



UNIVERSIDADE FEDERAL DE MINAS GERAIS

Faculdade de Medicina

Programa de Pós-Graduação em Medicina Molecular

**EXPRESSÃO DO MICRORNA 184 EM IDOSOS COM DEPRESSÃO  
MAIOR: UMA PERSPECTIVA TRANSLACIONAL**

Ana Paula Mendes-Silva

Belo Horizonte

2018

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MAIOR: UMA PERSPECTIVA TRANSLACIONAL**

Tese apresentada ao Programa de Pós-Graduação em Medicina Molecular, da Faculdade de Medicina da Universidade Federal de Minas Gerais, como pré-requisito para a obtenção do título de Doutor em Medicina Molecular.

Orientador: Prof. Dr. Breno Satler de Oliveira Diniz

Co-Orientador: Profa. Dra. Débora Marques de Miranda

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Dedico aos meus avós  
que me mostraram a dor e a delícia  
do envelhecer

**“Então, me inspiro e me animo  
Abasteço-me de coragem e fibra.  
Constato pela frente o desafio  
Do exato tamanho da força  
Que me sustenta e equilibra.”  
(Gilberto Ribeiro Vieira)**

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## RESUMO

A depressão é a desordem psiquiátrica mais comum e causa graves consequências em idosos. Os mecanismos biológicos envolvidos na depressão geriátrica (DG) são complexos e envolvem muitos genes. Os MicroRNAs (miRNAs) são pequenos RNAs não codificadores que regulam pós-transcricionalmente a expressão gênica. O desequilíbrio na expressão de miRNAs está relacionado a muitas doenças, incluindo DG. Nosso objetivo foi avaliar os miRNAs plasmáticos associados à DG e possivelmente à gravidade dos sintomas depressivos e ao desempenho cognitivo nesses indivíduos. Um total de 116 amostras de plasma de idosos (63 com DG e 53 controles) foram divididas aleatoriamente para fazer o sequenciamento de nova geração (SNG) e a validação por RT-qPCR. *Drosophila melanogaster* foi utilizado como modelo translacional para avaliar fenômenos comportamentais associados à superexpressão e ao nocaute do ortólogo de hsa-miR-184. A análise SNG encontrou o hsa-miR-184 ( $\log_2\text{foldchange}=-4,205$  e  $p\text{-valor}=1,195e-03$ ) e o hsa-miR-1-3p ( $\log_2\text{foldchange}=-3,444$  e  $p\text{-valor}=1,3251e-02$ ) diferencialmente expressos em indivíduos com DG, mas apenas o hsa-miR-184 (AUC=8378,  $p\text{-valor}<0,0001$ ) foi validado em uma amostra independente. hsa-miR-184 foi significativamente correlacionado com a gravidade dos sintomas depressivos ( $r=-0.425$ ,  $p\text{-valor}=0.001$ ) e com a sub-escala de iniciação/perseverança ( $r=0,292$ ,  $p\text{-valor}=0,032$ ) da Escala de Avaliação de Demência. O modelo translacional mostrou que o nocaute do ortólogo do hsa-miR-184 prejudicou o desempenho de locomoção e de memória sugerindo que esse miRNA pode desempenhar um importante papel fisiopatológico na DG.

**Palavras-chave:** Depressão maior, depressão geriátrica, miR-184, microRNAs, biomarcador, *Drosophila melanogaster*

## ABSTRACT

Late-life depression (LLD) is the most common psychiatric disorder and causes severe consequences in elderly. The biological mechanisms involved in LLD are complex and involve many genes. MicroRNAs (miRNAs) are small noncoding RNAs and post-translational regulators of gene expression. Imbalance in miRNAs expression is related to many diseases, including LLD. Our aim was to evaluate plasma miRNAs that are associated with LLD and possibly with the severity of depressive symptoms and cognitive performance in these individuals. A total of 116 older adults (63 with LLD and 53 elderly controls) were randomly split into discovery and validation set, using next generation sequencing (NGS) and RT-qPCR, respectively. *Drosophila melanogaster* was used as a translational model to evaluate behavioral phenotypes associated to the overexpression and knockout of the ortholog of hsa-miR-184. NGS analysis found hsa-miR-184 ( $\log_2\text{foldchange} = -4,205$  and  $p\text{-value} = 1,195e^{-03}$ ) and hsa-miR-1-3p ( $\log_2\text{foldchange} = -3,444$  and  $p\text{-value} = 1,3251e^{-02}$ ) differently expressed in LLD subjects, but only hsa-miR-184 (AUC=8378,  $p\text{-value} < 0.0001$ ) was validated in an independent sample. hsa-miR-184 was significantly correlated with the severity of depressive symptoms ( $r=-0.425$ ,  $p=0.001$ ) and the initiation/perseverance sub-score ( $r=0,292$ ,  $p=0,032$ ) of the Dementia Rating Scale. The translational model showed that the knockout of the ortholog of hsa-miR-184 impaired locomotor and memory performance. These results suggest that hsa-miR-184 may play a significant physiopathological role in LLD.

**Keywords:** Major depression, late life depression, miR-184, MicroRNAs, Biomarker, *Drosophila melanogaster*

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# Capítulo I

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Fundamentação teórica

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## **Fundamentação teórica**

### **1 Senescência e Senilidade**

A Organização Mundial da Saúde (OMS) considera o envelhecimento como um processo sequencial, individual, cumulativo, irreversível, universal, não patológico de deterioração de um organismo maduro, próprio a todos os membros de uma espécie, de maneira que o tempo o torne menos capaz de fazer frente ao estresse do meio ambiente e, portanto, aumente sua possibilidade de morte (OMS, 2016). Ainda para a OMS, o limite de idade entre o indivíduo adulto e o idoso é 65 anos em nações desenvolvidas e 60 anos nos países emergentes. Logo, o envelhecimento populacional é uma consequência do desenvolvimento econômico e social (OMS, 2016).

Em relação à saúde do idoso temos dois paradigmas, de um lado o envelhecer normal como um processo progressivo de diminuição de reserva funcional, e, do outro lado, um processo de envelhecimento anormal, podendo levar ao desenvolvimento de uma condição patológica. O envelhecer normal está relacionado à capacidade de adaptação do indivíduo às agressões do ambiente, não havendo prejuízo na independência da realização de atividades diárias, sendo esse processo caracterizado por senescência (Kempermann, 2015). O surgimento de doenças crônico-degenerativas, por exemplo, apresenta aspectos negativos como incapacidade progressiva para a vida saudável e ativa. Essa incapacidade progressiva é caracterizada como senilidade (Ciosak *et al.*, 2011). Ambos os processos de envelhecimento exigem intervenções dos profissionais de saúde, com atuações focadas nesse segmento populacional. Essas atuações devem prezar pela manutenção da qualidade de vida dos idosos, considerando os processos de perdas próprias do envelhecimento e as possibilidades de

prevenção, manutenção e reabilitação do seu estado de saúde (Blazer, 2003; Luppá *et al.*, 2008; Lyness *et al.*, 2009).

De acordo com o Instituto Brasileiro de Geografia e Economia (IBGE), em 2013, a população idosa ultrapassava 23,9 milhões de brasileiros, cerca de 11,7% da população brasileira, e em 2060 estima-se que essa população representará 33,7% dos brasileiros, num total de 73,5 milhões de pessoas (IBGE, 2013). Dentre os diversos transtornos que acometem os idosos, a Depressão Geriátrica (DG) merece atenção especial (Veras e Coutinho, 1994). No Brasil, estudos feitos nos estados do Rio de Janeiro, Rio Grande do Sul e Minas Gerais entre as décadas de 80 e 90, estimaram que as taxas de sintomas depressivos na população idosa eram de 35,1%, 20% e 32%, respectivamente (Veras *et al.*, 1987); Fundação João Pinheiro, 1993; Santos, 1999).

Segundo a OMS (2016), a depressão afeta em torno de 350 milhões de pessoas em todo o mundo. Na Alemanha, por exemplo, foi realizada uma pesquisa com 2663 sujeitos com uma média de idade de 81,2 anos, separados por depressão precoce e depressão tardia, na qual foi investigada a contribuição dos sintomas depressivos para uma subsequente demência. Nesse estudo, pacientes que tiveram depressão de início tardio apresentaram um risco maior de desenvolver demência, levando a indícios de que a depressão pode ser um fator importante no possível desenvolvimento de uma demência (Heser *et al.*, 2013).

A depressão maior tem sido relatada na literatura como um transtorno mental complexo desencadeado pela desregulação de vias biológicas de inúmeros genes que tornam o tecido cerebral mais susceptível aos danos celulares, como a apoptose, por exemplo (Panza *et al.*, 2010). O entendimento das modificações moleculares e celulares da depressão associadas às alterações características

do envelhecimento normal, e de como os fatores ambientais, como fatores sociais, podem modular as respostas do organismo é essencial para distinguir os fatores que determinam a senescência ou a senilidade (Mckinney *et al.*, 2012; Mckinney e Sibille, 2013).

## **1.1 Depressão Maior em Idosos**

Na população idosa os quadros depressivos têm características clínicas peculiares, como diminuição da resposta emocional (erosão afetiva), diminuição do sono, perda de prazer nas atividades habituais (Forlenza e Almeida, 1997). A depressão maior também está associada ao aumento da incapacidade funcional e da taxa de mortalidade dos idosos (OMS, 2016). Neste cenário em que o envelhecimento populacional aumenta conjuntamente com a taxa de sintomas depressivos nessa população, o estudo da DG torna-se cada vez mais importante, visto sua alta prevalência e impacto na qualidade de vida dessa população.

### **1.1.1 Sintomas, Classificação e Comorbidades Associadas a DG**

Episódios de depressão maior são caracterizados pela presença de sintomas relacionados ao humor (tristeza, anedonia, apatia, desesperança, ansiedade, irritabilidade, ideias e/ou planos suicidas), alterações psicomotoras (lentificação ou agitação), somáticas (dores e sintomas físicos em geral), cognitivas (relacionadas a atenção, cálculos, memória) e neurovegetativas (sono, apetite, libido e ritmos biológicos) (Manual Diagnóstico e Estatístico das Perturbações Mentais [DSM-IV], 1994).

Segundos os critérios da versão traduzida do DSM-IV (1994), para o diagnóstico de DM, pelo menos cinco critérios devem estar presentes por pelo menos duas

semanas e não mais que dois anos, havendo também prejuízo no funcionamento psicossocial ou sofrimento significativo. Dentre os sintomas relatados, o humor deprimido ou perda do interesse ou prazer devem se fazer presentes (APA, 1994). Para classificação do episódio depressivo é utilizado a Classificação Internacional de Doenças (CID-10, 10. Ed., 1994) (Löwe *et al.*, 2004), que subdivide o episódio depressivo em leve, moderado ou grave, avalia o número de episódios, a sua intensidade e a importância clínica dos sintomas (Almeida e Almeida, 1999). Em relação ao primeiro episódio depressivo, este pode ser caracterizado como (1) Episódio depressivo leve: dois ou três sintomas sem grave prejuízo nas atividades diárias; (2) Episódio depressivo moderado: quatro ou mais sintomas com sério prejuízo nas atividades diárias; (3) Episódio depressivo grave sem sintomas psicóticos: grande número de sintomas com alta intensidade, severo prejuízo nas atividades diárias, ideação suicida elevada e com ou sem sintomas somáticos; (4) Episódio depressivo grave com sintomas psicóticos: episódio depressivo grave acompanhado de alucinações, ideias delirantes, lentidão psicomotora ou de estupor de uma gravidade tal que todas as atividades sociais normais tornam-se impossíveis; pode existir o risco de suicídio, de desidratação ou de desnutrição (CID-10, 10. Ed, 1994).

A avaliação da intensidade dos sintomas depressivos é realizada na avaliação clínica médica com o auxílio de instrumentos como a Escala de Depressão de Hamilton (HAMD) (Hamilton, 1960), a Escala Hospitalar de Ansiedade e Depressão (HAD) (Zigmond e Snaith, 1983), e o Inventário de Depressão de Beck (BDI) (Beck *et al.*, 1961). A utilização de uma dessas escalas para avaliar a resposta do indivíduo ao longo do tratamento psiquiátrico, farmacológico e psicológico é de suma importância (Zimmerman *et al.*, 2013).

A idade na qual o primeiro episódio depressivo acontece nos idosos caracteriza a depressão como de início precoce ou tardio. Se o idoso apresentar episódios depressivos recorrentes antes dos 60 anos, caracteriza-se como depressão de início precoce. Enquanto os idosos que apresentam o primeiro episódio a partir dos 60 anos, são caracterizados como de início tardio (CID-10, 10. Ed., 1994).

