ADRIANE DALLANORA HENRIQUES

Expressão diferencial de microRNAs em amostras *post-mortem* de tecido cerebral de pacientes com Doença de Alzheimer

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Adriane Dallanora Henriques

EXPRESSÃO DIFERENCIAL DE MICRORNAS EM AMOSTRAS *POST-MORTEM* DE TECIDO CEREBRAL DE PACIENTES COM DOENÇA DE ALZHEIMER

Dissertação apresentada como exigência para obtenção do Grau de Mestre pelo Programa de Pós-Graduação em Ciências e Tecnologias em Saúde da Universidade de Brasília;

Área de concentração: Mecanismos Básicos e Processos Biológicos em Saúde

Linha de pesquisa: Mecanismos Moleculares e Funcionais da Saúde Humana

Orientador: Prof. Dr. Otávio de Toledo Nóbrega

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Data: 30 de Julho de 2019.

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"The greatest enemy of knowledge is not the ignorance, is the illusion of knowledge".

Sthephen Hawking

Sumário

Lista de abreviaturas

AKT /PKB	Proteína quinase B
ApoE	Apolipoproteína 8
BACE1	Enzima β-secretase 1
CASP8	Capase 8
cDNA	Ácido desoxirribonucleico complementar
CDR	Avaliação Clínica de Demência
CNS	Sistema Nervoso Central
DA	Doença de Alzheimer
dNTP	Desoxirribonucleotídeos
DSM-IV	Manual de Diagnóstico e Estatística de Doenças Mentais
ELOSA	Ensaio de Oligossorção ligado à enzima
FoxO	Proteína Forkhead box
GS3Kβ	Proteínas-quinase glicogênio sintase
HBB-BABSG	Biobanco Cerebral do *Brazilian Aging Brain Study Group*
INSR	Receptor de Insulina
KEGG	Enciclopédia de genes e genomas de Kioto
LCR	Líquido Cefalorraquidiano
MAPK	Proteínas-quinases ativadas por mitógenos
MAPT	Proteína Tau associada a microtúbulos
miRNA/miR	microRNA
mTOR	Proteína alvo da rapamicina em mamíferos
NIA-AA	Instituto Nacional do Envelhecimento e Associação de Alzheimer
PI3K	Fosfatidilinositol 3-cinase

PI3K	Quinase fosfoinositol-3
PIP2	Fosfatidilinositol-4,5-bifosfato
PIP3	Fosfatidilinositol-3,4,5-trifosfato
PMB	cérebro *post-mortem*
PPA	Proteína precursora amilóide
PTEN	Fosfatase homóloga à tensina deletada no cromossomo 10
qRT-PCR	Reação em cadeia da polimerase em tempo real
RNA	Ácido ribonucléico
RNAm	Ácido ribonucleico mensageiro
ROS	Espécies reativas de oxigênio
RT-PCR	Reação em cadeia da polimerase com transcrição reversa
Tau	Proteína Tau
TOMM40	Translocase da membrana mitocondrial externa
βA	Proteína β-amilóide

Resumo

Background: Como um distúrbio neurodegenerativo progressivo caracterizado pela morte de neurônios no sistema nervoso central com consequente deterioração funcional, a Doença de Alzheimer apresenta entre suas características bioquímicas a presença de depósitos extracelulares de proteínas β-amilóides e o acúmulo intracelular de filamentos de proteína Tau hiperfosforilados. Recentemente, microRNAs (miRNAs) emergiram como novos elementos reguladores, com até 70% de todos os miRNAs conhecidos rastreáveis no tecido cerebral, e cuja desregulação possui implicações patológicas. **Objetivo:** Realizamos uma ampla avaliação dos níveis de expressão de miRNAs em amostras de cérebro *post-mortem* de pacientes falecidos com e sem DA. Objetivamos identificar a expressão diferencial de miRNAs em pacientes DA, e assim, correlacionar com o processo fisiopatológico da neurodegeneração. **Métodos:** Por meio de uma plataforma de expressão de microarranjo, avaliamos o perfil cerebral de amostras de cérebros de 8 pacientes com DA e 8 pacientes controle, pareados por idade do óbito e sexo, obtidas do Biobanco Cerebral da Universidade de São Paulo. Identificamos 9 miRNAs potencialmente associados à neuropatologia da DA. Validamos o estudo com uma amostra independente de 18 pacientes controle e 16 pacientes DA com qRT-PCR e ensaios específicos com sistema TaqMan. **Resultados:** As análises resultaram em 6 miRNAs diferencialmente expressos entre os grupos (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-1202; miR-4286; miR-4449). A interação desse conjunto de miRNAs com genes específicos bem como com as vias de sinalização relacionados à DA foi explorada utilizando a ferramenta DIANA (mirPath v.3), resultando em 28 genes alvo possíveis e 11 vias de sinalização após análise via KEEG. **Conclusão:** Nossos resultados sugerem o envolvimento de miRNAs com uma gama de genes (especialmente PTEN) e vias que promovem morte neuronal por sinalização apoptótica, bem como autofagia e dano oxidativo, com ênfase nas vias PI3K-AKT, FoxO, MAPK e p53.

Palavras-chaves

Doença de Alzheimer; microRNA; biomarcadores; DIANA miRPath;

Abstract

Background: As a progressive neurodegenerative, age-related disorder characterized by neuron death of at the central nervous system with functional deterioration, the Alzheimer's disease so far has extracellular amyloid-β deposits and the intracellular filaments of hyperphosphorylated Tau as biochemical hallmarks. Recently, microRNAs (miRNAs) emerged as novel regulatory elements, with up to 70% of all known miRNAs tracked in the brain, and whose dysregulation have pathological implications. **Objective**: we performed a broad assessment on the expression levels of miRNAs in post-mortem brain (PMB) samples of patients deceased with or without AD. **Methods**: A high-throughput microarray expression platform was employed for profiling brains of 8 AD patients and 8 control subjects matched for age and sex, pulled from the University of São Paulo's Brain Biobank, by which 9 miRNAs were identified as potentially associated with the AD-specific neuropathology. Validation qRT-PCR used independent PMB samples from 18 control subjects and 16 AD patients, and specific TaqMan assays. **Results**: All together, these analyses yielded 6 miRNAs differentially expressed between groups (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-1202; miR-4286; miR-4449). The interplay of this set of miRNAs with specific AD-related genes as well as signaling pathways was explored using the DIANA Tools (mirPath v.3), resulting in possible 28 target genes and 11 pathways union among the KEGG analysis. **Conclusion**: Our results suggest involvement of miRNAs with a range of genes (specially PTEN) and pathways that promote neuronal death by apoptotic signaling, autophagy and oxidative damage as well, with emphasis to the PI3K-AKT, FoxO, MAPK and p53 pathways.

Key words

Alzheimer's disease; microRNA; biomarker; DIANA miRPath;

1. Introdução

A Doença de Alzheimer (DA) é uma das doenças mais comuns que atingem a população idosa, assim como é o distúrbio demencial de maior prevalência no mundo (1, 2). Estima-se que um novo caso de DA surja a cada 33 segundos, e que quase um milhão de novos casos são diagnosticados a cada ano (3). Atualmente afeta 35,6 milhões de indivíduos, e esse número tende a crescer para 65,7 milhões em 2030 e para 115,4 milhões em 2050, ou seja, acometerá cerca de 11,8% da população (4). Isso é extremamente importante no contexto do Brasil, já que o nosso país segue o padrão de envelhecimento populacional e consequentemente de casos de demência, o que requer grandes investimentos do governo no assunto.

Estudos investigaram a prevalência e incidência desta doença no contexto brasileiro, utilizando amostras de idosos de base comunitária (5-7). A taxa de incidência foi 7,7 por 1.000 pessoas-ano no estudo de São Paulo (6) e 14,8 por 1.000 pessoas-ano no estudo do Rio Grande do Sul (7). Projeções indicam que a prevalência média no Brasil apresenta-se mais alta que a mundial (8). Na população com 65 anos ou mais, passará de 7,6% para 7,9% entre 2010 e 2020, ou seja, 55.000 novos casos por ano (9).

O custo anual estimado para a demência é de US$ 818 bilhões, o equivalente a mais de 1% do produto interno bruto global. Até 2030, espera-se que esse valor mais que dobre, chegando a US$ 2 trilhões (10), montante que pode prejudicar o desenvolvimento social e econômico e sobrecarregar os serviços sociais e de saúde. O custo anual por pessoa com demência é variável, entre US$ 1.521 nos países de baixo rendimento, US$ 4.588 nos países de médio rendimento e US$ 17.964 em países de elevado rendimento (11).

A DA é uma pandemia relacionada ao processo de envelhecimento populacional. Trata-se de uma doença neurodegenerativa caracterizada pela morte de neurônios no sistema nervoso central (12), resultando em perdas funcionais (13). A progressão clínica da DA, marcada por perda progressiva da memória recente e outros comprometimentos cognitivos e comportamentais, culmina com incapacidade permanente para realizar atividades básicas e instrumentais da vida diária (14). Em seu processo patológico, a DA usualmente caracteriza-se pela presença no córtex cerebral de placas senis constituídas por depósitos extracelulares da proteína β-amilóide assim como por emaranhados neurofibrilares intraneuronais da proteína Tau hiperfosforilada (15-19).

