Legislação Brasileira, os seus dados somente poderão ser obtidos pelo senhor(a), pelo seu médico e pela equipe do estudo.

O(A) senhor(a) será identificado através de suas iniciais e de um número para garantir a confidencialidade dos seus dados.

INFORMAÇÕES ADICIONAIS

Caso o(a) senhor(a) tenha dúvidas relacionadas ao estudo, contate os pesquisadores responsáveis pelo estudo (Prof. Dra. Karina Braga Gomes Borges ou a doutoranda Carolina Antunes Magalhães) no seguinte telefone: (31) 3409-6895.

Para responder questões relacionadas a essa pesquisa, seus direitos como indivíduo participante e aspectos éticos da pesquisa o(a) senhor(a) poderá entrar em contato com o **Comitê de**

Ética em Pesquisa (COEP) da UFMG, Endereço: Av. Antônio Carlos, 6627, Unidade Administrativa II – 2º andar, Campus Pampulha, Belo Horizonte, MG – Brasil, CEP: 31270-901, tel: (31) 3409-4592, e-mail: coep@prpq.ufmg.br

FORMULÁRIO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Declaro que li as informações acima descritas.
Eu tive tempo suficiente para considerar minha decisão, oportunidade de fazer perguntas e todas as minhas questões foram respondidas.
Entendo que posso modificar minha decisão quanto à autorização de uso de meus dados a qualquer momento, devendo avisar ao pesquisador imediatamente da minha decisão.
Recebi uma via assinada deste Termo de Consentimento.

_____

Nome do participante

_____ _____

Assinatura **Data**

Caso o participante não tenha condições de compreender as informações contidas acima e estiver acompanhado de um representante legalmente aceito, o representante deverá assinar este Termo de Consentimento autorizando a participação no estudo. Se uma testemunha for necessária para leitura do Termo de Consentimento, esta também deverá assinar ao mesmo.

_____

Nome do Representante Legal

_____ _____

Assinatura do Representante Legal **Data**

_____ _____

Assinatura da Testemunha **Data**

ACORDO DO INVESTIGADOR

Declaro que todas as informações necessárias para participação foram esclarecidas ao paciente.
O estudo será conduzido conforme diretrizes e legislação vigente para condução de pesquisa clínica no Brasil.

_____

Nome do Investigador que aplicou o Termo

_____ _____

Assinatura do Investigador Responsável pela obtenção **Data**