Estudos revelaram que idosos com depressão de início tardio, quando comparados aos de início precoce, tinham uma menor frequência de histórico familiar de transtorno de humor, uma maior prevalência de demência, um maior comprometimento em testes neuropsicológicos e aumento dos ventrículos laterais do cérebro (Alexopoulos, 2005; Gatz *et al.*, 2005; Byers e Yaffe, 2011). A depressão tardia também está associada ao comprometimento funcional e cognitivo significativo, sendo um importante fator de risco para a demência, em particular a doença de Alzheimer (DA) (Wilson *et al.*, 2002; Gatz *et al.*, 2005) e demência vascular (Diniz, Butters, *et al.*, 2013). Comorbidades como hipertensão arterial, insuficiência cardíaca, infarto do miocárdio, acidente vascular cerebral e diabetes mellitus encontram-se fortemente associadas à depressão (Oslin *et al.*, 2002; Kales *et al.*, 2005; Chew *et al.*, 2016). No entanto, embora esses estudos apontem altas taxas de depressão associadas às condições médicas crônicas supracitadas, não fica claro se qualquer uma das doenças tem uma associação única, específica e com relativa significância em relação à depressão (Dhar e Barton, 2016; Wang *et al.*, 2016).

### **1.1.2 Alterações de Vias Biológicas associadas à Depressão**

Pesquisas sugerem que o transtorno depressivo é impulsionado por desregulação de vias biológicas importantes, particularmente as que contribuem

para a remodelação na neurotransmissão glutamatérgica (Popoli *et al.*, 2012; Sanacora *et al.*, 2012), mecanismos de neurogênese e neuroplasticidade (Tardito *et al.*, 2006; Pittenger e Duman, 2008), e funções neuroimunes (Müller e Schwarz, 2007).

Ding e colaboradores (2015) fizeram uma caracterização molecular das funções cerebrais alteradas em 101 indivíduos com depressão maior, através de uma meta-análise da expressão diferencial de 8 estudos de sequenciamento gênico de três regiões corticolímbicas. Os resultados evidenciaram alteração em genes associados à plasticidade cerebral, sinalização neuronal e neurogênese. As funções biológicas mais representadas entre os genes identificados na meta-análise concentraram-se em duas funções principais, especificamente morte e sobrevivência celular e sinalização célula-a-célula. Esses resultados incluem a expressão diminuída de genes codificadores de neuropeptídeos sinalizadores (CORT, VEGFA, TGFB2), assim como do hormônio que libera corticotrópicos (CRH), do fator neurotrófico derivado do cérebro (BDNF) e do fator de crescimento do nervo (VGF), três genes com evidência prévia de envolvimento em distúrbios relacionados ao estresse e depressão maior como também relatado nos estudos de De Kloet e colaboradores (2005), Hunsberger e colaboradores (2007) e Sen e colaboradores (2008). Outros estudos também mostram que mecanismos celulares e moleculares envolvidos na neuroplasticidade e neurogênese estão alterados em sujeitos deprimidos (Pittenger e Duman, 2008; Alexopoulos e Morimoto, 2011).

A depressão tem sido relacionada a um estado de envelhecimento acelerado que afeta a região do hipocampo e os sistemas cardiovascular, cerebrovascular, neuroendócrino, imune e metabólico (Heuser, 2002; McIntyre *et al.*, 2007; Bauer,

2008; Bauer *et al.*, 2009; McIntyre *et al.*, 2009). Indivíduos deprimidos possuem uma maior incidência de comorbidades frequentemente associadas com o avanço da idade, como a diabetes do tipo II, síndrome metabólica, osteoporose, doença cardiovascular, acidente vascular encefálico e envelhecimento cognitivo patológico incluindo a DA e outras demências (Brown *et al.*, 2004; Evans *et al.*, 2005; Ownby *et al.*, 2006; McIntyre *et al.*, 2007; Vogelzangs *et al.*, 2007; McIntyre *et al.*, 2009; Brown *et al.*, 2011).

Várias explicações para o envelhecimento acelerado na depressão têm sido propostas, tais como a hipótese da cascata de glucocorticóide (Oitzl *et al.*, 2010; Sachs-Ericsson *et al.*, 2013) e acúmulo de carga alostática (Mcewen, 2000; Mcewen e Wingfield, 2003; Kapczinski *et al.*, 2008; Walker *et al.*, 2014). A descoberta dos processos patológicos na depressão, a nível celular, pode auxiliar na identificação de novos alvos tanto para o tratamento da depressão quanto das comorbidades a ela associadas (Wolkowitz *et al.*, 2010).

O processo de envelhecimento normal é acompanhado de declínio cognitivo gradual e progressivo. Após a quarta década de vida, observa-se um declínio das habilidades cognitivas nas quais os tempos de reação ficam mais lentos até mesmo nas tarefas mais simples (Owsley, 2011). Já em atividades mais complexas que requerem processamento mental, os tempos de reação declinam rapidamente a partir dos 60 anos (Cerella *et al.*, 1990).

Em estudos recentes, a DG têm sido associada a alterações no funcionamento cognitivo (memória, atenção, fluência verbal, e outros aspectos do processamento cognitivo), não condizentes ao declínio cognitivo esperado pelo processo normal de envelhecimento (Rosenberg *et al.*, 2010; Papazacharias e

Nardini, 2012). Além disso, as alterações no funcionamento cognitivo são associadas a episódios depressivos recorrentes nessa população (Panza *et al.*, 2010). Dessa forma, com o aumento do número e duração dos episódios depressivos os déficits cognitivos podem tornar-se permanentes, sendo evidentes mesmo quando há melhora do humor. Tais mudanças no funcionamento cognitivo conectam a DG ao Comprometimento Cognitivo Leve (CCL) e, possivelmente, com doenças neurodegenerativas (Bondi *et al.*, 2014; Galecki *et al.*, 2015).

No estudo realizado por Roberts e colaboradores (2014), 534 participantes foram avaliados num intervalo de 15 meses em que 153 deles (28,7%) tiveram progressão do CCL para demência (média de 71,3 para cada 1000, por ano). Petersen e colaboradores (2001) ressaltaram em seu estudo que a taxa de conversão de pacientes com CCL para demência ou Doença de Alzheimer (DA) variou entre 10 e 15%, sugerindo que essa condição seria um fator de risco para demência (Petersen *et al.*, 2001).

Há indícios de que o comprometimento das funções executivas na depressão maior e no CCL tem sido considerado um estágio de transição entre o envelhecimento cognitivo normal e estágios demenciais (Brown *et al.*, 2011; Risacher *et al.*, 2013; Lin *et al.*, 2014).

Estudos baseados em proteômica mostraram que indivíduos com DG apresentaram anormalidades significativas nas vias biológicas relacionadas ao controle da resposta inflamatória, remodelação tecidual, função endotelial e vascular, controle da proteostase, suporte neurotrófico e padrões moleculares relacionados à senescência (Diniz *et al.*, 2015; Diniz *et al.*, 2016; Diniz *et al.*, 2017). Uma meta-análise feita por Mitchell e Shiri-Feshki (2009) analisou 41

estudos clínicos, com um total de 10119 sujeitos, e encontrou a taxa anual de conversão de CCL para demência de 9,6%. Sendo que, 8,1% dos sujeitos analisados convergiram de CCL para DA no período de um ano. Dessa forma, tem sido proposto que a depressão maior em idosos e o CCL precedem o desenvolvimento de demências e a identificação de vias metabólicas alteradas, genes e moléculas associadas a essas podem servir como indicativos do início do desenvolvimento de doenças neurodegenerativas (Tabert *et al.*, 2006; Brainerd *et al.*, 2014).

Mendes-Silva e colaboradores publicaram uma revisão sistemática (2016) que identificou 7 microRNAs (miRNAs) comuns a depressão maior e à doença de Alzheimer (hsa-miR-664a-3p, hsa-miR-361-5p, hsa-miR-26b-5p, hsa-miR-191-5p, hsa-let-7d-5p, hsa-let-7g-5p, hsa-let-7f-5p). O interessante foi que todos os 7 miRNAs comuns encontrados foram superexpressos em indivíduos com depressão maior e hipoexpressos em indivíduos com a doença de Alzheimer. Esses miRNAs estão associados com 45 genes validados e as principais vias biológicas e processos regulados por eles são relacionadas ao controle da proteostase, manutenção da integridade genômica, regulação da atividade transcricional, controle imunoinflamatório e suporte neurotrófico.

No entanto, muito há que se esclarecer de como essa relação ocorre e quais são os fatores que podem atuar nessa importante tríade envelhecimento normal-depressão-demência. Uma das linhas crescentes nesses estudos é a busca por biomarcadores plasmáticos como os miRNAs.

## 1.2 MiRNAs

Os miRNAs são moléculas pertencentes à família dos RNAs não codificadores e possuem a capacidade de regular a expressão de muitos genes simultaneamente, influenciando as funções celulares de inúmeras vias biológicas. Seus transcritos são sequências curtas de RNA fita-dupla de 18 a 25 nucleotídeos, e podem induzir o silenciamento de seus genes-alvos por complementariedade de bases. Sabe-se que mais de dois terços dos genes codificadores de proteína em humanos são regulados por miRNAs (Rucker e McGuffin, 2014) e há evidências de que essas moléculas atuam na modulação de inúmeros processos cerebrais, incluindo o desenvolvimento e proliferação neuronal, diferenciação celular, metabolismo e apoptose (Bocchio-Chiavetto *et al.*, 2013).

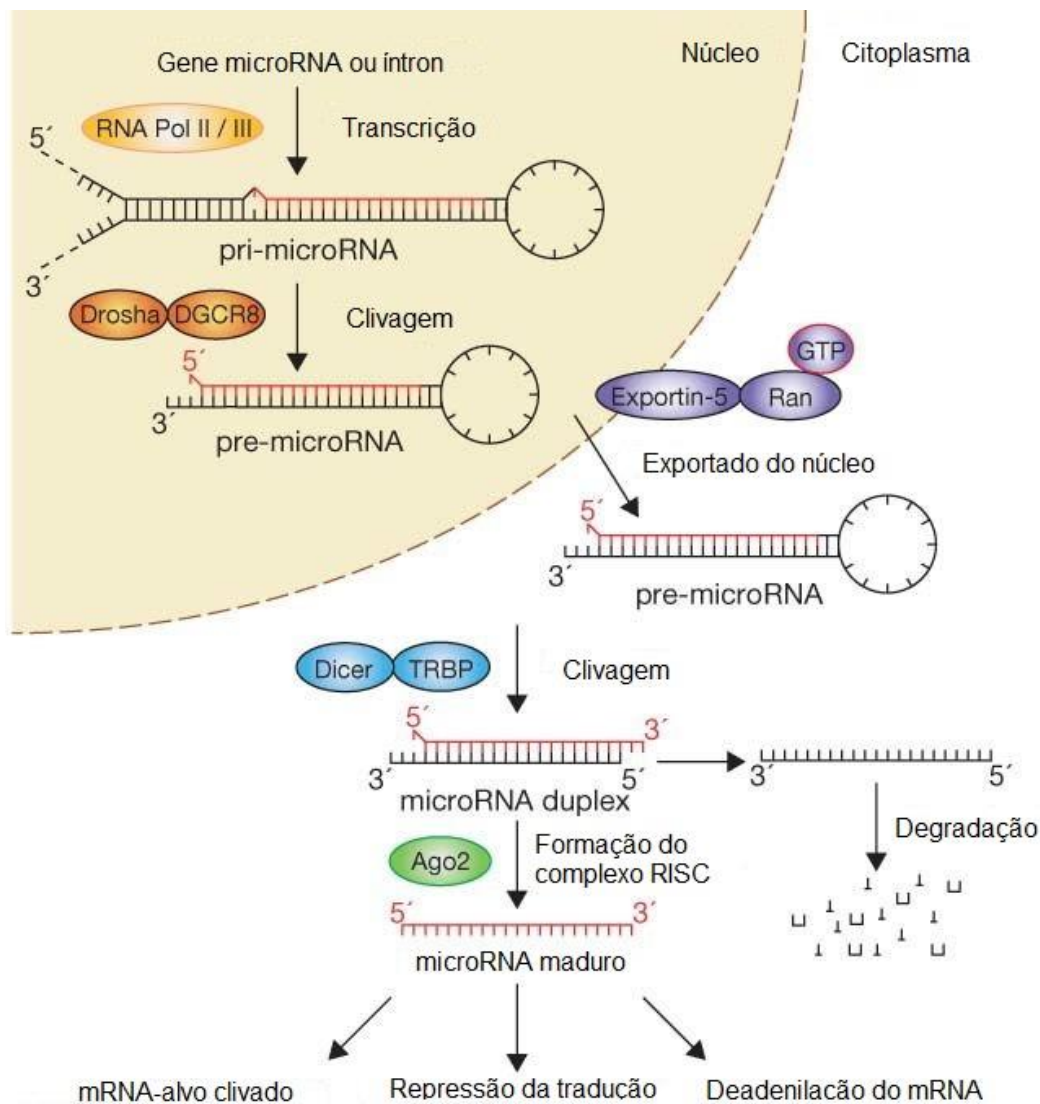
Os mecanismos e processos conhecidos até o momento pelos quais os miRNAs regulam a expressão gênica envolvem a inibição da tradução, desestabilização do RNA-alvo, silenciamento transcricional, promoção da transcrição e aumento da eficiência da tradução (Huntzinger e Izaurralde, 2011). Todos esses mecanismos regulatórios são capazes de influenciar vias biológicas inteiras dos genes regulados (Geaghan e Cairns, 2015).