Até o presente, a DA não pode ser diagnosticada enquanto o fenótipo de demência não for clinicamente estabelecido (15), e ainda, não há tratamentos para mudar o curso progressivo desse distúrbio cerebral (15, 20, 21). Apesar disso, algumas mudanças celulares possivelmente associadas à doença podem ser identificadas anos, e até décadas, antes da apresentação dos primeiros sinais de perda de memória e/ou desvio de comportamento (22-25). A partir desses achados, pesquisas tem intensificado a investigação de novos biomarcadores para DA capazes de identificar alterações fisiológicas que possibilitem predizer risco de acometimento pela doença, inclusive em pessoas sem sintomas (26, 27). A detecção pré-sintomática da DA é crucial e pode facilitar a implementação de tratamentos eficientes, assim como, modificar o curso natural da doença. Além da importância no diagnóstico, novos biomarcadores tem sido estudados para compreender melhor os aspectos fisiopatológicos relacionados à desordem.

Diversos ensaios sobre biomarcadores estão atualmente sendo conduzidos e, neste contexto, destacam-se aqueles com foco sobre elementos de microRNAs (miRNAs). MiRNAs tem emergido como uma nova classe de elementos de regulação gênica, com função central no desenvolvimento fisiológico e de patologias (28, 29).

MiRNAs são caracterizados como um grupo de pequenos RNAs de cadeia simples não codificadores de proteínas, com 20 a 22 nucleotídeos de comprimento (12). Essa variedade de RNA destaca-se pela grande habilidade de modular uma complexa rede regulatória de expressão gênica e desempenha papel importante na regulação a nível pós-transcricional do genoma (30-32). Atuam sobre genes-alvo usualmente por ligação à região 3’ não traduzida do RNA mensageiro e consequente inibição da sua tradução ou a degradação da região alvo. Os miRNAs regulam mais de 50% de todos os genes codificadores de proteínas implicando consequências em todos os processos biológicos, incluindo inflamação, proliferação e apoptose (33-35).

Mais de 70% de todos os miRNAs conhecidos podem estar presentes nos tecidos cerebrais, e evidências da literatura enfatizam que os miRNAs tem papel crucial na regulação neuronal e nas funções da glia (36). MiRNAs tem sido descritos como os principais reguladores da homeostase neuronal e a desregulação desses pode resultar em condições patológicas cerebrais (31). Em geral, a maioria dos miRNAs expressos no tecido cerebral tem se mostrado como chaves em processos de regulação das funções

biológicas neuronais, como plasticidade neuronal, neurogênese e diferenciação neuronal (12, 37).

Estudos tem demostrado que a desregulação da expressão gênica causada por alterações na expressão de miRNAs tem implicações em doenças neurodegenerativas (38, 39). Evidências sugerem que até uma pequena alteração na atividade de miRNAs pode causar detrimento na função neuronal (36, 40, 41). MiRNAs são associados com doenças neurodegenerativas como a DA e são reguladores de muitos aspectos relacionados a sua patogênese (29, 38, 42). Alinhado a isso, muitos miRNAs tem sido identificados como relevantes por alterar a expressão ou a função de moléculas relevantes para a patogênese da DA, como a proteína precursora amilóide (PPA) (43), a enzima de clivagem da proteína β-amilóide (44) e a proteína Tau (45, 46).

Há cerca de uma década foram iniciados estudos em pequena escala de perfis para fornecer as primeiras pistas sobre mudanças de produção cerebral de miRNA em DA (47). Desde então, vários grupos têm realizado estudos genômicos em maior escala que demonstram padrões diferenciais de expressão de miRNA não só apenas no tecido cerebral como também em biofluidos (12, 48-50).

No contexto patológico da DA, tem sido demostrado que o perfil de expressão de miRNAs em amostras de cérebro obtidos de substância branca de pacientes diagnosticados com a patologia mostra-se diferente do encontrado em tecidos saudáveis de pacientes controle (51). Dessa forma, após realizarmos uma ampla avaliação dos níveis de expressão desses miRNAs no tecido cerebral *post-mortem* de pacientes que vieram a óbito com e sem DA, nos propusemos a identificar os miRNAs expressos diferencialmente nos pacientes doentes. Ainda, pretendemos estabelecer a relação entre os miRNAs desregulados com alterações na fisiologia cerebral relacionados à patologia. É importante ressaltar que a fisiopatologia da DA ainda não foi completamente elucidada, portanto, estudos que possibilitem um maior entendimento do processo neurodegenerativo são informativos e podem permitir possibilidades de rastreio, de diagnóstico ou ainda terapêutico a longo prazo (52). Atualmente o diagnóstico com precisão da DA somente é feito após exames post-mortem (53).

2. Artigo Científico

Genome-wide expression and predicted significance of *post-mortem* brain microRNA profiling in Alzheimer's disease.

Short title: microRNA expression in AD brains

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Author Contributions

All authors contributed to the study conception, design and commented on the first version of the manuscript. All authors read and approved the final manuscript. Sample preparation and clinical procedures were executed by R.E.P. Leite, L.T. Grinberg and C.K. Suemoto. Microarray processing and validation assays were performed by A.D. Henriques and W. Machado-Silva. Advising on study execution was performed by K.R.M. Leite, M. Srougi and Wilson Jacob-Filho. Data analysis and interpretation of results were performed by A.D. Henriques and O.T. Nóbrega.

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Abstract

Background: As a progressive neurodegenerative disorder characterized by neuron dysfunction and death at the central nervous system, Alzheimer's disease (AD) has extracellular amyloid-β deposits and intracellular hyperphosphorylated Tau as biochemical hallmarks. MicroRNAs (miRNAs) emerged as novel regulatory elements, with up to 70% of all known miRNAs tracked in the brain, and whose dysregulation have pathological implications. **Objective**: to perform a broad assessment on the expression levels of miRNAs in *post-mortem* brain (PMB) samples of patients deceased with or without AD. **Methods**: A high-throughput microarray expression platform was used to profiling brain samples of AD cases with senil plaques confirmed by neuropathological examinations and compare to control samples matched for age and sex, pulled from the University of São Paulo's Brain Biobank. The miRNAs identified as potentially associated were subjected to validation with specific TaqMan qRT-PCR assays employing independent PMB samples. **Results**: All together, these analyses yielded 6 miRNAs differentially expressed (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-1202; miR-4286; miR-4449). The interplay of this set of miRNAs with specific AD-related genes as well as signaling pathways was explored using bioinformatics analyses (including the KEGG package, mirPath v.3), resulting in possible 28 target genes and 11 pathways. **Conclusion**: Our results suggest involvement of this set of miRNAs with a range of genes (notably PTEN) and pathways (emphasis to the PI3K-AKT) to explain neuronal death by apoptotic signaling, autophagy and/or oxidative damage, among other potentially involved genes and signaling routes.

Key words: Alzheimer's disease; microRNA; array analysis; DIANA miRPath.

Introduction

Alzheimer's disease (AD) is one of the most common illnesses of later life as well as the first cause of dementia worldwide [1] and currently affects 35.6 million individuals with such number expected to increase to 65.7 million in 2030 and to 115.4 million in 2050 [2]. AD has become pandemic as the population continues to age, with no known cure or widely effective prevention measures presently [3, 4]. As a multifactorial, age-related neurodegenerative disorder characterized by death of neurons at the central nervous system that unfolds into functional deterioration [5], AD's biochemical hallmarks are the extracellular amyloid-β (Aβ) deposits (senile plaques) and the intracellular arousal of filaments of hyperphosphorylated Tau (neurofibrillary tangles) [6, 7].

Recently, micro-RNAs (miRNAs) have emerged as a novel class of gene regulatory elements with conserved roles in development and disease. Studies have shown dysregulation of gene expression due to altered expression of miRNAs in the physiopathological context of neurodegenerative diseases [8-10]. MiRNAs are a class of small non-coding RNAs of 20–22 nucleotides in length that regulate the genome at the posttranscriptional level [11, 12], affecting expression of more than 50% of all protein-coding genes and implicated in all biological processes [13]. Regulation of gene expression by miRNA is usually achieved by its binding to the 3' untranslated region of target mRNA(s), with due cleaving or inhibition of its translation [14, 15].

Up to 70% of all known miRNAs could be tracked in the brain and a growing body of evidence emphasizes on miRNAs as crucial regulators of neuron and glial functions [16, 17], being described as main regulators of neuronal differentiation, patterning, homeostasis and plasticity along with synaptogenesis [18-21] and involved in the pathophysiology of different disorders in the brain [11, 22]. Evidence suggests that even slight aberrations in miRNA activities can be detrimental to neuronal function [23-25]. In line, miRNAs are associated with the AD pathogenesis [11, 26, 27], with several miRNAs affecting the expression of AD-relevant molecules such as the amyloid precursor protein (APP) [28], the β-site amyloid precursor protein cleaving enzyme 1 (BACE1) [29] and the Tau protein [30]. Nonetheless, the precise causative elements underlying the AD-related biochemical flaw are not completely known or understood [3] and the pathophysiology of the condition is yet to be elucidated. At present, autopsy is still necessary to diagnose AD accurately [31].

Understanding the main causes of cell dysfunction and death in many neurodegenerative diseases is a challenging task that might also provide important insights into the mechanisms of neurodegeneration.

In this pathological context, it has been demonstrated that the miRNA expression profile in white matter-enriched brain samples is altered from the pattern found in the normal tissue [32]. Herein, we propose to perform a broad assessment on the expression levels of these miRNAs in the post-mortem brain (PMB) of patients deceased with or without AD, and to establish relationships with the pathophysiology of the disease.

Materials and methods

Study population

In the present study, 50 specimens collected at the Human Brain Bank of the Brazilian Aging Brain Study Group (HBB-BABSG) of the University of São Paulo Medical School, from 2005–2012, were studied [33]. Tissue donations were obtained in the municipal São Paulo Autopsy Service, in Brazil. Exclusion criteria included age at death of less than 50 years, causes of death or tissue condition that impeded neuropathological analysis, informants without knowledge on the functional status of subjects and violent/criminal deaths. All tissue donations were necessarily made by a next-of-kin after providing informed consent, and the study was approved by the institutional review boards of all participating institutions.

Neuropathological assessment

The clinical diagnosis was made in a consensus meeting with the participation of gerontologists and of one neurologist with expertise in dementia, using all of the available information. At the brain bank, the diagnosis of dementia was based on DSM-IV criteria. Cognitive status was assessed *post-mortem* using information provided during the clinical interview with the next-of-kin. The Clinical Dementia Rating (CDR) scale was used to evaluate cognitive function [34]. In the control group, donation was done from individuals to whom no signs of cognitive impairment (CDR 0) were described prior to death. The present study included subjects with severe dementia (CDR $\geq$ 2) summed to an equivalent number of paired control cases, matched for age and sex.

Neuropathological procedures

Clinical and neuropathological examinations were performed in a blinded manner, and neuropathological diagnoses were made by two experienced neuropathologists. Brains were inspected

macroscopically, and lesions of Alzheimer's disease (senil plaques and neurofibrillary tangles) were scored according to the accepted criteria [35]. Control cases enclosed only subjects lacking such neuropathological findings. For this study, post-mortem tissues were typically harvested from superior and middle temporal gyrus (Brodmann Areas 21/22), and archived and stored at − 80 °C, typically after being snap frozen in cryogenic liquid. The mean timespan for post-mortem harvesting of the samples used was of 10.4 h (range of 4–20h).

MicroRNA Isolation from post-mortem tissues

MiRNA was isolated from 100 mg samples of snap-frozen brain tissue using mirVana™ PARIS™ Kits (Life Technologies, Lithuania), used according to the manufacturer's recommendations with one slight modification in the protocol consisting of one additional step of organic extraction with Acid Phenol:Chloroform after tissue lysis. MiRNA quality was confirmed using the A_{260}/A_{280} ratio with NanoDrop Lite Spectrophotometer (Thermo Scientific™, China). For further assays, only samples with A_{260}/A_{280} ratio within 1.9 – 2.1 were performed. Samples were stored at -20°C until further analysis.

Microarray methods

The microarray expression plataform chosen was the *Applied Biosystems miRNA Array kit*® by Thermo Fisher Scientific. This high-density array provides a sensitive measurement of a wide range of small non-coding RNA transcripts, including complete coverage of miRBase v20 encompassing 2,578 mature and 2,025 imature human miRNAs. For the direct labeling of the miRNAs, the *FlashTag™ Biotin HSR Labeling Kit* (Thermo Fisher Scientific) was used for 16 samples (8 from AD and 8 from control samples) following the manufacturer's recommendations. To confirm whether biotinylation of microRNA samples occurred properly, an Enzyme Linked Oligosorbent Assay (ELOSA) was used to specifically detect the binding of biotinyled samples to spike, complementary control RNA oligos adsorbed to a Nunc® plate.

Hybridization and capture of the fluorescence spots was performed with the *GeneTitan*™ instrument (Thermo Fisher Scientific), with the *Array Strip Hybridization*® (Thermo Fisher Scientific) kit as support. To control the microarray procedure, the *GeneChip*® Expression 3'-Amplification Reagents Hybridization Control™ (Thermo Fisher Scientific) was used. The results obtained in *GeneTitan*™ were analyzed for quality purposes using the *Expression Console*™ software, version 4.1 (Thermo Fisher

Scientific) (Figure 1). The standard data exported by *Expression Console*™ (CPH.) was processed in the *Transcriptome Analysis Console*™ software version 3.1 (Thermo Fisher Scientific), which allows statistical analysis to obtain a list of differentially expressed genes. This latter applied the unpaired Student's *t*-test to identify miRNAs differentially expressed between sample pairs and probes, set at $p < 0.05$ and fold-change values ≥ 65% as parameters to define miRNAs with different mean concentrations across samples, with the requirement that the miRNA was expressed by a minimum of 75% of all participants of at least one of the groups.

Validation of differential expression by RT-PCR

To validate the previous microarray results, we have performed quantitative RT-PCR with 16 DA and 18 control independent samples. Specific quantitation of each microRNA indicated by the array technique used commercial *TaqMan*® qRT-PCR assays (Applied Biosystems, Foster City, CA, USA). The *TaqMan*® MicroRNA Reverse Transcription Kit was used to prepare complementary DNA (cDNA) with each reaction containing 10 ng of total post-mortem brain RNA. Briefly, reactions were set up with 1 ng/μL of RNA, 0,5X stem-loop RT primer, 3.33 U/μL reverse transcriptase, 0.25 U/μL RNase inhibitor, 0.25 mM dNTPs, and 1X reaction buffer in a total reaction volume of 10 μL, being incubated at 16°C for 30 min, and 42°C for 30 min, and 85°C for 5 min in a thermocycler.

Following the RT step, 4 μL of the RT reaction was combined with 0.5 μL of a 20X TaqMan MicroRNA Assay (containing forward primer, reverse primer, and probe) and 5 μL of TaqManR Universal PCR Master Mix (without UNG for cycling) completed with ultra-pure water to another 10-μL final volume. All reactions were performed on the QuantiStudio 3 Thermal Cycler (Applied Biosystems, CA, USA) in its standard mode.

To evaluate the efficiency of each microRNA assay and the endogenous reference, standard curves were developed with serial, 10-fold dilutions (from $1:10^0$ to $1:10^{-5}$) of known miRNA concentrations, with reactions performed as described above. From each standard curve, a linear regression analysis was performed to yield the correlation coefficient (R^2) and the slope value. Efficiency (E) of each reaction was assumed as $= 10^{(-1/slope)} - 1$, with values of 1 meaning efficiency of 100% [36].

The total tissue level of each miRNA was evaluated by normalization to levels of a small nucleolar RNA (RNU43), member of a family that has been proven useful to normalize expression of miRNAs from post-mortem brain tissue samples [37]. The relative quantification was determined by the $2^{-\Delta Ct}$ method,

where $\Delta Ct = (Ct_{miRNA} - Ct_{endogenous})$ [36]. All reactions were performed in duplicate, determining the mean value of the amplification threshold (CT) of each sample, with amplification of targets and internal control run alongside to avoid inter-assay variation. The data from amplifications were obtained from QuantStudio™ Design and Analysis Software v1.4.3.

Statistical analysis

Initial exploratory analyzes of the data evaluated the normality of distribution of the discrete dependent variables, using the Kolmogorov-Smirnov test. Where appropriate, the data was expressed as averages ± standard deviation, medians (with interquartile range) or frequencies (%). The Student's t-test was used for parametric data whereas for the non-parametric measures, the Mann-Whitney test was used. Frequencies were tested with the chi-square test.

Beyond the statistical steps described to test the microarray and validation outputs, correction of these latter analyses for the multiple investigations was done by determining *p* values in analyses adjusted using the false discovery rate of each miRNA in univariate analysis of covariation [38]. All analyzes were performed using the Statistical Package for Social Sciences (SPSS) for Windows (version 17.0). An association was considered significant assuming p value <0.05.

Results

The present study used brain samples from subjects with *post-mortem* diagnosis for AD (including senil plaques under neuropathological examinations) as well as from counterparts matched for age and sex and rendered as control cases based on absence of AD-related neuropathological finding and devoid of report of cognitive decline whilst alive. We identified specific miRNAs differentially expressed between samples that conform into a signature of the disease stage. Our first analysis consisted in the miRNA profiling phase by means of the hybridization procedure. Results from this microarray analysis appeared robust due to expected levels and homogeneous pattern of expression signals in a sample-to-sample comparison. In this context, we identified 9 miRNAs potentially associated with the AD-specific neuropathology (Figure 2).

It is noteworthy that the miRNA expression pattern of two AD cases happened to cluster separately from their mates in the heatmap. Even so, the Euclidean-based hierarchical clustering allowed discriminating the miRNA repertoire expressed in the tissues of AD and non-demented subjects, mostly

relying on the relative underexpression observed within the pathological samples. To confirm the elements with expression most different between groups, the miRNAs indicated by the array we validated using specific qRT-PCR assays with independent, array-unrelated samples from other 18 control subjects and 16 AD patients, with results shown in Table 1. Six miRNAs proved themselves differentially expressed, with five being importantly downregulated among AD patients (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-4286; miR-4449), whereas the miR-1202 alone was up-regulated in the pathological samples, in straight agreement to each miRNA's individual profile in the clustering map.

Since no prior reports support a relationship between this miRNA signature and AD, we sought to understand this possible interplay by means of bioinformatics analyses. To evaluate whether the miRNAs could play a role as regulators of AD-related genes, the search mode of the prediction tool www.mirdb.org [39, 40] was used to yield all possible targets of those six microRNAs, and each list was checked across AD-relevant genes derived from genomic wide association studies (www.alzgene.org) [41, 42]. Based on this strategy, we unraveled a handful of AD-related gene candidates, as depicted in Table 2.

Subsequently, the whole set of differentially expressed miRNAs (not each individually) was taken into account in another analysis to reveal signaling pathways possibly affected by this scenario of miRNA deregulation. This was done using the TarBase v7.0 mode of the KEGG analysis package (DIANA Tools (mirPath v.3) [43] set for human elements, what resulted in 28 target genes implicated and 11 pathways affected. When using their likely target genes (from Table 2) as filters in the same analysis, associated genes and pathways were narrowed down to an absolute count of 7 targets and 11 signaling routes, and significantly implicating only three (miR-30e_3p; miR-365b_5p; miR-4286) out of the six deregulated miRNAs, as depicted in Table 3.