### 1.2.1 Biogênese dos microRNAs

Os genes dos miRNAs estão dispersos ao longo do genoma, algumas vezes distantes dos genes alvos, embora muitos sejam intrônicos (Geaghan e Cairns, 2015) (Figura 1). Inicialmente, os miRNAs são transcritos pela RNA polimerase II como um miRNA primário (pri-miRNA) formando uma molécula com aproximadamente 70 a 130 pares de base (pb). Após ser processado pelo

complexo enzimático DROSHA-DGCR8, o pri-miRNA é transformado em pré-miRNA com 60 a 70 pb, sendo exportado do núcleo para o citoplasma. No citoplasma esse pré-miRNA é processado por outra enzima chamada DICER, formando uma molécula fita-dupla conhecida como miRNA maduro com 18-25 nucleotídeos. Estas pequenas moléculas fita-dupla ligam-se ao complexo protéico RISC (do inglês *RNA-induced silencing complex*) havendo a degradação de uma das fitas do miRNA e a ligação da outra fita à região 3' UTR do mRNA-alvo (Ameres e Zamore, 2013; García-López *et al.*, 2013). A seleção da fita ativa depende dos nucleotídeos na extremidade 5' e da assimetria termodinâmica do miRNA maduro (Kawamata e Tomari, 2010). Quando um miRNA e um mRNA exibem complementaridade total, este mRNA será degradado pela ação catalítica da enzima Argonata. Quando o pareamento entre a fita do miRNA e o mRNA-alvo é incompleta, o mRNA tem a tradução prejudicada, levando a baixos níveis de proteína (Bartel, 2004; 2009). A interação entre o miRNA e o mRNA-alvo é mediada pela região constituída pelos nucleotídeos na posição 2 a 8 da extremidade 5' do miRNA responsável pela sua hibridização com seu alvo. Recentes descobertas revelaram que apesar do grau de complementaridade entre o miRNA e o mRNA-alvo ser um parâmetro importante, ele não é o único determinante da forma como miRISC atua, pois miRNAs parcialmente complementares ainda podem levar à clivagem do RNA-alvo (Ameres e Zamore, 2013; Hausser e Zavolan, 2014; Dornseifer *et al.*, 2015). Estudos sugerem que a competição entre os miRNAs para a ligação com o mRNA-alvo é um princípio fundamental da regulação pós-transcricional. Não somente os miRNAs podem ser sequestrados e neutralizados pelos alvos com os quais interagem, mas competir entre os sítios de ligação de diferentes mRNAs

também podem levar a uma regulação cruzada entre os transcritos (Ebert *et al.*, 2007; Gardiner *et al.*, 2015; Jens e Rajewsky, 2015).



**Figura 1** Biogênese do microRNA e mecanismos de regulação do mRNA-alvo, adaptado de Winter e colaboradores (2009)

Os processos de inibição da tradução podem ocorrer a partir de mecanismos de competição pelo 5'CAP (Pestova *et al.*, 2001; Sonenberg e Dever, 2003; Martínez-Salas *et al.*, 2013), inibição da montagem dos ribossomos (Chendrimada *et al.*, 2007) ou pela deadenilação do mRNA seguida pelo bloqueio do início da tradução.

A produção dos miRNAs é realizada naturalmente pelas células, sendo esses liberados nos fluidos corporais quando contidos em microvesículas ou associados a proteínas ou lipídios (Guarnieri e Dileone, 2008). Os miRNAs, uma vez transportados por todo o corpo, podem mediar a comunicação intercelular atuando na regulação da expressão gênica, por meio da inibição da tradução ou da clivagem de vários mRNAs-alvos (Bartel, 2009).

O transporte horizontal dos miRNAs nos fluidos corporais permite o monitoramento sistêmico de processos metabólicos associados a células específicas, além de ser coletados facilmente. A análise de miRNAs circulantes é considerada uma via potencial para o desenvolvimento de biomarcadores periféricos para inúmeras doenças como as associadas a disfunções no Sistema Nervoso Central (SNC) (Sheinerman e Umansky, 2013).

### **1.2.2 MicroRNAs na Depressão**

Os miRNAs por serem modeladores da expressão gênica, são de suma importância na regulação de vias biológicas da neurogênese e plasticidade sináptica do SNC (Mehler e Mattick, 2006; Guarnieri e Dileone, 2008; Mellios *et al.*, 2008; Bartel, 2009; Belzeaux *et al.*, 2012; Lausted *et al.*, 2014; Kim *et al.*, 2015; Walker *et al.*, 2015; Dwivedi, 2016; Hu *et al.*, 2017). Estudos *post-mortem* de tecidos cerebrais de indivíduos diagnosticados com esquizofrenia, transtorno afetivo bipolar e depressão maior mostraram alterações nos níveis de vários miRNAs reguladores da plasticidade e desenvolvimento neuronal (Mellios *et al.*, 2008; Beveridge *et al.*, 2010; Kim *et al.*, 2010; Smalheiser *et al.*, 2012).

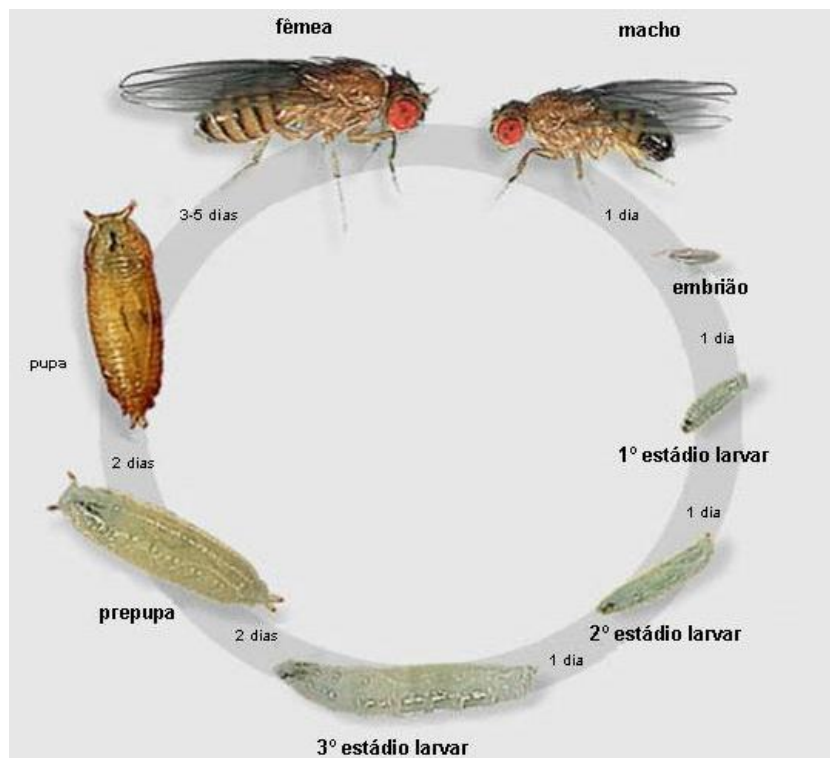
Indivíduos com o diagnóstico de depressão maior têm apresentado uma regulação anormal de diversos miRNAs, incluindo os da família hsa-let-7, hsa-

miR-125a, hsa-miR30d e hsa-miR-191. Nos estudos que avaliaram os níveis plasmáticos de miRNAs em indivíduos saudáveis e os diagnosticados com depressão maior, foram identificados 31 miRNAs diferencialmente expressos com  $p\text{-valor} \leq 0,05$  e  $\log_2\text{foldchange} \geq |1,5|$  (*hsa-miR-130b*, *hsa-miR-505*, *hsa-miR-29b-2*, *hsa-miR-26b*, *hsa-miR-22*, *hsa-miR-26a*, *hsa-miR-664*, *hsa-miR-494*, *hsa-let-7d*, *hsa-let-7g*, *hsa-let-7e*, *hsa-let-7f*, *hsa-miR-629*, *hsa-miR-106b*, *hsa-miR-103*, *hsa-miR-191*, *hsa-miR-128*, *hsa-miR-502-3p*, *hsa-miR-374b*, *hsa-miR-132*, *hsa-miR-500*, *hsa-miR-589*, *hsa-miR-183*, *hsa-miR-574-3p*, *hsa-miR-335*, *hsa-miR-361-5p*, *hsa-miR-579*, *hsa-miR-941*, *hsa-miR-133a*, *hsa-miR-107*) (Belzeaux *et al.*, 2012; Bocchio-Chiavetto *et al.*, 2013; Liu, X. *et al.*, 2014). Os genes-alvos desses miRNAs estão relacionados com processos biológicos essenciais na manutenção da homeostase neuronal, assim como na função mitocondrial, estresse de retículo endoplasmático, ciclo celular, apoptose e metabolismo de lipídios (Niikura *et al.*, 2002; Tsukamoto *et al.*, 2003; Blasko *et al.*, 2004; De La Monte e Wands, 2005; Rivera *et al.*, 2005; De La Monte e Wands, 2006).

Estudos recentes identificaram miRNAs como biomarcadores de resposta ao tratamento na depressão maior (Fiori *et al.*, 2017; Roy *et al.*, 2017). A maioria dos estudos que avaliaram miRNAs associados a depressão maior incluíram na amostra jovens e adultos. O número de estudos que avaliam alterações na expressão de miRNAs em DG é reduzido, prejudicando o entendimento de como esses miRNAs atuam nas vias biológicas, funções cerebrais e comportamentais dessa população. Dessa forma, uma das ferramentas empregadas nos dias de hoje para examinar melhor o papel da expressão dos miRNAs é a utilização de modelos translacionais como a *Drosophila melanogaster*.

### 1.3 *Drosophila melanogaster*

*Drosophila melanogaster* é um dos organismos mais estudados na biologia e serve como um sistema modelo para a investigação de muitos processos celulares e de desenvolvimento comuns aos eucariotos superiores, incluindo os humanos (Adams *et al.*, 2000). A *D. melanogaster* foi um dos primeiros organismos a ter o genoma completamente sequenciado. Popularmente conhecida como a mosca da fruta (El-Sherif *et al.*, 2012), é um inseto da ordem díptera que vem sendo amplamente empregado como organismo modelo nos atuais estudos da área de genética. A mosca da fruta possui ciclo de vida curto (Figura 2), baixos custos de manutenção e quase 77% dos genes causadores de doenças humanas têm um homólogo funcional na mosca (Reiter *et al.*, 2001).



**Figura 2** Ciclo de vida da *Drosophila melanogaster*

Com um rápido ciclo de vida as fêmeas podem produzir várias dezenas de descendentes resultantes de uma única fecundação, em apenas uma semana. E,

após atingirem a fase adulta as moscas têm uma expectativa média de vida de 60 dias (Roote e Prokop, 2013).

Seu genoma compacto e simples, com apenas quatro cromossomos, permitiu o desenvolvimento de modelos transgênicos de doenças humanas com genes candidatos expressos em regiões específicas se necessário (Hales *et al.*, 2015). Para doenças neurodegenerativas, por exemplo, existem milhares de linhagens transgênicas disponíveis. Utilizando o sistema binário de expressão Gal4/UAS, um ativador de transcrição de leveduras, genes humanos podem ser expressos no cérebro da drosófila de forma precisa (Jackson, 2008; Jenett *et al.*, 2012).

Nos últimos 50 anos, estudos genéticos utilizando a mosca da fruta vêm sendo aplicados com sucesso para decifrar os principais mecanismos que sustentam uma variedade de fundamentos biológicos e seus processos, incluindo sinalização celular (Miron e Sonenberg, 2001; Cadigan e Peifer, 2009), ciclo celular (Igaki *et al.*, 2002; Lee e Orr-Weaver, 2003; Kanda *et al.*, 2011; Ma *et al.*, 2014), sistema nervoso (Koushika *et al.*, 1996; Chan e Bonini, 2000; Zhang *et al.*, 2012; Lang *et al.*, 2013), comportamento (Shimada *et al.*, 1993; Bellen *et al.*, 2010), desenvolvimento (Dammai *et al.*, 2003; Kumita *et al.*, 2012) e aspectos moleculares de doenças humanas (Chien *et al.*, 2002; Igaki *et al.*, 2002; Bier, 2005; Igaki *et al.*, 2011).

#### **1.3.1.1 Linhagens transgênicas**

A geração de linhagens transgênicas de *D. melanogaster* através da inserção de elementos transponíveis/transposons tem possibilitado a análise e o estudo de inúmeros processos biológicos (Toba *et al.*, 1999; Duffy, 2002; Roote e Prokop, 2013). Esses elementos construídos se inserem no genoma da *Drosophila* como

um elemento endógeno e a partir de então é mantido na mesma posição nas gerações seguintes (Roote e Prokop, 2013).