Discussion

Our investigation scanned miRNAs from samples of *post-mortem* brain tissue employing a high-throughput array technique in order to assess candidate signature(s) related to the Alzheimer's Disease. Despite being a technique that presents some limitations and raises questions regarding accuracy, precision and specificity [44, 45], microarrays display importance as valuable informative strategy for understanding diseases [46]. Furthermore, microarray technology has improved in the past 4 years, including the addition of many newly annotated miRNAs [47].

From six differentially expressed miRNAs found by our study, five were shown down-regulated (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-4286; miR-4449) whereas one as up-regulated (miR-1202) in AD samples. To our knowledge, this investigation is the first to implicate this set of miRNAs all together with the disease, even though a few of these elements (miR-365b_5p [22]; miR-664_3p [48] and miR-4449 [49]) have been individually associated. We now discuss how these 6 miRNAs are linked to important pathways that contribute to the disease pathophysiology. In general terms, this overall down-regulation of miRNAs may lead to an overexpression of amyloidogenic and taupathic genes so to yield a pathological status. From the miRNA set unveiled here, namely the miR-30e_3p, -365b_5p and -4286 have been demonstrated to control genes whose dysregulation relates directly to the AD's pathophysiology, boosting precisely amyloidogenesis and taupathy. However, knowledge on the direct impact of these miRNA on the regulation of AD-related genes remains to be extended.

First of all, it is important to considerer that the National Institute on Aging and Alzheimer's Association (NIA-AA) in 2018 updated guidelines to define AD as a pathophysiologic construct by establishing the proposed ATN classification that consider Aβ deposits (A), pathologic Tau (T) and neurodegeneration (N) as hallmarks of the condition, with A+ statuses as pivotal grounds [6, 50]. Accordingly, the AD samples used herein derived exclusively from patients with confirmed amyloidosis in histopathological examination, therefore consistent with the A+ phenotype of the new NIA-AA guideline. Possibly due to this homogeneity in phenotype, it does not surprise that the miRNAs indicated by our methods relate to pathways that contribute to the onset of amyloid β plaques and P-tau.

Out of the 6 miRNAs confirmed as differentially expressed, the 3 miRNAs most likely involved as pointed out by bioinformatic analyses (mir-30e-3p, -365b-5p and -4286) relate with cell death mechanisms (as the PI3K-AKT, FoxO, MAPK and p53 signaling pathways) that contribute to neurodegeneration [51-54]. The literature indicates, for instance, the involvement of multiple miRNAs in the regulation of genes as PTEN [55], INSR [56], PRKAA1 [57], SOD2 [58] and MAPT [59], all genes involved in neuronal death by apoptotic signaling, autophagy and/or oxidative damage, processes that remarkably account to the AD-related neuropathy [60-62]..

In this complex scenario, the Phosphatase and Tensin Homolog on Chromosome 10 (PTEN) gene, characterized as a ubiquitous modulator of cell growth and proliferation [63], stands out from the analysis with the KEGG package by being implicated in 7 of the 11 AD-related pathways disclosed therein. Due to its lipid phosphatase activity, PTEN produces lipid-signaling molecules whose dysregulation has

been implicated not only with cancer but with a wide variety of non-oncogenic illnesses, including Alzheimer's disease [64]. Deregulation of PTEN-AKT signaling pathway has been implicated with AD, with prior report on mRNA and protein expression having demonstrated elevated PTEN levels in human AD brains [62, 65].

Despite that the actual role of PTEN in AD pathogenesis was not fully elucidated, a hint raised by Knafo *et al*. [64] shows that PTEN silencing reduces β-amyloid aggregation and prevents Alzheimer-related cognitive decline in animals. PTEN regulates neuronal survival processes by arresting cell cycle and triggering apoptosis by antagonizing the PI3K-AKT pathway in the CNS [66, 67]. Studies shown that suppression of PTEN expression provides neuroprotection [68, 69], particularly of hippocampal cells against apoptosis [70]. Among possible venues, attenuation of the PI3K-AKT signaling pathway seems the most likely route [71], in line with our KEGG outputs and with evidence from which the PI3K-AKT signaling is suppressed in the brains of AD patients [52, 72]. To the author's best knowledge, it is plausible that the down-regulated miR30e-3p might allow an overexpression of the gene (see target prediction on Table 2), yielding a state compatible with the onset of the disease. Therefore, we hypothesize that exuberant PTEN action due to the suppressed miR30e-3p may contribute to a PI3K-AKT-deficiency-mediated process of neurodegeneration in Alzheimer's disease.

Apart from the PI3K-AKT signaling pathways, PTEN also interact with other important routes for neurodegeneration, as the FoxO pathway for cell cycle arrestment [73] and p53 activation to induce ROS generation [74], both pinpointed by our bioinformatics analyses. On what concerns the latter, PTEN and p53 interact by mutual regulation at transcriptional and translational levels [75, 76]. Regardless of how neuronal injury is triggered, p53 is highly elevated in a context of PTEN-enriched AD neurons [54], leading to cell death [77]. Also, study in animals has demonstrated that PTEN knockdown leads to a pronounced decrease in the cellular level of ROS and prevention of neuronal apoptosis [70]. All things considered, an interplay between the mir-30e-3p and Alzheimer's based on a PTEN-centered exacerbation of relevant apoptotic, autophagic and/or oxidative pathways appear plausible based on the rationale above. Afterall, the mir-30e-3p predominates in the output of our KEGG analyses, being implicated in 9 of the 11 pathways. The relationship of the miR-30 family with neurodegeneration was already described elsewhere. Nunez-Iglesias [78] pointed out that miR-30e-5p is down-regulated in lobe cortex of disease patients [10]. In line with this body of evidence for a miR30e effect on neuropathological conditions, the miR30e-3p was shown

to be up-regulated in Parkinson's Disease serum [49]. In brief, elements of the miR-30e family have been related to neurodegenerative diseases so far, however by mechanisms not yet fully understood.

Another fact that relates PTEN to the neuropathology of AD is the finding that its suppression markedly reduces AKT-GSK3-mediated Tau hyperphosphorylation yielding a pro-survival effect [65]. However, the literature is less abundant in evidence for a PTEN contribution to Tau phosphorylation whereas dysregulation of the actual MAPT gene expression rate is more likely to affect Tau metabolism. Some studies indicate that miRNA affect Tau production and processing, potentially contributing to several neuropathological features of AD [79-82]. In regard to this, our study found out that the miR-365b-5p is downregulated in AD brains and likely to regulate MAPT as indicated by the KEGG analyses. An overall reduction in miRNA production in Dicer-knockout mouse brains induces a severe neurodegenerative phenotype with altered Tau by either an alternative processing of the MAPT gene or an AD-resembled Tau hyperphosphorylation phenotype [83, 84]. Another study with Dicer-knockout mice [81] found that MAPK signaling pathways (ERK1/MAPK3 and ERK2/MAPK1) act as major regulators of neuronal Tau phosphorylation *in vivo*, settling with our bioinformatics outputs in which the MAPT gene and the MAPK routes interrelate.

The MAPT gene has a relatively large (4.2 Kb) 3' UTR with multiple conserved predicted miRNA recognition sites [85], including for miR-365b-5p. Even though MAPT is regulated post-transcriptionally, it is not known whether miRNA play a direct role, and we hypothesize that miR-365b-5p can regulate human Tau via its 3'UTR so to prevent an overexpression of Tau under physiological conditions. In line, down-regulation of some miRNA as the miR-9 and miR-124 have shown to contribute to an altered Tau composition in AD brains [79]. But one should not ignore that a number of Tau kinases (including GSK3β and JNK/MAPK8) are targets for miRNA regulation as well, as demonstrated in various cell types [86, 87].

Also from our analysis, the miR-4286 was found down-regulated in AD samples, and implicated the insulin receptor (INSR) gene. The involvement of INSR in AD has long been described [88-90], and relates to the fact that $A\beta_{42}$ peptides directly compete with insulin for binding [91, 92], blocking the receptor and provoking insulin resistance and glycemic unbalance that reduces efficiency in the CNS and impairs cellular clearance of the neurotoxic $A\beta_{42}$ oligomers [93]. Compensatory upregulation of INSR expression have been shown in cerebral regions as diverse as the hippocampus and the entorhinal cortex of AD patients [94-96], having also been observed in peripheral samples of prodromal as well as of fully symptomatic AD carriers [95, 97]. Also, significant alteration in mRNA expression of genes related to insulin signaling in

the cortex and hippocampus has been described [98]. Decreased insulin action leads to dephosphorylation and activation of glycogen synthase kinase GSK3β, the main responsible for Tau hyperphosphorilation, as expected in a context of inhibition of the PI3K-AKT pathway [99]. This way, it is well known that altered insulin signaling in the brain leads to appearance of classic AD hallmarks [92] and that some critical cell survival pathways in neurons are regulated by the activity of INSR [100-102].

A recent meta-analysis implicated the miR-4286 with Parkinson's neurodegeneration, but without proposing a role in the pathophysiology [103]. In line, different reports describe age-related reduction of miRNAs predicted to target INSR expression during the mouse brain aging [104, 105]. Therefore, a possible causal effect of the under-expressed miR-4286 in the adaptative INSR overexpression in AD cannot be discarded. Since this enhancement does not necessarily translate into efficient signaling due to INSR blockage by Aβ oligomers, it is conceivable that such biochemical setting may account to inhibit the PI3K-AKT pathway so to trigger neuronal death [92].