Para obtenção de transgênicos de *D. melanogaster*, transposons são modificados geneticamente. O gene da transposase é removido e substituído por outros genes que se deseja introduzir no genoma da *Drosophila* (Toba *et al.*, 1999; Roote e Prokop, 2013). Esses constructos contêm genes marcadores de seleção em bactérias que permitem a seleção de clones contendo o elemento P. O acesso aos transgênicos é possível através dos grupos de pesquisa que o criaram, mas também estão disponíveis em centros de estoques de *Drosophila* como os centros Bloomington (Cook *et al.*, 2010). Há uma grande variedade de linhagens disponíveis.

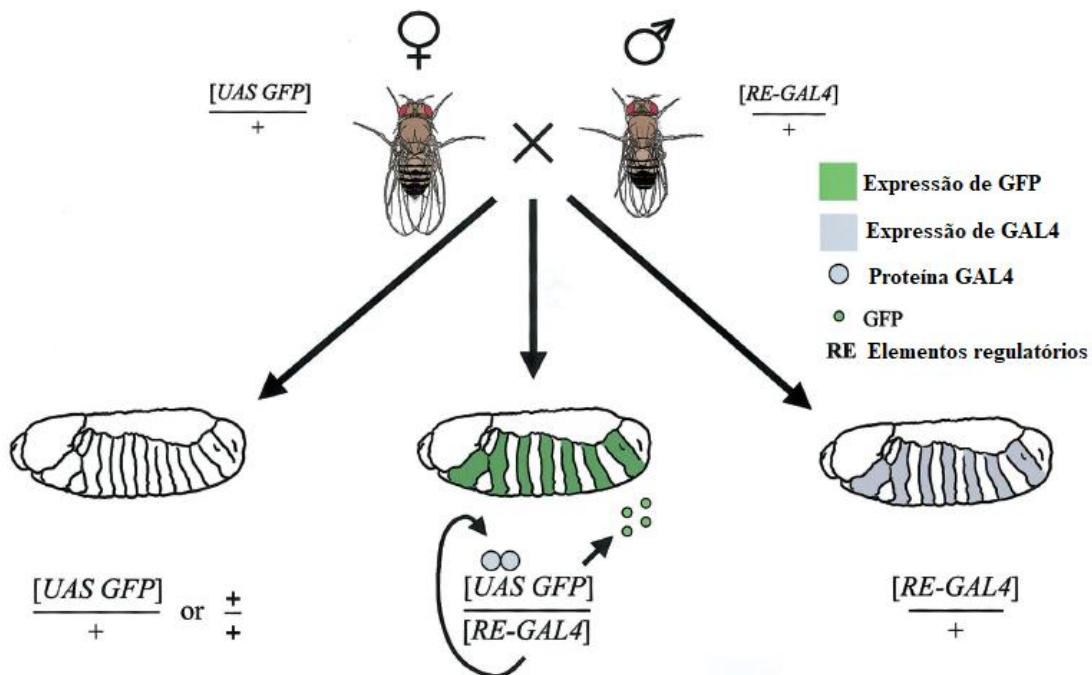
As linhagens *driver* são construídas para direcionarem a expressão de um gene de interesse a um tecido específico como por exemplo, as linhagens GMR-Gal4 (olho, pernas e asa) (Giorgi *et al.*, 1993; Li *et al.*, 2012) e *yolk-Gal4* (corpo gorduroso de fêmeas adultas) (Giorgi *et al.*, 1993).

As linhagens *responder* são caracterizadas por um sistema de expressão gênica cuja transcrição do gene-alvo está sob o comando de uma sequência de ativação antecedente (UAS) dependente de Gal4 (Cook *et al.*, 2010). Alguns exemplos de linhagens *responder* são: UAS-*eiger* (ortólogo do TNF-alfa) e UAS-*eiger-IR* (RNA de interferência para o gene *eiger*) (Igaki *et al.*, 2002).

### **1.3.1.2 O sistema Gal4/UAS**

O sistema Gal4/UAS é um método bioquímico usado para estudar a expressão e a função de genes em organismos como a mosca da fruta. UAS e Gal4 não existem naturalmente em *Drosophila* (Duffy, 2002). A ativação do sistema

acontece pelo simples cruzamento entre linhagens expressando Gal4 (*drivers*) com linhagens contendo o elemento UAS (*responder*). Como resultado do cruzamento, a prole irá expressar o gene ligado ao UAS sob um padrão de expressão dirigido por Gal4 (Figura 3) (Duffy, 2002; Elliott e Brand, 2008). Os genes de interesse podem ser repórteres, diferentes isoformas ou de outras espécies e RNAs de interferência. De modo geral, o sistema Gal4/UAS possibilita o resultado da expressão gênica mediante uma análise temporal e espacial (Elliott e Brand, 2008).



**Figura 3** O sistema GAL4/UAS em *Drosophila melanogaster*. Fêmeas portando o responder (UAS-GFP) são cruzadas com machos expressando o driver GAL4 (RE-GAL4), resultando em uma prole que contenha ambos os elementos do sistema. A presença de GAL4 em segmentos embrionários alternados dirige a expressão do responder (UAS-GFP) para esses locais. Figura adaptada de Duffy (2002).

### **1.3.1.3 Explorando os mecanismos genéticos da *Drosophila* para entender a função e regulação do miRNA**

O elav-Gal4 é um dos *drivers* pan-neuronais mais utilizados para conduzir a expressão gênica em todo o sistema nervoso. Manipulações genéticas direcionadas especificamente para neurônios com o sistema binário Gal4/UAS (Brand e Perrimon, 1993) têm sido usadas para indução controlada de genes em *Drosophila melanogaster*. Com o objetivo de verificar os efeitos biológicos e comportamentais nas atividades locomotoras, de aprendizagem e de memória que podem estar associadas a anormalidades de expressão de miRNAs, pode-se fazer a avaliação da superexpressão e do knockout do ortólogo de miRNAs específicos em moscas transgênicas. Estudos que avaliam especificamente os níveis de expressão de miRNAs utilizando a *Drosophila* como modelo têm proporcionado uma maior compreensão da regulação gênica (Smibert e Lai, 2010).

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## **Objetivos**

### **Objetivo Geral**

Avaliar os miRNAs plasmáticos que possam estar associados à DG e possivelmente com a severidade dos sintomas depressivos e a performance cognitiva desses indivíduos;

### **Objetivos Específicos**

- Identificar os miRNAs já descritos na literatura como associados a depressão maior e que também possam estar envolvidos em performance cognitiva;
- Identificar e avaliar a expressão dos miRNAs plasmáticos em idosos com depressão maior;
- Validar por RT-qPCR os miRNAs diferencialmente expressos identificados por SNG;
- Verificar os genes-alvos e as vias biológicas;
- Avaliar os efeitos da expressão dos ortólogos dos miRNAs no comportamento das moscas.

## Capítulo II

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Shared Biologic Pathways Between Alzheimer`s  
Disease and Major Depression: A Systematic Review  
of MicroRNA Expression Studies

## LIST OF FIGURES

**Figure 1** Possible mechanisms linking MDD and AD. A major depressive episode (in young and older adults) leads to activation of distinct biologic events, such as increased proinflammatory status, increased oxidative and nitrosamine stress, mitochondrial dysfunction, cerebrovascular disease, reduced neurotrophic support, impaired intracellular signaling, and hypothalamus-pituitary-adrenal axis (HPA) dysfunction. These biologic events interact with normal aging processes, leading to an enhanced molecular and cellular senescent pattern, shown as genomic instability, cell cycle abnormalities, loss of proteostasis control, and cell death. These changes can lead to self-perpetuating iterative processes that culminate in reduced brain reserve and increased vulnerability to the emergence of neurodegenerative pathology and the early manifestation of dementia in these individuals. PIS: proinflammatory status; ONS: oxidative and nitrosamine stress; MiD: mitochondrial dysfunction; NS: neurotrophic support; IIS: impaired intracellular signaling; CVD: cerebrovascular disease..... 70

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## **2 Shared Biologic Pathways Between Alzheimer Disease and Major Depression: A Systematic Review of MicroRNA Expression Studies**

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### **ABSTRACT**

**Objective:** The clinical–epidemiological relationship between major depressive disorder (MDD) and Alzheimer disease (AD) suggests that they may share common neurobiologic abnormalities. **Methods:** The authors conducted a systematic review and identified microRNAs abnormally expressed in both AD and MDD. The pattern of microRNA regulation in each disorder and the genes regulated by each microRNA and the biologic processes and pathways regulated by these genes were identified. **Results:** Seventy-four microRNAs were abnormally expressed in AD and 30 in MDD; 7 were common for both disorders (hsa-let-7f-5p, hsa-miR-664a-3p, hsa-miR-361-5p, hsa-let-7g-5p, hsa-let-7d-5p, hsa-miR-191-5p, hsa-miR-26b-5p). These microRNAs interact with 45 validated genes, and the main biologic pathways and processes regulated by them were proteostasis control, maintenance of genomic integrity, regulation of transcriptional activity, immune-inflammatory control, and neurotrophic support. **Conclusion:** The current results suggest that the maintenance of genomic integrity, proteostasis control, immune-inflammatory regulation, and neurotrophic support are key neurobiologic links between these conditions. A comprehensive hypothetical model for the interaction between MDD, aging, and the development of AD is provided. (*Am J Geriatr Psychiatry* 2016; 24:903–912)

**Keywords:** Alzheimer disease, major depressive disorder, geriatric depression, microRNAs, biologic pathways, biomarkers.

## 2.1 INTRODUCTION

Alzheimer disease (AD) and major depressive disorder (MDD) are common neuropsychiatric disorders and major causes of disability and premature death in the general population (Murray *et al.*, 2012). Despite clear differences in their clinical manifestations, they have a complex and probably a bidirectional relationship. Cognitive impairment is common during depressive episodes in young and older adults (Butters *et al.*, 2004; Rock *et al.*, 2014) and can persist even after successful antidepressant treatment (Butters *et al.*, 2000; Nebes *et al.*, 2003). On the other hand, patients with AD have higher rates of depressive symptoms than those observed in older adults or elderly people in the general population (Zubenko *et al.*, 2003). Meta-analyses showed that lifetime history and current depressive episodes in the elderly significantly increase the risk of developing AD and vascular dementia (Ownby *et al.*, 2006; Diniz, Butters, *et al.*, 2013).

The complex relationship between MDD and AD suggest that these disorders may share common neurobiologic abnormalities. Several lines of evidence show that subjects with AD or MDD (young and older adult onset) have higher rates of cerebrovascular disease, decreased gray matter, and hippocampal volume (Drevets, 2001; 2004; Smith *et al.*, 2007; Dotson *et al.*, 2009; De La Torre, 2013; Stratmann *et al.*, 2014; Snyder *et al.*, 2015). Data show enhanced peripheral proinflammatory and decreased anti-inflammatory status, reduction of neurotrophic support, increased oxidative stress markers, insulin resistance, and

glycogen synthase kinase (GSK)-3 $\beta$  hyperactivation (Hooper *et al.*, 2008; Dowlati *et al.*, 2010; Swardfager *et al.*, 2010; Teixeira *et al.*, 2010; Diniz *et al.*, 2011; Diniz e Teixeira, 2011). However, no study so far has provided a comprehensive and integrated analysis of biologic abnormalities in both conditions, limiting the understanding of how these conditions are related from a neurobiologic perspective.

MicroRNAs are small noncoding RNAs approximately 22 nucleotides long (Lee *et al.*, 1993). They bind to mRNA, leading to its temporary inactivation or degradation (Filipowicz *et al.*, 2008). Because of its small length, microRNA can bind with low or high affinity to several different mRNAs (Guo *et al.*, 2010; Hogg e Harries, 2014). Additionally, they can be secreted to body fluids (e.g., plasma, cerebrospinal fluid) and may act as paracrine and endocrine factors, influencing gene function at distant sites (Mcdonald *et al.*, 2013; Pacifici *et al.*, 2014; Srivastava *et al.*, 2015). Because of its broad net effect on gene functioning and the ability to modulate a broad network of biological processes, abnormalities in microRNA expression may be associated with different diseases. In fact, several studies have suggested the involvement of microRNAs in various types of cancer and cardiovascular diseases (Lu *et al.*, 2005; Van Rooij *et al.*, 2008; Seok e Wang, 2010; Quiat e Olson, 2013; Condorelli *et al.*, 2014; Maegdefessel, 2014; Pink *et al.*, 2015). Few studies have evaluated the microRNA expression in AD and MDD. Subjects with AD showed abnormal expression of several microRNA, including the hsa-miR-9, hsa-miR-125b, hsa-miR-181c, hsalet-7, and hsa-let-26 families (Cogswell *et al.*, 2008; Lukiw e Alexandrov, 2012; Hébert *et al.*, 2013; Leidinger *et al.*, 2013). Patients with MDD also showed abnormal regulation of different microRNAs, including the hsa-let-7 family, hsa-miR-125a, hsa-miR-191.

However, no study so far has provided a comprehensive and integrated analysis of common biologic pathways related to both AD and MDD, based on microRNA expression studies.

Thus, we carried out a systematic review to identify common microRNAs between MDD and AD and the expression profiles of these diseases. We further evaluated the biologic pathways shared by MDD and AD based on the gene expression regulated by microRNAs abnormally expressed in MDD and AD. Despite that we do not have an a priori hypothesis, we expected to uncover biologic processes and pathways common to both conditions, indicating potential neurobiologic links between these disorders.

## **2.2 METHODS**

### **Search Strategy**

We carried out a comprehensive literature search in the electronic databases PubMed/Medline, Web of Science, and Scopus. These databases provide a broad coverage of biomedical literature. The literature search was conducted in September 2015, and we limited the research to articles published in English. The search terms were “microRNA or miRNA and Alzheimer’s Disease”; “microRNA or miRNA and Major depressive disorder or Depressive disorder or Depression.” Although the link between dementia and major depression may be more evident among older adults, no study evaluated microRNA expression among late-life depression individuals, and we opted to expand our search to include individuals with major depression across the lifespan. Also, past meta-analysis provides strong evidence that major depressive episodes among younger adults is also a significant risk factor for AD (Ownby *et al.*, 2006).

Additionally, we carried out a manual search for relevant articles in the references of the original articles included, as well as in review articles about the role of microRNA in neuropsychiatric disorders.

Two investigators (APM and ESN) independently reviewed the title and abstract of each retrieved article to identify potentially relevant studies based on eligibility criteria. The selected articles were reviewed to verify whether they fulfilled the inclusion criterion for data extraction. Two investigators independently extracted data (APMS and ESN) using a standardized data extraction form. In the case of study selection or data extraction disagreement, a third investigator (BSD) made the final decision on the inclusion of the selected article. Supplementary Figures S1 and S2 show a flowchart detailing the search strategy in each separate research.

The studies were included if the following criterion were fulfilled:

1. Original article
2. Articles that reported human blood microRNAs expression
3. Clinical evaluation for AD and MDD diagnosis
4. Included a comparison group
5. In the case of articles with overlapping samples, the article with the largest sample size was included.

### **Selection of Differentially Expressed MicroRNAs and Target Genes**

For each study (AD or MDD), we selected the microRNA with a  $\log_2\text{foldchange}$  higher or equal  $\pm 1.5$  and  $p \leq 0.05$ . We checked the authenticity of the mature

microRNAs based on the Mirbase database (<http://www.mirbase.org/>). We searched the DIANA (DNA Intelligent Analysis, <http://diana.cslab.ece.ntua.gr/>) databases to identify the validated gene targets of each microRNA. In the DIANA database search, we selected validated genes with a miRNA target genes (miTG) score  $\geq 0.99$  for the binding of the microRNA to the mRNA. The miTG score is a parameter that indicates the accuracy of the predicted microRNA–mRNA interaction target (Sato e Tabunoki, 2011).

### **Pathway Enrichment Analysis**

The gene set enrichment analysis was performed by using ClueGO and CluePEDIA Cytoscape plugin, and the Mirwalk 2.0 databases (Bindea *et al.*, 2009; Dweep *et al.*, 2011; Bindea *et al.*, 2013) from the selected list of common microRNAs in MDD and AD. The functional enrichment of specific Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) (Kanehisa e Goto, 2000) terms used the right-sided hypergeometric test, followed by the Bonferroni-Hochberg correction to control the false discovery rate at  $p < 0.01$ . The p-value is the exact hypergeometric probability distribution of random matches of a subsequence (from the 5' end of the miRNA sequence) in a given sequence (gene) based on a Poisson distribution. The GO assignments were made using the KEGG dataset released 73.0 September 2015 (Kanehisa e Goto, 2000) and the Molecular Signatures Database v5.1 (<http://software.broadinstitute.org/gsea/index.jsp>).

## **2.3 RESULTS**

We included two MMD studies and five AD studies for data extraction (Supplementary Figures S1 and S2). The characteristics of included studies are

shown in Table 1. We identified 30 microRNAs differently expressed in MDD patients and 74 microRNAs differently expressed in AD patients with a fold change  $\geq 1.5$  and  $p \leq 0.05$  (Supplementary Table S1). The microRNAs related to MDD regulate 277 different genes, whereas those related to AD regulate the expression of 442 different genes.

Seven microRNAs were common to MDD and AD (Table 2). All were upregulated in patients with MDD and downregulated in patients with AD. A total of 45 validated genes is regulated by the seven microRNAs, based on the DIANA database (Supplementary Table S2).

Pathway enrichment analysis identified a total of 291 unique biologic pathways and processes associated with the seven common microRNA differentially expressed in AD and MDD. Table 3 shows the top most significant pathways found in the analysis (please refer to Supplementary Table S3 for a full list of biologic pathways and processes). The biologic pathways and processes are mainly related to the modulation of transcriptional regulation, maintenance of genomic integrity and telomere length, proteostasis control, synaptic signaling, immuneinflammatory control, calcium signaling pathway, cell cycle re-entry, commitment and arrest, apoptosis, energy metabolism, neurotrophic signaling pathway, and MAPK signaling pathway.

Because different microRNAs can regulate genes related to the same biologic process or pathway, we evaluated which ones were over-represented in the pathway enrichment analysis. In this context, overrepresentation indicates biologic pathways and processes that are regulated by distinct microRNAs and, thus, may be more relevant to the relationship between MDD and AD. We defined

an over-represented biologic process or pathway if it was associated to three or more distinct microRNAs. This was an arbitrary definition. A total of 98 biological pathways and processes was over-represented in the analysis (Supplementary Table S4). The biologic processes and pathways in this analysis can be grouped into general biologic functions related to the maintenance of genomic integrity, proteostasis control, regulation of apoptotic processes, and neurotrophic support. It is worth noting that these biologic pathways and processes were also among those highly statistically significant in the analysis of individual microRNAs.

## **2.4 DISCUSSION**

This systematic review, aligned with bioinformatics approaches, identified seven microRNAs that were abnormally expressed in both MDD and AD. These microRNAs regulate several biologic functions, and the most significant and over-represented biologic processes and pathways found in the current study were related mainly to the maintenance of genomic integrity, proteostasis control, regulation of apoptotic processes, and neurotrophic support. This is the first comprehensive study that sought to identify common neurobiologic links that help to explain why young and older adults with MDD are at increase the risk of AD. It is worth noting that the seven microRNAs common to AD and MDD showed different patterns of regulation in each disease. They were upregulated in MDD and downregulated in AD subjects. Our results suggest that the regulation dynamics of gene expression and biologic pathways related to these microRNAs are distinct for each disorder.

Loss of genomic integrity (i.e., the cellular machinery responsible for the verification and correction of potential replication and transcriptional errors in the

cells) and of proteostasis control (i.e., the set of integrated biologic pathways that control the biogenesis, folding, trafficking, and degradation of proteins present within and outside the cell) are key features of the senescence process (López-Otín *et al.*, 2013; Chow e Herrup, 2015). The initiating cascades for the accumulation of genomic damages and abnormal proteins in neurons are multifactorial. They involve the interaction between intrinsic repairing mechanisms (e.g., non-homologous end joining, autophagy) and cell level stressors (e.g., activation of stress-related cascades, oxidative stress, mitochondrial dysfunction, increased proinflammatory status, reduced neurotrophic support). The accumulation of genomic and protein damages can lead to the activation of cell cycle re-entry and/or apoptosis cascades and, finally, to the emergence of AD in vulnerable individuals (Borgesius *et al.*, 2011).

Decreased neurotrophic support and impaired neurotrophic signaling pathways are also common features of both MDD and AD (Teixeira *et al.*, 2010). Reduced neurotrophic support can decrease neuroplasticity, impair long-term potentiation, and reduce neuronal gene transcription and resilience (Diniz e Teixeira, 2011). In recent studies, we showed that individuals with late-life depression have significantly lower cerebrospinal fluid and plasma levels of brain derived neurotrophic factor (BDNF) and other growth factors (e.g., nerve growth factor (NGF)) (Diniz, Teixeira, *et al.*, 2013; Diniz, Reynolds, Begley, *et al.*, 2014; Diniz, Teixeira, *et al.*, 2014). The reduction in neurotrophic support was more significant in those with cognitive impairment and correlated with cognitive decline upon follow-up. Likewise, reduced neurotrophic support is an independent predictor of conversion from mild cognitive impairment to AD in older adults (Forlenza, Diniz, *et al.*, 2015; Forlenza, Miranda, *et al.*, 2015). These data, together with the results

of this systematic review, suggest that reduced neurotrophic support is an early event in the cascade of biologic events that render neurons more vulnerable to neurotoxicity insults, including the buildup of  $\beta$ -amyloid in the brain, leading to increased risk of cognitive decline and AD in older adults.

The current results are in line with recent proteomic and transcriptome studies in patients with MDD and with AD. For instance, a study involving community-based adult subjects with MDD showed significant abnormalities in cell communication and signal transduction, protein metabolism, and immune response (Bot *et al.*, 2015). Another study with drug-naive, first-episode MDD subjects showed increased proinflammatory and oxidative stress response, hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, and dysregulation of growth factor pathways (Stelzhammer *et al.*, 2014). In a recent study, our group evaluated the circulating proteomic profile of older adults with MDD and mild cognitive impairment (late-life depression + mild cognitive impairment) compared with those without mild cognitive impairment. We found that subjects with late-life depression + mild cognitive impairment had significant abnormalities in biologic processes related to proteostasis control, immune-inflammatory control, apoptosis, intracellular signaling, lipid metabolism, and clotting processes (Diniz *et al.*, 2015). A meta-analysis of transcriptome and genome wide association study (GWAS) studies showed that altered neurotrophic support, brain plasticity, and neuronal signaling is a core feature of MDD (Ding *et al.*, 2015). Likewise, the emergence of AD-related neurodegenerative changes was associated with abnormal regulation of cellular metabolic processes, maintenance of genomic integrity and telomere length, and immune-inflammatory response (Silva *et al.*, 2012; Begcevic *et al.*, 2013). Therefore, growing evidence from multiple biologic

levels suggests that certain biologic processes, in particular, loss of proteostasis control, loss of genomic maintenance capacity, and decreased neurotrophic support, are common features of both MDD across the lifespan and AD.

The different expression patterns observed in MDD and AD suggests that both diseases have distinct regulation dynamics for the same microRNAs. Because microRNAs are major post-transcriptional regulators of gene expression, the upregulation of microRNA observed in MDD may be a compensatory response to the hyperactivation of related biologic pathways (e.g., increased proinflammatory state, cerebrovascular changes, oxidative and nitrosamine stress). The persistence of this allostatic overload state (e.g., depressive episode chronicity, unsuccessful treatment, relapse, or recurrence of depressive episodes) may lead to exhaustion of repairing mechanisms and enhanced cellular damage (e.g., genomic instability, loss of proteostasis control, shortened telomere length). These events result in lower brain reserve and increased vulnerability to the emergence and manifestation of neurodegenerative diseases such as AD. Also, a biologic consequence of the exhaustion of these compensatory mechanisms might be the downregulation of microRNAs observed in patients with AD (Figure 1).

Other biologic processes can be involved in the association between major depression and AD, in particular, proinflammatory changes and increased cerebrovascular burden. A mild increase in proinflammatory status is a common feature of MDD across the lifespan and of AD (Alexopoulos e Morimoto, 2011; Heneka *et al.*, 2015). Some lines of evidence suggest that the mild increase in the proinflammatory status is one of the main drivers of senescence-related biologic changes, a process named “inflammaging” (Franceschi, 2007). An

increased cerebrovascular burden is also a feature of brain aging. Past studies suggest that individuals with late-life depression and AD show increased cerebrovascular burden, independent of age and presence of cardiovascular risk factors (Taylor *et al.*, 2013). Enhanced proinflammatory status and increased cerebrovascular burden can lead to increased oxidative stress levels, mitochondrial dysfunction, and stimulation of cell death by inducing apoptosis, rendering patients with MDD more vulnerable to the development of AD and other dementia syndromes, for example, vascular dementia (Diniz, Butters, *et al.*, 2013). Our results did not find evidence that the common microRNAs between MDD and AD regulate these biologic processes. A possible explanation is that changes in inflammatory regulation and emergence of cerebrovascular disease are related to biologic abnormalities that do not involve changes in microRNA expression in these patients.