We should not ignore the other miRNAs revealed by our study, but much is yet to be unveiled about their basic biology. To name a few of these candidates with solid grounds to play roles in the AD onset, the miR-1202 (which is specific of primates, enriched in the human brain [106] and associated with the pathophysiology of depression [107]) is known to regulate genes involved with glutamatergic, dopaminergic, GABAergic and serotonergic neurotransmission [108, 109]. Also, this miRNA regulates the ATP-binding cassette transporter A1 (ABCA1) gene, that encodes a pump of cholesterol and phospholipids from cells to lipid-poor ApoE-containing lipoprotein particles, as to form HDL. ABCA1 deficiency was shown to increase amyloid deposition and memory deficits in different AD model mice, whereas transgenic mice overexpressing ABCA1 in brain have fewer amyloid plaques [110]. Up-regulation of miR-1202 (as observed herein) could contribute to the scenario.

On its turn, the miR-4449 was also related with neurodegenerative conditions as Huntington's disease and Schizophrenia [4, 111]. In line, Denk *et al*. [22] identify miR-4449 as down-regulated in CSF samples of AD patients, finding its level in correspondence to those of the $A\beta_{42}$ peptide, total Tau and phospho-Tau, allowing to hypothesize the miR-4449 as candidate biomarker for AD. The gene TOMM40 (Translocase of the Outer Membrane of 40), which has been related to late-onset AD [112-114], is targeted by the miR-4449. TOMM40, adjacently-linked to ApoE in chromosome 19, is associated with the AD onset and progression possibly by affecting vicinal transcription or by interacting in a complex way with apoE isoforms [115, 116]. Also, TOMM40 codes for an essential mitochondrial protein, suggesting that

mitochondrial integrity and energy metabolism could play an important role in pathophysiology of AD [117]. APOE and TOMM40 may interact to affect aspects of mitochondrial function although mechanistically it is unclear.

Lastly, Burgos *et al.* [49] have profiled the miRNAs from CSF and serum of AD patients and observed correlation of miR-664b-3p expression with aspects of disease severity such as Braak stage, but not with plaque density or neurofibrillary tangle scores. MiR-664b-3p was associated with signaling pathways related to FoxO, MAPK, and mTOR [118]**,** implicating this miRNA with outcomes such as apoptosis. Not surprisingly, one target of miR-664b-3p is CASP8, the apoptosis-related cysteine protease and known genetic risk factor for AD [119, 120]. In addition to its role in amyloid processing [121], caspase-8 and its downstream effector caspase-3 are involved in synaptic plasticity, learning, memory and control of microglia pro-inflammatory activation, indicating additional mechanisms that might contribute to AD [122].

Among the limitations of our study, we can cite the lack of assays to confirm the relationships indicated herein between the miRNAs, target genes and signaling pathways, mostly interpreted out of the operation of bioinformatic tools/databases and of the appropriate literature. In addition, no previous calculation on sample size was performed to evaluate the statistical power of our analyses. A larger sample in the microarray procedure could have evidenced miRNAs with smaller effect size but also relevant to the AD pathogenic process.

Conclusion

Being abundant in brain and highly stable in biofluids as CSF, it is settled that the miRNA content influences the onset and progression of neurodegenerative diseases. This exploratory investigation takes an approach to detect miRNA differentially expressed in the typical A+ phenotype of the Alzheimer's disease, with six miRNAs identified as deregulated in the advanced stage of the disease. Based on the literature available on these miRNAs and their known and possible target genes, our work point out to a number of signaling pathways in the neuronal milieu (mainly the PI3K-AKT, FoxO, MAPK and p53 pathways) and hypothesize on plausible avenues of dysfunction and neurotoxicity with connection to the entity's neurochemistry. But, the actual role of these miRNAs as players for the AD onset needs to be assessed by well-controlled studies of experimental nature whereas the utility of these elements as disease markers

demand future, well-powered work with larger sample sizes to ensure detection of miRNAs with smaller effect sizes.

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Conflict of interest

The authors declared that there is no conflict of interests.

Ethical approval

All procedures performed in studies involving human participants were in accordance with ethical standards of the institution and/or national research committee (Ethics Committee on Research, São Paulo University number 1.072.651/2015) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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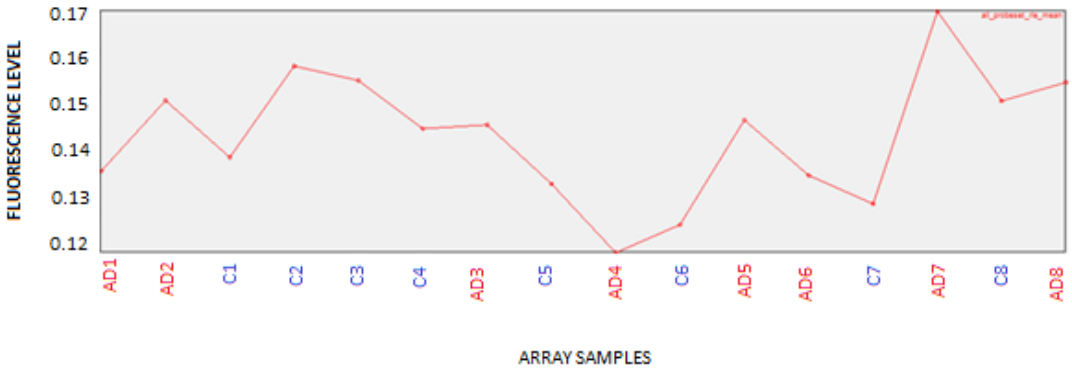

Figure 1. Result from *Expression Console*™ software version 4.1 analysis showing homogeneity from samples in the microarray. AD, Alzheimer's disease; C, control.

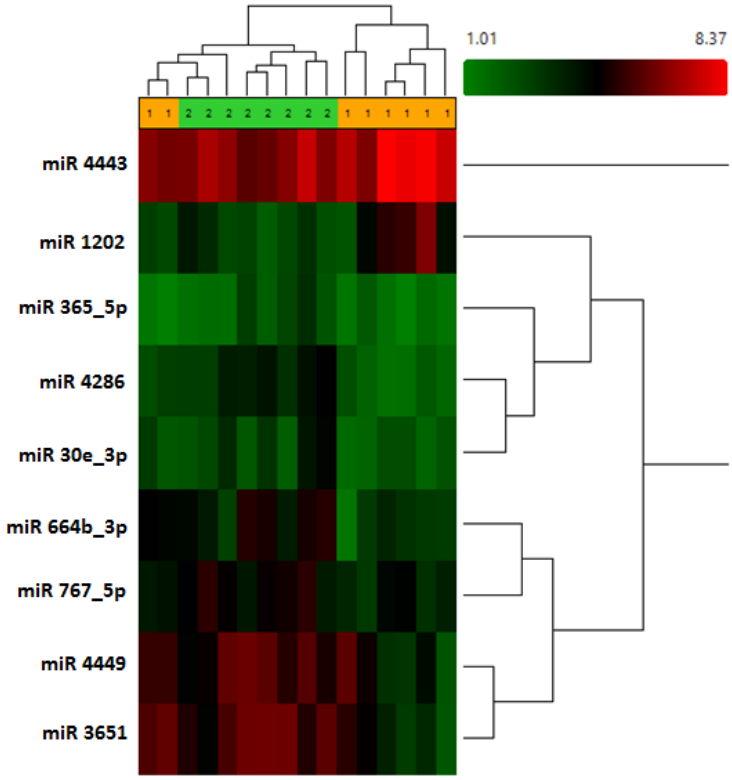

Figure 2. Hierarchical clustering from microarray resulted from *Transcriptome Analysis Console*™ software version 3.1. *hsa* filter, fold-change [± 1.65] and $p < 0.05$. Number 1 means Alzheimer's disease sample; number 2 refers to controls.

Table 1. Description of the clinical variables and relative quantification of miRs in the validation sample.

Variables	Control (n = 18)	AD (n = 16)	*p*
Age of death, years †	77.9 ± 9.9	80.9 ± 7.9	0.350
Male, % §	27.8	25.0	0.855
CDR = 3, % §	-	81.2	-
miR-30e_3p ($.10^{-2}$)	36.4 (24.5, 46.1)	21.2 (6.7, 32.8)	0.015
miR-365b_5p ($.10^{2}$)	4.2 (2.6, 24.4)	2.7 (1.3, 3.4)	0.023
miR-664_3p ($.10^{0}$)	30.1 (26.0, 38.2)	1.2 (0.6, 22.6)	<0.001
miR-767_5p ($.10^{0}$)	8.6 (6.8, 14.8)	8.4 (4.2, 13.0)	0.512
miR-1202 ($.10^{2}$)	2.0 (0.8, 10.8)	15.0 (6.0, 45.4)	0.006
miR-3651 ($.10^{0}$)	0.9 (0.5, 1.6)	1.0 (0.4, 1.3)	0.678
miR-4286 ($.10^{-5}$)	42.3 (21.7, 112.2)	18.0 (10.4, 47.4)	0.032
miR-4443 ($.10^{0}$)	20.4 (12.6, 35.0)	12.8 (2.5, 32.5)	0.133
miR-4449 ($.10^{0}$)	41.8 (18.9, 83.6)	10.4 (3.2, 40.1)	0.013

Data are expressed as average ± standard deviation† for continuous parameters, as relative frequencies§ for categorical features, or as median and interquartile interval for continuous traits with non-Gaussian distribution. The Student's t test, the chi-square test or the Mann-Whitney test were used, respectively. AD = Alzheimer's disease. miR = microRNA.

Table 2. AD relevant genes targets prediction using microRNA.org and AlzGene.org for miRNAs, ordered alphabetically.

miR-30e_3p	miR-4286	miR-664b_3p	miR-1202	miR-365b_5p	miR-4449
ABCA1	ABCG4	ARSB	ABCA1	APOM	TOMM40
ADAM10	ADCYAP1R1	CASP8	ABCG4	BACE1	
AKAP8	ADRA1A	CD2AP	APOB	CELF2	
APBB2	ATXN1	DKK1	CAND1	MAPT	
APH1B	CCR2	ERCC4	CCR5	NCOA2	
AR	CDX2	FOXO3	F11R		
ARID4A	CHRNA2	HECTD2	HNF4A		
CAMK2G	DLD;	KIF11	SAR1A		
CAST	EGR2	KLF5	SH3PXD2A		
CAV1	F11R	LRAT	SMAD3		
DLST	GAB2	LRP6	SOS1		
DOPEY2	HK2	LRRK2	TAP2		
GLO1	HSPA5	LRRTM3	TNMD		
GMEB1	INSR	MARCH5	TRAK2		
GOLM1	LDLR	SIRT1			
HSPA5	MME	SMAD3			
ILB1	NDUFB8	SRP72			
LRP8	SEMA4D	TANC2			
LSS	SERPINA3				
NDUFC2	SH3PXD2A				
NR3C1	SNCA				
NTRK2	SYN3				
NUMB	TANC2				
PCGF5	TGFB1				
PICALM	TMEM132C				
PLCE1	TMEM63C				
POU2F1	TP73				
PPP1R3A	TREM2				
PRKAA1	TRPC4AP				
PTEN	VDR				
RUNX1	WNT8B				
SLC6A3					
SOD2					
SORT1					
SOS2					
TET1					

ABCA1 - ATP binding cassette subfamily A member 1; **ABCG4** - ATP binding cassette subfamily G member 4; **ADAM10** - ADAM metallopeptidase domain 10; **ADCYAP1R1-** ADCYAP receptor type I; **ADRA1A** adrenoceptor alpha 1A; **AKAP8** – A-Kinase anchoring protein 8; **APBB2** - Amyloid beta precursor protein binding family B member 2; **APH1B** - Aph-1 homolog B, gamma-secretase subunit; **APOB** - apolipoprotein B; **APOM** - apolipoprotein M; **AR** – Androgen receptor; **ARID4A** - AT-rich interaction domain 4A; ARSB - arylsulfatase B; **ATXN1** - ataxin 1; **BACE1** - beta-secretase 1; **CAMK2G** - calcium/calmodulin dependent protein kinase II gamma; **CAND1** - cullin associated and neddylation dissociated 1; **CASP8** - caspase 8; **CAST** – calpastatin; **CAV1** - caveolin 1; **CCR2** - C-C motif chemokine receptor 2; **CCR5** - C-C motif chemokine receptor 5**; CD2AP** - CD2 associated protein; **CDX2** - caudal type homeobox 2; **CELF2** - CUGBP Elav-like family member 2; **CHRNA2** - cholinergic receptor nicotinic alpha 2 subunit; **DKK1** - dickkopf WNT signaling pathway inhibitor; **DLD** - dihydrolipoamide dehydrogenase; **DLST** - dihydrolipoamide S-succinyltransferase; **DOPEY2** - dopey family member 2; **EGR2** - early growth response 2**; ERCC4** - ERCC excision repair 4, endonuclease catalytic subunit; **F11R** - F11 receptor; **FOXO3** - forkhead box O; **GAB2** - GRB2 associated binding protein 2; **GLO1** - glyoxalase I; **GMEB1** - glucocorticoid modulatory element binding protein 1; **GOLM1** - golgi membrane protein 1; **HECTD2** - HECT domain E3 ubiquitin protein ligase 2; **HK2** – hexokinase 2; **HNF4A** - hepatocyte nuclear factor 4 alpha; **HSPA5** - heat shock protein family A (Hsp70) member 5; **INSR** - Insulin receptor; **LDLR** - low density lipoprotein receptor; LRAT - lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase); **LRP6** - LDL receptor related protein 6; **LRP8** - low density lipoprotein receptor-related protein 8, apolipoprotein e receptor; **LRRK2** - leucine rich repeat kinase 2; **LRRTM3** - leucine rich repeat transmembrane neuronal 3; **LSS** - lanosterol synthase; **MAPT** - microtubule associated protein tau; **MARCH5** - membrane associated ring-CH-type finger 5; **MME** - membrane metalloendopeptidase; **NCOA2** - nuclear receptor coactivator; **NDUFB8** - ubiquinone oxidoreductase subunit B8; **NDUFC2** - NADH:ubiquinone oxidoreductase subunit C2; **NR3C1** - nuclear receptor subfamily 3 group C member 1; **NTRK2** - neurotrophic receptor tyrosine kinase 2; **NUMB** - NUMB endocytic adaptor protein; **PCGF5** - polycomb group ring finger 5; **PICALM** - phosphatidylinositol binding clathrin assembly protein; **PLCE1** - phospholipase C epsilon 1; **POU2F1** - POU class 2 homeobox 1; **PPP1R3A** - protein phosphatase 1 regulatory subunit 3A; **PRKAA1** - protein kinase AMP-activated

catalytic subunit alpha 1; **PTEN** – phosphatase and tensin homolog; **RUNX1 -** RUNX family transcription factor 1; **SAR1A** - secretion associated Ras related GTPase 1A; **SEMA4D** - semaphorin 4D; **SERPINA3** - serpin family A member 3; **SH3PXD2A** - SH3 and PX domains 2A; **SH3PXD2A** - SH3 and PX domains 2A; **SIRT1** - sirtuin 1; **SLC6A3** - solute carrier family 6 member 3; **SMAD3** - SMAD family member 3; **SNCA** - synuclein alpha; **SOD2** - superoxide dismutase 2; **SORT1** - sortilin 1; **SOS1** - SOS Ras/Rac guanine nucleotide exchange factor 1; **SOS2** - SOS Ras/Rho guanine nucleotide exchange factor 2; **SRP72** - signal recognition particle 72; **SYN3** - synapsin III; **TANC2** - tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2; **TAP2** - transporter 2, ATP binding cassette subfamily B member; **TET1** - tet methylcytosine dioxygenase 1; **TGFB1** - transforming growth factor beta 1; **TMEM132C** - transmembrane protein 132C; **TMEM63C** - transmembrane protein 63C; **TNMD** – tenomodulin; **TOMM40** - translocase of outer mitochondrial membrane 40; **TP73** - tumor protein p73; **TRAK2** - trafficking kinesin protein 2; **TREM2** - triggering receptor expressed on myeloid cells 2; **TRPC4AP** - transient receptor potential cation channel subfamily C member 4 associated protein; **VDR** - vitamin D receptor; **WNT8B** - Wnt family member 8B;

Table 3. AD's relevant miRNAs, genes and pathways.

KEGG Pathways	Code	Genes	miRNA	p-value
PI3K-Akt signaling pathway	hsa04151	INSR	miR-4286	3.6×10^{-2}
		PRKAA1	miR-30e_3p	
		PTEN	miR-30e_3p	
FoxO signaling pathway	hsa04068	INSR	miR-4286	3.9×10^{-2}
		PRKAA1	miR-30e_3p	
		PTEN	miR-30e_3p	
		SOD2	miR-30e_3p	
MAPK signaling pathway	hsa04010	MAPT	miR-365b_5p	3.9×10^{-2}
p53 signaling pathway	has04115	PTEN	miR-30e_3p	6.0×10^{-4}
Prion disease	hsa05020	HSPA5	miR-30e_3p; miR-4286	2.7×10^{-19}
Hepatitis B	hsa05161	PTEN	miR-30e_3p	6.8×10^{-5}
Steroid biosynthesis	hsa00100	LSS	miR-30e_3p	1.0×10^{-3}
Glioma	hsa05214	PTEN	miR-30e_3p	1.0×10^{-3}
Pathways in cancer	hsa05200	PTEN	miR-30e_3p	1.0×10^{-3}
Small cell lung cancer	hsa05222	PTEN	miR-30e_3p	1.0×10^{-3}
Adherens junction	hsa04520	INSR	miR-4286	2.0×10^{-3}

Data from DIANA (mirPath v.3) KEGG analysis with genes union mode, FDR correction on, p-value threshold = 0.05 and filtered genes from Table 2. **HSPA5 -** heat shock protein family A (Hsp70) member 5; **INSR** - Insulin receptor; **LSS** - lanosterol synthase; **MAPT** - microtubule associated protein tau; **PRKAA1** - protein kinase AMP-activated catalytic subunit alpha 1; **PTEN** – phosphatase and tensin homolog; **SOD2** - superoxide dismutase 2;

3. Discussão geral e conclusões

Em nosso estudo, validamos seis miRNAs diferencialmente expressos no grupo doente. Dentre eles, cinco mostraram-se hipo-expressos (miR-30e_3p; miR-365b_5p; miR-664_3p; miR-4286; miR-4449), enquanto um mostrou-se super expresso (miR-1202) na DA. Sugerimos que esses seis miRNAs relatados estão implicados em importantes rotas relacionadas à DA e dessa forma contribuem para a fisiopatologia da doença. Em geral, a hipo-expressão desses miRNAs pode culminar na super-expressão de genes que contribuem para a formação de placas amiloidóticas e deposição de proteínas Tau, características essenciais da DA.