The current results must be viewed with caution. This is a hypothesis-generation study, and the microRNAs and results described herein need to be validated in large, independent longitudinal studies including MDD subjects across the lifespan trajectory and with a comprehensive cognitive assessment to ascertain the development of AD or other neurodegenerative diseases. The studies included in the systematic review used different laboratory methodologies and statistical approaches for the discovery and validation of differentially expressed microRNA. Furthermore, the sample sizes were relatively small for all studies. These factors may significantly influence the individual study results and therefore compromise the results of the systematic review. To overcome this limitation, we took a very conservative approach to select the differentially expressed microRNAs in MDD and AD. The more conservative approach, in turn,

may incur a risk of missing potentially significant microRNAs in both conditions. Moreover, it should be noted that the MDD studies included in the systematic review evaluated young and middle-aged adults, limiting the generalization of finding to older adults with major depression. Finally, despite analyzing the biologic processes and pathways from different sources and algorithms, it relies on databases that, in spite of providing comprehensive coverage of known biologic processes and molecular functions, are under continuous updating as novel biologic processes and molecular functions related to the microRNAs are described.

MicroRNAs are attractive therapeutic targets for the development of new interventions for MDD and AD. Although the field of microRNA-based therapeutics is still in its early stages, already a few compounds are being developed and tested for cancer and cardiovascular diseases (Van Rooij e Kauppinen, 2014). There is no current active study on microRNA-based therapeutics in MDD or AD ([www.clinicaltrials.gov](http://www.clinicaltrials.gov); accessed on December 3, 2015). Our results describe new potential targets for the development of intervention strategies aiming to prevent the development of cognitive impairment and AD in subjects with MDD. However, these targets need to be validated in future studies to confirm its therapeutic potential.

In conclusion, the current results provide a comprehensive neurobiologic model for the interaction between MDD, aging, and the development of AD. Our data suggest that biologic processes related to the maintenance of genomic integrity, proteostasis control, immune-inflammatory regulation, and neurotrophic support are key neurobiologic links between these conditions. Future studies are

necessary to confirm the role of microRNAs as biomarkers and therapeutic targets for MDD and AD.

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#### **APPENDIX: SUPPLEMENTARY MATERIAL**

Supplementary data to this article can be found online at [doi:10.1016/j.jagp.2016.07.017](https://doi.org/10.1016/j.jagp.2016.07.017).

**Table 1** Characteristics of Studies Included in the Systematic Review

Articles	Patients (N in parentheses)	Age (mean $\pm$ SD)	Sex (M/F)	Biologic Samples	NGS (Discovery Phase)	RT-qPCR (Validation Phase)	Statistical Methods
Leidinger et al., 2013 <sup>39</sup>	AD (106), HC (22), DEP (15)	72.7 $\pm$ 10.4 (AD) 67.1 $\pm$ 7.5 (HC) 45.2 $\pm$ 9.1 (MDD)	53/53 (AD) 11/11 (HC) 15/0 (MDD)	Blood samples	Illumina HiSeq 2000 sequencer Cohort size AD (48), HC (22)	miScript PCR System (Qiagen) Cohort size AD (106), HC (22), DEP (15)	miRDeep2 pipeline, support vector machines (SVM) from the R package e1071, linear kernels
Galimberti et al., 2014 <sup>45</sup>	AD (22), HC (8)	72.77 $\pm$ 0.65 (AD) 66.54 $\pm$ 0.22 (HC)	8/14 (AD) 2/6 (HC)	Serum and CSF samples	Applied Biosystems 7500 FAST system Cohort size AD (22), control subjects (8)	SABiosciences PCR Array Cohort size AD (22), control subjects (8)	$\Delta\Delta$ Ct method with Bonferroni correction, Spearman test
Tan et al., 2014 <sup>46</sup>	Cohort NGS AD (50), HC (50) Cohort validation AD (158), HC (155)	Cohort NGS 72.65 $\pm$ 4.51 (AD) 71.64 $\pm$ 5.28 (HC) Cohort validation 77.43 $\pm$ 7.40 (AD) 76.86 $\pm$ 7.00 (HC)	Cohort NGS 25/25 (AD) 25/25 (HC) Cohort validation 78/80 (AD) 70/85 (HC)	Blood samples	Illumina HiSeq 2000 sequencer	CFX96 Touch Real-Time PCR Detection System (Biorad)	McDonald's method, $\Delta\Delta$ Ct method
Bhatnagar et al., 2014 <sup>47</sup>	Cohort 1 AD (110), HC (123) Cohort 2 AD (25), HC (27)	Cohort 1 80.0 $\pm$ 6.17 (AD) 72.0 $\pm$ 1.31 (HC) Cohort 2 82.0 $\pm$ 4.11 (AD) 80.0 $\pm$ 3.26 (HC)	—	Blood samples	—	7500 Real-Time PCR System (Applied Biosystems) Cohort 1 e Cohort 2	$\Delta\Delta$ Ct method
Kumar et al., 2013 <sup>48</sup>	Cohort 1 AD (11), MCI (9), HC (20) Cohort 2 AD (20), HC (17)	Cohort 1 79.64 $\pm$ 5.97 (AD) 77.44 $\pm$ 6.16 (MCI) 66.61 $\pm$ 5.78 (HC) Cohort 2 69.3 $\pm$ 6.18 (AD) 65.58 $\pm$ 4.44 (HC)	Cohort 1 6/5 (AD) 3/6 (MCI) 10/8 (HC) Cohort 2 10/10 (AD) 9/8 (HC)	Plasma samples	nCounter Digital Analyzer (v.20081009) Cohort 1 AD (11), MCI (9), NC (20)	ABI7500 Cohort 1 e Cohort 2	$\Delta\Delta$ Ct method
Belzeaux et al., 2012 <sup>49</sup>	MDD (16), HC (13)	55.3 $\pm$ 2.71 (MDD) 55.5 $\pm$ 2.55 (HC)	9/7 (MDD) 6/7 (HC)	PBMCs	Agilent DNA Microarray Scanner (G2505C)	ABL PRISM 7900 HT thermocycler (Applied Biosystems)	Discriminant function analysis
Liu et al., 2014 <sup>50</sup>	HC (14), MDD (16)	32.6 $\pm$ 9.6 (HC) 34.8 $\pm$ 13 (MDD)	4/10 (HC) 5/11 (MDD)	Blood samples	miRCURY LNA Array system	ABL PRISM 7900 HT thermocycler (Applied Biosystems)	t test

Notes: SD: standard deviation; NGS: next-generation sequencing; RT-qPCR: quantitative real-time polymerase chain reaction; HC: healthy control; DEP: depression; CSF: cerebrospinal fluid; MCI: mild cognitive impairment; PBMC: peripheral blood mononuclear cells.

**Table 2** List of the Common MicroRNAs between MDD and AD

MicroRNAs	MDD			AD		
	p-value	FC	Regulation	p-value	FC	Regulation
hsa-miR-664a-3p	0.02	3.67	Up	0.002	1.50	Down
hsa-miR-361-5p	0.04	2.40	Up	9.16E-07	7.08	Down
hsa-miR-26b-5p	0.02	3.94	Up	0.038	6.3	Down
hsa-miR-191-5p	0.04	2.27	Up	7.41E-06	2.22	Down
				<0.0001	5.15	
				<0.0001	6.76	
hsa-let-7d-5p	0.03	2.90	Up	2.69E-12	2.03	Down
				<0.0001	3.03	
				<0.0001	4.73	
hsa-let-7g-5p	0.03	3.46	Up	1.38E-24	2.30	Down
				<0.0001	2.62	
				<0.0001	3.92	
hsa-let-7f-5p	0.03	4.02	Up	<0.0001	1.68	Down

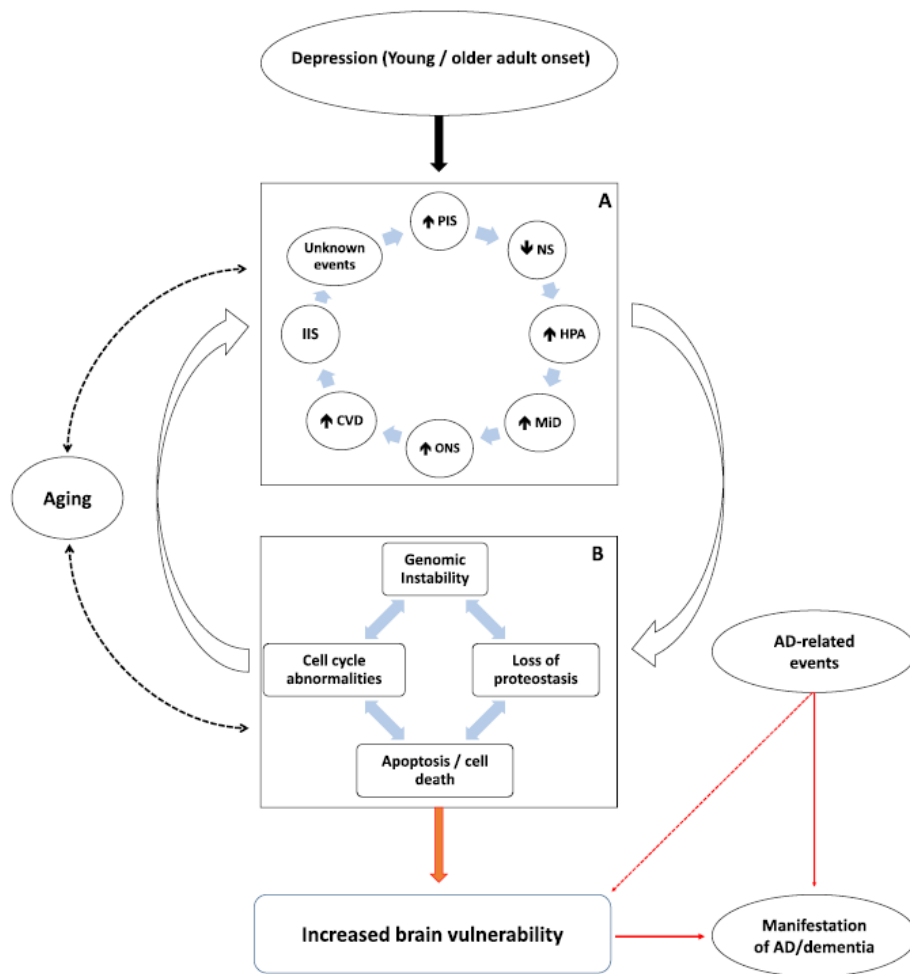
Note: FC: fold change.

**Table 3** Pathway Enrichment Analysis for the Common MicroRNAs in MDD and AD (Top 12 Biological Pathways and Processes)

MicroRNA	PathName	PathFg	PathBg	GenomeFG	GenomeBG	Exact Hypergeometric p-value (BH Correction)	GO/KEGG
hsa-let-7d-5p	Actin cytoskeleton organization	97	126	11000	19747	1.83E-09	GO
hsa-miR-26b-5p	Small cell lung cancer	56	84	7547	19747	2.21E-09	KEGG
hsa-let-7d-5p	Endocytosis	90	116	11000	19747	2.24E-09	GO
hsa-miR-361-5p	Epidermal growth factor receptor signaling pathway	85	130	8586	19747	2.59E-09	GO
hsa-miR-664a-3p	Palate development	45	61	8483	19747	4.41E-09	GO
hsa-miR-664a-3p	Ubiquitin mediated proteolysis	87	134	8483	19747	4.42E-09	KEGG
hsa-miR-26b-5p	Intracellular signal transduction	132	247	7547	19747	4.53E-09	GO
hsa-miR-26b-5p	Regulation of rho protein signal transduction	41	59	7547	19747	4.93E-09	GO
hsa-let-7g-5p	Adherens junction	59	76	9716	19747	6.31E-09	KEGG
hsa-miR-26b-5p	Colorectal cancer	56	86	7547	19747	7.17E-09	KEGG
hsa-miR-361-5p	Ion transport	84	131	8586	19747	7.18E-09	GO
hsa-let-7d-5p	wnt receptor signaling pathway	103	138	11000	19747	7.42E-09	GO

*Notes:* PathName is the biologic pathway associated, PathFg is the number of genes predicted as putative targets in a given pathway, PathBg is the number of genes in a given pathway, GenomeFG is total number of target genes within genome, GenomeBG is the total number of genes within genome (nomenclature based on the MiRwalk 2.0 database outputs). The p-value is based on a Poisson distribution and is the probability distribution of random matches of a subsequence (from the 5' end of miRNA sequence) in a given sequence (gene) is calculated by using Poisson distribution, where a low probability implies a significant hit.

**Figure 1** Possible mechanisms linking MDD and AD. A major depressive episode (in young and older adults) leads to activation of distinct biologic events, such as increased proinflammatory status, increased oxidative and nitrosamine stress, mitochondrial dysfunction, cerebrovascular disease, reduced neurotrophic support, impaired intracellular signaling, and hypothalamus-pituitary-adrenal axis (HPA) dysfunction. These biologic events interact with normal aging processes, leading to an enhanced molecular and cellular senescent pattern, shown as genomic instability, cell cycle abnormalities, loss of proteostasis control, and cell death. These changes can lead to self-perpetuating iterative processes that culminate in reduced brain reserve and increased vulnerability to the emergence of neurodegenerative pathology and the early manifestation of dementia in these individuals. PIS: proinflammatory status; ONS: oxidative and nitrosamine stress; MiD: mitochondrial dysfunction; NS: neurotrophic support; IIS: impaired intracellular signaling; CVD: cerebrovascular disease.