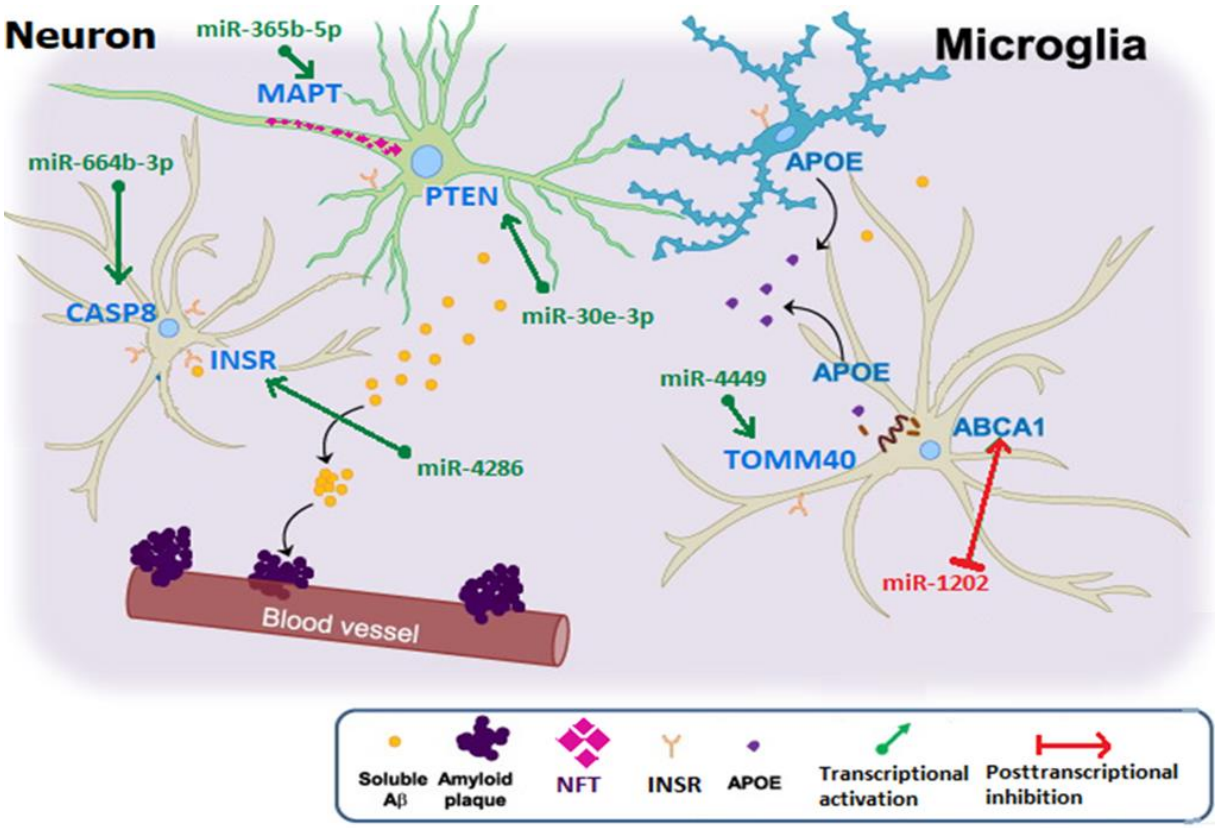

Figura 3. miRNAs desregulados na Doença de Alzheimer. Figura desenvolvida pelo autor.

Os miRNAs -30e_3p, -365b_5p e -4286 têm sido relacionados a genes cuja desregulação sabidamente tem relação com a fisiopatologia da DA, como os genes PTEN (54), MAPT (55) e INSR (56), respectivamente, todos envolvidos com morte neuronal, seja por atuarem sobre rotas de apoptose, autofagia ou danos oxidativos (57-59), figura 3. As rotas de sinalização apontadas pelo nosso trabalho estão envolvidas com processo de morte celular,

como as vias PI3K-AKT (incluindo a rota GS3Kβ), FoxO, MAPK e p53, e são fortemente associadas a processos neurodegenerativos (60-63).

Ainda em relação aos miRNAs encontrados como relevantes na nossa amostra (miR-1202; miR-4449 e miR-664_3p) é possível observar que eles atuam em importantes processos relacionados a fisiopatologia da DA, seja pelo envolvimento com a deposição da proteína β-amilóide, por promover alteração na integridade e metabolismo mitocondrial, ou ainda, por induzir processos apoptóticos, já que atuam sobre genes relevantes para a DA, conforme apresentado na figura 3.

Em 2018, o Instituto Nacional do Envelhecimento e Associação de Alzheimer (NIA-AA) atualizou as diretrizes para definir a DA como um construto fisiopatológico estabelecendo a classificação ATN. Essa proposta considera depósitos de β-amilóide (A), proteína Tau patológica (T) e neurodegeneração (N) como marcas condicionantes à patologia, com A+ como pré-requisito essencial (64, 65). Por conseguinte, as amostras de DA utilizadas no nosso trabalho derivaram exclusivamente de pacientes com amiloidose confirmada em exame histopatológico, portanto consistentes com o fenótipo A+ da nova diretriz NIA-AA. Possivelmente, devido a essa homogeneidade no fenótipo, não surpreende que os miRNAs indicados por nossos métodos estejam relacionados a vias que contribuem para o surgimento de placas de β-amilóide.

Sendo abundante no cérebro e altamente estável em biofluidos, está estabelecido que o conteúdo de miRNA tem influência no início e na progressão das doenças neurodegenerativas. Esta investigação exploratória utilizou uma abordagem para detectar miRNAs diferencialmente expressos de acordo com um fenótipo específico da DA, onde seis miRNAs foram identificados no estágio da doença, conectando esses miRNAs com a neuroquímica da entidade, revelando potenciais biomarcadores dessa patologia cerebral. Com base nas evidências disponíveis sobre esses miRNAs e seus genes-alvo, nosso trabalho aponta para várias vias de sinalização no meio neuronal, especialmente as vias PI3K-AKT, FoxO, MAPK e p53, para hipotetizar possíveis vias de disfunção e neurotoxicidade.

Ademais, devemos destacar a potencial importância do miR-30e-3p de interferir na neurodegeneração, já que este parece estar implicado em nove das onze vias de sinalização apontadas. O gene-alvo destacado, PTEN, mostrou-se relevante em sete das onze vias de sinalização após análise pelo KEEG. A centralidade de PTEN e MAPT pode ser observada por meio da análise realizada na plataforma String, v.11.0, figura 4. Nessa análise reiteramos a

convergência das rotas de sinalização apontadas pela tabela 3 como possivelmente desreguladas e implicadas com o processo fisiopatológico da DA.

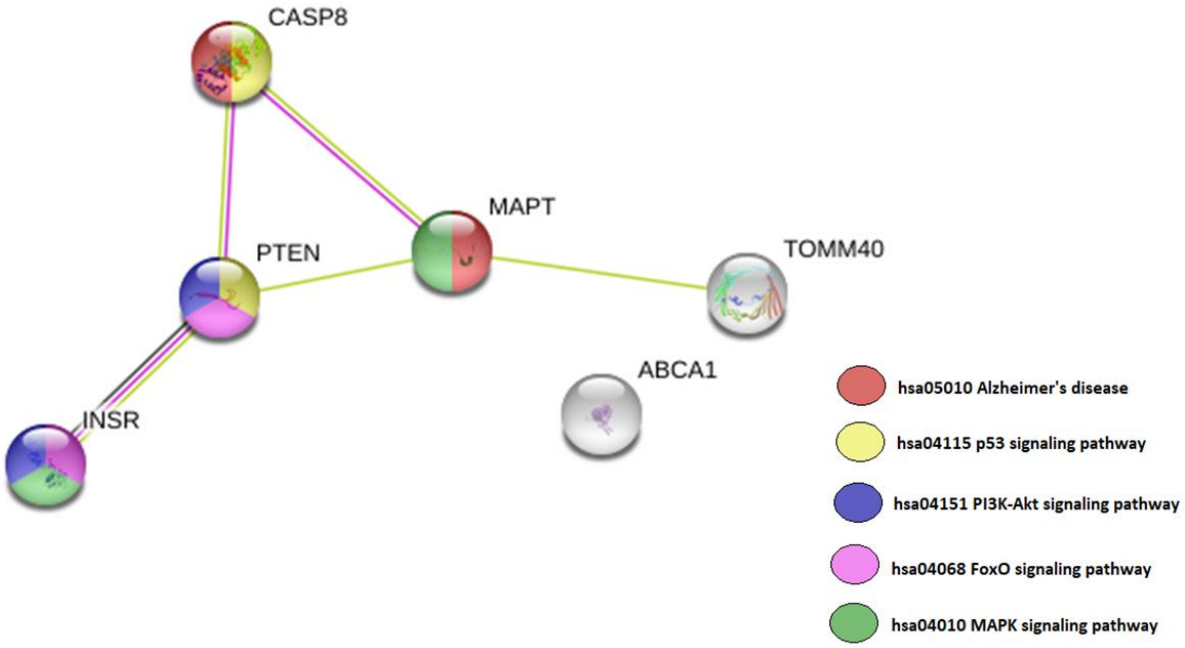

Figura 4. Rede e Análise de Enriquecimento. Vias metabólicas do termo KEGG detectadas como reguladas pela plataforma String v11.0. As ligações entre as proteínas representam interações. p-value: 0.00547

O conhecimento de biomarcadores de processos neurodegenerativos ainda estão em processo de estudos, dessa forma, moléculas que participem do processo neurodegenerativo, que tenham poder para orientar e predizer sobre os danos celulares ocorridos na DA, são atualmente de grande valia e contribuem para o entendimento da doença.

Por isso, esse trabalho visou apontar miRNAs com potenciais de envolvimento com a fisiopatologia da DA. E, apesar de alguns miRNAs apontados terem sido relatados de forma escassa na literatura com relação à neurodegeneração, pudemos embasar nossos achados a partir dos genes-alvo destacados pelas análises de bioinformática, assim como com as vias de sinalização. Isso reforça que o painel de miRNAs pode realmente ter forte relação com o processo neurodegenerativo provocado pela DA. O papel real desses miRNAs no início e progressão da DA ainda necessita ser avaliado por estudos controlados de natureza experimental, enquanto a utilidade desses elementos como marcadores de doença demanda trabalho futuro e equiparado com amostras maiores para assegurar a detecção de miRNAs com seus efeitos.

Dentre as limitações do nosso estudo, uma amostra mais ampla poderia ter evidenciado outros miRNAs também importantes para a patologia da DA. Ainda não utilizamos cálculos amostrais prévios para avaliar o mínimo de amostras necessárias para a análise ter poder estatístico, trabalhamos com as amostras de conveniência cedidas pelo Biobanco. Podemos citar ainda a falta de ensaios *in vitro* para testar a relação entre os miRNAs com os genes-alvo e suas correspondentes vias de sinalização, avaliando, por exemplo, o potencial desses miRNA em induzir processos apoptóticos.

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Anexos

Parecer do Comitê de Ética em Pesquisa

FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO - FMUSP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Biomarcadores potenciais para distúrbios crônicos do envelhecimento humano: microRNAs circulantes e teciduais.

Pesquisador: Otávio de Tolêdo Nóbrega

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 42256214.4.3001.0065

Instituição Proponente: Faculdade de Medicina da Universidade de Brasília - UNB

Patrocinador Principal: MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

DADOS DO PARECER

Número do Parecer: 1.072.651

Data da Relatoria: 20/05/2015

Apresentação do Projeto:

Trata-se de projeto de estudo de microRNAs em sangue periférico de pacientes idosos de vários grupos avaliados prospectivamente em Brasilia com particpação do Banco de Encéfalos e da Urologia FMUSP.

Objetivo da Pesquisa:

Identificar marcadores prognósticos por biologia molecular de sangue periférico de pacientes geriátricos com diversas síndromes clínicas.

Avaliação dos Riscos e Benefícios:

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SAO PAULO, 21 de Maio de 2015

Assinado por:
Roger Chammas
(Coordenador)

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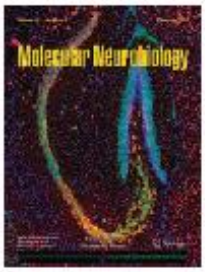

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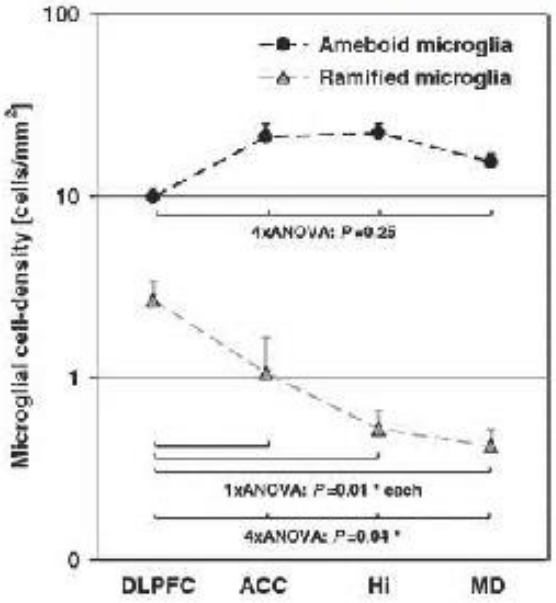

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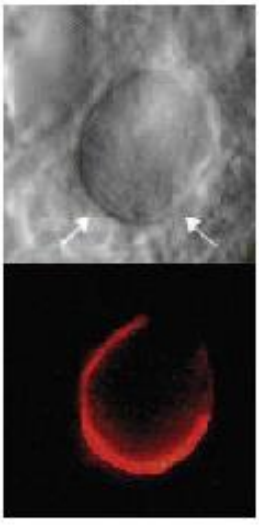
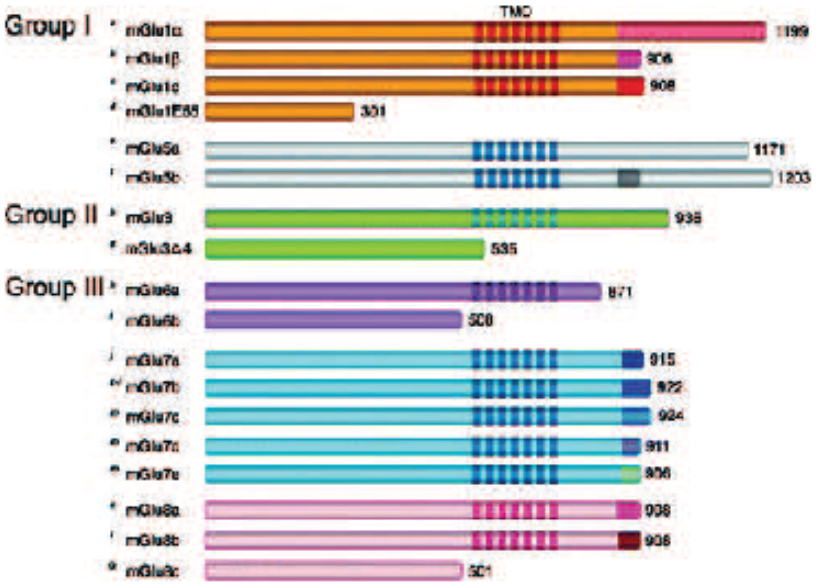


Combination Art



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Full author list: Adriane D. Henriques; Wilcelly Machado-Silva; Renata E. P. Leite; Lea T. Grinberg; Claudia K. Suemoto; Kátia R. M. Leite; Miguel Srougi; Wilson Jacob-Filho; Otávio T Nóbrega

Dear Ms. Adriane Henriques,

We have received the submission entitled: "Genome-wide expression and predicted significance of post-mortem brain microRNA profiling in Alzheimer's disease" for possible publication in Molecular Neurobiology, and you are listed as one of the co-authors.

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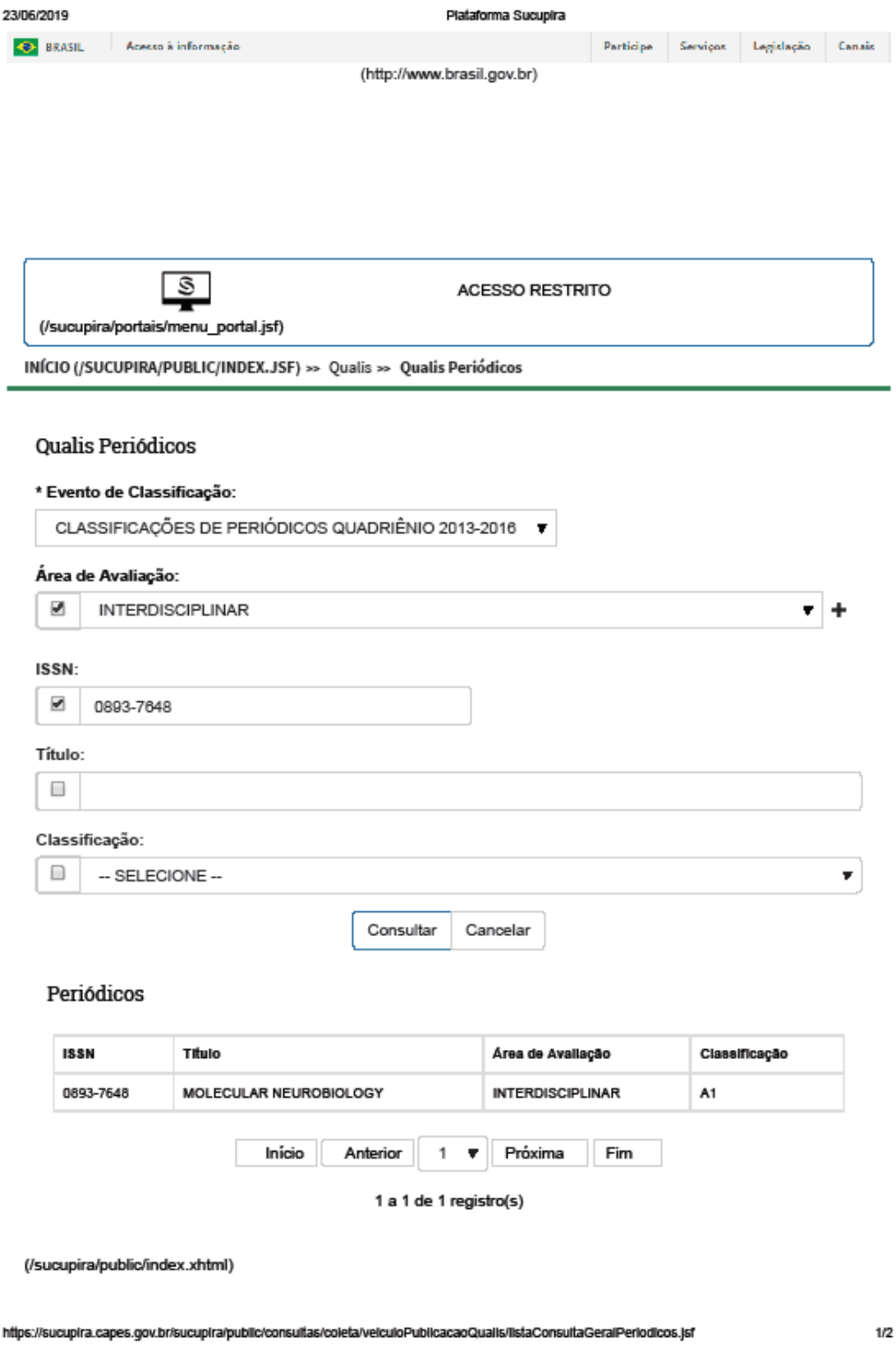
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