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## Capítulo III

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Brain enriched MicroRNA-184 is downregulated in older adults with major depressive disorder: a translational study

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### **3 Brain enriched MicroRNA-184 is downregulated in older adults with major depressive disorder: a translational study.**

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#### **ABSTRACT**

Late-life depression (LLD) is the most common psychiatric disorder and causes severe consequences in elderly. The biological mechanisms involved in LLD are complex and involve many genes. MicroRNAs (miRNAs) are small noncoding RNAs and post-translational regulators of gene expression. Imbalance in miRNAs expression is related to many diseases, including LLD. Our aim was to evaluate plasma miRNAs that are associated with LLD and possibly with the severity of depressive symptoms and cognitive performance in these individuals. A total of 116 older adults (63 with LLD and 53 elderly controls) were randomly split into discovery and validation set, using next generation sequencing (NGS) and RT-qPCR, respectively. *Drosophila melanogaster* was used as a translational model to evaluate behavioral phenotypes associated to the overexpression and knockout of the ortholog of hsa-miR-184. NGS analysis found hsa-miR-184 ( $\log_2\text{foldchange} = -4,205$  and  $p\text{-value} = 1,195e^{-03}$ ) and hsa-

miR-1-3p ( $\log_2\text{foldchange} = -3,444$  and  $p\text{-value} = 1,3251e^{-02}$ ) differently expressed in LLD subjects, but only hsa-miR-184 (AUC=8378,  $p\text{-value} < 0.0001$ ) was validated in an independent sample. hsa-miR-184 was significantly correlated with the severity of depressive symptoms ( $r=-0.425$ ,  $p=0.001$ ) and the initiation/perseverance sub-score ( $r=0,292$ ,  $p=0,032$ ) of the Dementia Rating Scale. The translational model showed that the knockout of the ortholog of hsa-miR-184 impaired locomotor and memory performance. These results suggest that hsa-miR-184 may play a significant physiopathological role in LLD.

**Keywords:** Major depression, late life depression, miR-184, MicroRNAs, Biomarker, *Drosophila melanogaster*

### 3.1 INTRODUCTION

Late-life depression (LLD) is one of the most common mental disorders in the elderly, with prevalence rates ranging from 1%-5% (for major depressive episodes) (Byers *et al.*, 2010). Its occurrence increases the risk of adverse health outcomes, including higher rates of medical morbidity (Blazer, 2003), mortality (Diniz, Reynolds, Butters, *et al.*, 2014), and increased risk of Alzheimer's disease and Vascular dementia (Diniz, Butters, *et al.*, 2013). Beyond disease, LLD has been linked to decreased health span as measured by fewer years lived without frailty, significant functional impairments, elevated direct and indirect costs (Butters *et al.*, 2004; Ferrari *et al.*, 2013; St John *et al.*, 2013; Zivin *et al.*, 2013).

The biological mechanisms of LLD are not fully understood, but they are probably heterogeneous and involve the interaction of abnormalities in multiple biological pathways (Alexopoulos e Morimoto, 2011; Diniz *et al.*, 2011; Naismith *et al.*, 2012; Pomara *et al.*, 2012). Recent comprehensive, multi-modal biomarkers

studies showed that subjects with LLD had significant abnormalities in biological pathways related to inflammatory response control, tissue remodeling, endothelial and vascular function, proteostasis, neurotrophic support, and enhanced molecular senescence abnormalities (Diniz *et al.*, 2015; Diniz *et al.*, 2016; Diniz *et al.*, 2017).

MicroRNAs (miRNAs) are small (18-23 nucleotides) non-coding RNAs that are involved in the posttranscriptional regulation of gene expression by base-pairing with complementary mRNA sequences (Bartel, 2009). They can modulate the expression of multiple different genes and, thus, can have to influence multiple biological pathways. MiRNAs are produced inside the cells and can be secreted and transported by proteins, lipids, or be packed into exosomes or microvesicles, being able to modulate gene expression in different cells and tissues (Weber *et al.*, 2010; Gallo *et al.*, 2012; García-López *et al.*, 2013; Salido-Guadarrama *et al.*, 2014). They are less susceptible to RNA degradation and can be detected in different body fluids such as plasma, serum and cerebrospinal fluid (Turchinovich *et al.*, 2012). Therefore, they can be viewed as noninvasive biomarkers that can detect and monitor the dysregulation of multiple biological pathways in different disorders.

Recent studies have identified several miRNAs enriched in human brain (Maffioletti *et al.*, 2014; Azevedo *et al.*, 2016). They regulate genes that are involved in the regulation of neural processes, such as neurogenesis, neuroplasticity, as well as more general processes like neuroinflammatory response, metabolism, and cell-cycle regulation (Olde Loohuis *et al.*, 2012; Mendes-Silva *et al.*, 2016). Accordingly, abnormalities in the miRNA expression

patterns can be major players in the pathophysiology of psychiatric disorders (Geaghan e Cairns, 2015).

Previous studies have identified changes in miRNAs expression (e.g., miR-124a, miR-16, miR-26b, miR-34 family, and the miR-let-7 family) in young and middle-aged adults with major depressive disorder (Bocchio-Chiavetto *et al.*, 2013; Lopez *et al.*, 2014; Wan *et al.*, 2015; Azevedo *et al.*, 2016). More recently, they were identified as biomarkers of treatment response in major depression (Fiori *et al.*, 2017; Roy *et al.*, 2017). However, these studies did not screen for miRNAs using unbiased, whole-genome sequencing methods, and included relatively small sample sizes of patients and controls. Moreover, they mostly included younger adult population, and no study has evaluated miRNA expression changes in LLD.

Therefore, we aimed to investigate changes in miRNA expression in subjects with LLD. We used next-generation sequencing (NGS) for the unbiased discovery of miRNA differentially expressed in these subjects. We validated the NGS findings using quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis in an independent sample of LLD and healthy controls (HC). Bioinformatics analyses were carried out to predict miRNA target genes and to evaluate the biological pathways associated with miRNA expression changes. Finally, we used the *Drosophila melanogaster* as a translational model to validate the role of miRNA expression in the brain in behavioral changes.

## 3.2 METHODS AND MATERIALS

### Sample recruitment and assessment

We included a convenience sample of 116 older adults (63 with LLD and 53 HC) in this study. All patients with LLD were recruited and evaluated at the Psychogeriatric Outpatient Clinic at the Federal University of Minas Gerais, Belo Horizonte, Brazil after referral for the assessment of depressive symptoms. The subjects in the control group were recruited as part of an ongoing cohort study of healthy cognitive aging at the Federal University of Minas Gerais. All participants were above 60 years old at the time of study recruitment.

All participants underwent a comprehensive psychiatric, clinical, and neurological assessments. The psychiatric assessment included the administration of the Mini Neuropsychiatric Interview (MINI) (Sheehan *et al.*, 1998). The diagnosis of LLD was based on the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders – Fifth Edition) criteria for major depressive episode (single and recurrent). The severity of depressive symptoms was rated with the Hamilton Depression Rating Scale - 21 items (HDRS-21) (Hamilton, 1960). Inclusion criteria for controls were no history of major depressive disorder or other major psychiatric disorder, and no evidence of cognitive impairment based on the DRS scores. All participants (LLD and controls) were not under current antidepressant treatment at the time of psychiatric assessment and blood collection. Additional exclusion criteria for this study were the presence of unstable medical illness, history of auto-immune disease, chronic use of anti-inflammatory drugs medication, history of substance abuse disorder in the past year.

We administered the Mattis Dementia Rating Scale (DRS) for neurocognitive assessment and to exclude potential dementia cases in this population (Marson *et al.*, 1997). The DRS provides scores for different cognitive domains (executive function, attention, visuoconstructional ability, language, and episodic memory) and also a total score that reflects global cognitive performance.

These subjects were randomly split into two sets: discovery set (24 with LLD and 19 HC); validation set (39 LLD and 34 HC). The discovery set sample was used in the NGS analysis for the discovery of miRNAs differentially expressed between LLD and control groups. The findings on the discovery set sample were independently validated in the validation set sample using RT-qPCR.

The sociodemographic and clinical and characteristics of the sample are shown in Table 1. The study and its procedures were approved by the ethics committee of the Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

### **RNA extraction from plasma samples**

Whole blood was collected by venipuncture in EDTA coated vacutainers and plasma was separated by centrifugation immediately after collection and stored at -80°C until further use. Total RNA containing microRNA was extracted from 200 µl plasma using mirVana PARIS kit (Ambion, Austin, TX/USA) (Yoon *et al.*, 2017). Extracted RNA was eluted in 50 µl of preheated nuclease-free water and measured on Qubit (Thermofisher, Waltham, MA/USA), then immediately stored at -80°C.

### **NGS sequencing**

Sequencing large number of individuals, which is often needed for population genetics studies, is economically challenging despite falling costs of NGS. *Pool-seq* is an alternative cost- and time-effective option to analyze several individuals (Liu, Y. *et al.*, 2014; Anand *et al.*, 2016). Plasma samples of 43 individuals (19 HC and 24 LLD) was used the discovery set in NGS. Control subjects were divided into 3 pools (6 to 7 individuals per pool) and LLD subjects were divided into 6 pools (3 to 5 individuals per pool). Each pooled sample was processed, and libraries were prepared using Total Ion RNA-Seq v2 kit (Life Technologies, Carlsbad, CA/USA) and were carried on Ion PI (Life Technologies) chip prepared and loaded in an automated Ion Chef (Life Technologies) system. Sequencing was performed on the Ion Proton sequencer (Life Technologies) using the Ion PI™ Hi-Q™ Sequencing 200 Kit, and all procedures were according to the manufacturer's instructions

FASTQ sequencing data was obtained using Ion Torrent Suite. Sequenced single-end reads were trimmed and filtered using PRINSEQ (Schmieder e Edwards, 2011). Low-quality sequences were trimmed from the ends until a base pair of *Phred* quality score  $\geq 20$  (at least 99% accurate) was not found.

### **Small RNA-Seq analysis**

miRDeep2 was used to align mapped reads to the human reference genome sequence hg19 via the mapper.pl script (Friedländer *et al.*, 2012). Mature and hairpin miRNA sequences were obtained from miRBase release 21 (<http://www.mirbase.org/>). A collapsed reads of the mapped reads were processed by miRDeep2.pl script, and miRNA read counts were generated. The miRNA read counts (at least 1 read) were used as input into the Bioconductor

DESeq v. 2.0 (DESeq2) package (Anders e Huber, 2010) for further differential expression analysis. Differential expression of LLD and HC miRNAs was based on a negative binomial distribution using adjusted  $p < 0.05$  and  $\log_2$  values of fold difference.

### **MiRNA extraction and RT-qPCR**

For validation analysis, miRNA was extracted from 200  $\mu\text{l}$  of plasma samples (34 HC and 39 LLD) using mirVana PARIS kit (Ambion, Austin, TX/USA). Before extraction, a 200 fmol of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) was spiked into each plasma sample, according to the manufacturer's recommendations and previous studies (Farina *et al.*, 2014; Yoon *et al.*, 2017). Extracted miRNA was eluted in 50  $\mu\text{l}$  of preheated nuclease-free water and measured on NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA/USA), then immediately stored at  $-80^\circ\text{C}$ .

TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA/USA) was used for determining miRNA levels in plasma samples for seven candidate miRNAs (hsa-miR-100-5p, hsa-miR-184, hsa-miR-1-3p, hsa-miR-140-3p, hsa-miR-5572, hsa-miR-125b-5p, hsa-let-7a-5p) based in NGS results. U6 expression and a spike-in (cel-miR-39) were used as an endogenous and exogenous reference controls, respectively. These assays target only a mature form of the specific miRNA, which ensures a biologically relevant result. Reverse transcription (RT) was performed using TaqMan miRNA RT kits according to the instructions from manufacturer's recommendations. Briefly, the cDNA was synthesized from total miRNA (10 ng) using miRNA-specific primers in a 15- $\mu\text{l}$  reaction volume. The RT reaction was performed using the following thermal cycling program: 30 min at

16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The RT product was diluted to 100ng/ µl, and 4 µl of the product was used in a total reaction volume of 20 µl for relative quantification by real-time PCR using an ABI 7500HT system (Applied Biosystems). The thermal cycling program used for the quantification was as follows: 50°C for 2 min, 95°C for 10 min and followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The cycle threshold (Ct) number is defined as the cycle number at which the fluorescence crossed the fixed threshold. The Ct number was calculated using the second derivative method in the ABI software.

### **Data analysis of RT-qPCR**

TaqMan gene expression assay raw Cq values of miRNAs were imported into the qbasePLUS (Biogazelle, Ghent, Belgium) software (Hellemans *et al.*, 2007). The same cDNA samples were used for all of these analyses. The qbasePLUS algorithm enabled normalization to more than one reference gene and also employed inter-run calibrator signals to minimize inter-run variability (Hellemans *et al.*, 2007) distinguishing it from the  $\Delta\Delta\text{Ct}$  method (Livak e Schmittgen, 2001). All data were expressed as calibrated normalized relative quantities (CNRQs). GraphPad 5 (GraphPad Software, San Diego, CA/USA) was used for statistical analysis. Differences between variables were examined for statistical significance using the Student's t-test and p-values < 0.05 were considered statistically significant. The receiver operating characteristic (ROC) curve and the area under curve (AUC) were used as an accuracy index for evaluating the diagnostic performance of the selected miRNA panel.

We carried out a Student t-test to evaluate differences in sociodemographic, HDRS-21 and DRS scores, and miRNAs levels between LLD and control

subjects. We also carried out Pearson correlation analysis to investigate the association between miRNAs, cognitive performance, and depressive symptoms.

### **Pathway enrichment analysis**

The gene set enrichment analysis was performed by using ClueGO and CluePEDIA Cytoscape plugin (Shannon *et al.*, 2003; Bindea *et al.*, 2009; Bindea *et al.*, 2013), and the Mirwalk 2.0 databases (Dweep *et al.*, 2011) from the selected microRNA that was differently expressed in HC and LLD in NGS and RT-qPCR results. The functional enrichment of specific Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (Kanehisa e Goto, 2000) used the right-sided hypergeometric test, followed by the Bonferroni-Hochberg correction to control false-discovery rate (FDR) at a p-value of < 0.01. The GO assignments were made using the KEGG dataset released 73.0 September 2015 (Kanehisa e Goto, 2000) and the Molecular Signatures Database v5.1 (<http://software.broadinstitute.org/gsea/index.jsp>). A density score evaluated the significant categories (Bader e Hogue, 2003). Subsequently, we investigated the substructure of the main protein interaction network extracted from intersecting networks and focused on highly connected nodes, known as clusters, using the molecular complex detection algorithm (Bader e Hogue, 2003). A cluster is a complete n-node sub-graph representing highly interconnected regions, which were obtained by using the parameter set: K-core = 4, node score cut-off = 0.3 and max depth up to 100.

**Drosophila melanogaster:** To further examine the role of miRNA expression in brain function, we evaluated the behavioral phenotypes of *Drosophila melanogaster* flies with knockout or hyper-expressing the ortholog of miRNAs that were differentially expressed in LLD. *D. melanogaster* models of human diseases provide several unique features such as powerful genetics, short life cycle, highly conserved disease pathways, and low maintenance costs. Many basic biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*, and nearly 75% of human disease-causing genes have a functional homolog in the fly (Reiter *et al.*, 2001). We used the elav-Gal4 and GAL4–UAS system to control the miRNA expression in the flies. Methodological details of the genetic manipulation and behavioral assays are shown in the Supplementary Material 1.

### 3.3 RESULTS

#### **miRNA discovery with NGS**

We first examined the miRNA expression from plasma from 43 participants (24 LLD and 19 HC) using NGS. The NGS analysis generated a total of 33,264,170 raw reads which were used to construct the sRNA libraries of the HC and LLD samples. The valid reads of the LLD and HC libraries were obtained after removal of the corrupted adapter sequences, reads with length <13 and >26 nt and junk reads.

Using miRdeep2, which identifies known microRNAs from small RNA-seq, we identified a total of 105 circulating miRNAs in LLD and controls (Figure 1 and Supplementary Table 1). Most of the miRNAs were expressed in both HC and

LLD. Subjects with LLD showed a significant downregulation of the expression of hsa-miR-184 ( $\log_2\text{foldchange} = -4.21$ ,  $p = 1.2 \times 10^{-3}$ ) and hsa-miR-1-3p ( $\log_2\text{foldchange} = -3.45$ ,  $p = 1.3 \times 10^{-2}$ ), after adjustment for multiple analyses (false-discovery rate q-value < 0.05).

### **Validation of miRNA expression with RT-qPCR**

We selected the two miRNAs that showed statistically significant differences in expression levels between LLD and controls (hsa-miR-184 and hsa-miR-1-3p) in NGS analysis to validate. However, the small sample size of the discovery set sample and the pooling strategy for NGS analysis can lead to a small variance in the miRNA count and, as a consequence, findings of non-statistically significant differences even for miRNA with large effect size differences (i.e., statistical error type II). To avoid the risk of false-negative results from the NGS analysis, we also included more 5 miRNAs whose *log<sub>2</sub>foldchange* was greater than 1.5 and read count of more than 10 in both groups (hsa-miR-100-5p, hsa-miR-125b-5p, hsa-miR-5572, hsa-miR-140-3p, hsa-let-7a-5p). The miRNAs specific forward primers used in RT-qPCR are shown in Supplementary Table 2.

The expression levels of the 7 miRNAs selected for validation were examined in plasma by RT-qPCR. All miRNAs were detected in our samples (Ct value < 35). Of note, cel-miR-39 and endogenous control U6 expression were stable, with no significant differences between LLD and controls.

The relative quantification of circulating miRNAs hsa-miR-184, hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p in plasma are shown in Figure 2. MiRNAs hsa-miR-1-3p, hsa-miR-125b-5p, and hsa-let-7a-5p were detected in less than 30% of total samples and were not included in the statistical analysis.

The hsa-miR-184 was significantly downregulated in LLD compared to controls ( $p < 0.001$ ) (Figure 2). There were no statistically significant differences in the expression of hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p between LLD and controls. ROC curve analysis in plasma samples showed that hsa-miR-184 can discriminate HC from LLD subjects (AUC = 0.84,  $p < 0.0001$ ) (Figure 3A). The ROC curve analysis of the other three miRNAs was not statistically significant (Figures 3B, 3C, and 3D).

Correlation analysis using the whole sample showed that hsa-miR-184 expression was significantly correlated with the severity of depressive symptoms ( $r = -0.425$ ,  $p = 0.001$ ), worse scores in the DRS Initiation/Perseveration item ( $r = 0.292$ ,  $p = 0.03$ ), and years of education ( $r = 0.260$ ,  $p = 0.04$ ). There were no significant correlations between hsa-miR-184 and other demographic and clinical variables.

### **Bioinformatics analyses**

Mirwalk 2.0 dataset found 463 genes target genes of miR-184 (3' and 5'UTRs). Protein-protein interaction network build using the genes targets showed 1042 nodes and 68 clusters. The biological pathways and processes were mainly related to the regulation of the immune-inflammatory processes (MAP kinase, Calmodulin-kinases, NF-kappa B signaling pathway), second messenger signaling (TRP channels activation, regulation of activation of G protein in gated potassium channels calcium signaling pathway, GABA receptor activation), metabolic control (insulin signaling pathway), neurotrophic support (neurotrophic signaling pathway, VEGF signaling pathway), proteostasis and senescence-related pathways (mTOR signaling pathway) (Supplementary tables 3 and 4).

**Validation of behavioral phenotype in genetically modified *Drosophila melanogaster* knockout-expressing and over-expressing the ortholog of hsa-miR-184.**

We used the negative geotaxis RING assay to evaluate the locomotor activity of the of flies. We include 3 groups of flies:  $\Delta$ miR-184 (knockout of the ortholog of hsa-miR-184), elav-Gal4>UAS-miR-184 (overexpression of the ortholog of hsa-miR-184), and elav-Gal4 flies (parental control). We assessed locomotor activity in three different ranges of days post-eclosion (d.p.e), 0-4 d.p.e, 11-15 d.p.e and 21-25 d.p.e.

Figure 4 shows the results for the flies' locomotor activity. We observed an age-dependent effect of miR-184 knockout expression in the flies' locomotor activity. Younger knockout flies (0-4 d.p.e.) did not show significant differences in locomotor activity, while middle-aged flies (11-15 d.p.e.) had higher locomotor activity, and older flies (21-25 d.p.e.) had significantly worse locomotor activity when compared to the control flies (Figure 4A). Flies overexpressing the miR-184 showed a significantly higher locomotor activity in all ages when compared to control flies (Figure 4B). As expected, flies with miR-184 knockout expression had significantly worse locomotor activity compared to flies with miR-184 overexpression (Figure 4C).

We used the aversive odor stimulus training to investigate the effects miR-184 expression on memory retention. The flies were evaluated 6 hours (short-term memory) and 24 hours (long-term memory) after the aversive odor stimulus.

Flies knockout of miR-184 showed worse short-term and long-term memory compared to control flies in an age-dependent fashion (Figure 5A). The flies

overexpressing miR-184 showed better short and long-term memories in the 11-15 d.p.e. group when compared to control flies (Figure 5B). Finally, flies knockout of miR-184 showed significantly worse short and long-term memories compared to flies overexpressing miR-184 at all d.p.e. ranges. (Figure 5C).

Together these data from genetically modified *Drosophila melanogaster* model suggest that the knockout of the ortholog of hsa-miR-184 had a depressive-like behavioral phenotype, i.e., reduced locomotor activity and worse memory performance. Conversely, the overexpression of the miR-184 significantly reversed these phenotypes.

### **3.4 DISCUSSION**

Our study provides the first NGS analysis, with independent validation, of whole-genome circulating microRNA expression in LLD. We showed that miR-184 was significantly downregulated in LLD, and significantly correlated with the severity of depressive symptoms and executive dysfunction in this sample. Our results are in line with a previous study showing the downregulation of miR-184 in the anterior cingulate cortex (post-mortem brain tissue) of a small sample of middle-aged adults with major depression (Azevedo *et al.*, 2016). We further biologically validated the role of miR-184 to induce behavioral changes in flies. We found that miR-184 knockout flies had less locomotor activity and worse memory capacity that were more intense at older flies. The over-expression of miR-184 reversed these phenotype changes. Our results suggest that the down-regulation of miR-184 is a potential mechanism for the development of depressive symptoms and cognitive impairment in older adults.

The miR-184 is most abundantly expressed in the brain<sup>52,53</sup>, and our study is the first to identify changes in expression in LLD. Enrichment pathway analysis showed that this miRNA is involved in the control of immune-inflammatory cascades, maintenance of neuronal structural integrity and plasticity, metabolic control, proteostasis and senescence regulation. Abnormalities in these cascades have been previously described in subjects with LLD using proteomics approach. For instance, our group and others have shown that individuals with LLD have greater activation of pro-inflammatory cascades, proteostasis dysregulation, reduced neurotrophic support and enhanced senescence molecular changes (Diniz *et al.*, 2016; Diniz *et al.*, 2017). Moreover, changes in these biological cascades are more intense in those subjects with LLD and comorbid cognitive impairment (Diniz *et al.*, 2015). Interestingly, in our study, the miR-184 was significantly associated with the severity of depressive symptoms and executive dysfunction, a common feature of LLD. Thus, the downregulation of miR-184 may be a common mechanism underlying the severity of depressive symptoms and cognitive impairment in older adults. Moreover, it can be a potential therapeutic target for both conditions.

Based on the findings from subjects with LLD, we used genetically modified model *Drosophila melanogaster* to further evaluate the impact of miR-184 expression on brain function and phenotype. *Drosophila melanogaster* is a powerful translational model to evaluate the effect of gene modification on biological functions, physiological and behavioral changes related. The fly shares around 75% of gene homology with humans, its maintenance is low cost and has a short lifespan. We used a well-established genetic manipulation tool (the *GAL4-UAS system*) to provide spatial control of miR-184 expression *in central nervous*

system (Reiter *et al.*, 2001). *Drosophila* with knockout miR-184 showed a significant reduction in the locomotor activity and worse learning and memory, indicating a depressive-like behaviors. These changes were more intense in older flies (21 – 25 d.p.e.) compared to younger counterparts. On the other hand, the overexpression of miR-184 significantly improved locomotor activity, learning and memory capacity of the flies, even at later d.p.e. Therefore, we provide robust evidence, from a translational perspective, that the downregulation of miR-184 is a biological mechanism that contributes to the development of depression, especially at older ages. Moreover, we also demonstrate that increasing the miR-184 expression can significantly improve the depressive-like behaviors of flies.

Major depression is a biologically heterogeneous condition throughout the lifespan. Previous studies including younger subjects with major depression have shown that miR-124, miR-1202, let-7 family and other miRNAs were significantly downregulated or upregulated during depressive episodes (Bocchio-Chiavetto *et al.*, 2013; Lopez *et al.*, 2014; Azevedo *et al.*, 2016; Fiori *et al.*, 2017). In contrast, our study did not identify such miRNAs as differentially expressed in LLD. One possible explanation is that the pathophysiological mechanisms of major depression are distinct across different ages. Therefore, we can hypothesize that the biological processes of aging interact and moderate the pathophysiological changes of major depression (age\*disease interaction)<sup>55</sup>. Likewise, our results suggest that the biological mechanisms of major depression are age-dependent what can help to explain differences observed in long-term prognosis and treatment response in young, middle-aged and older adults. These evidence of age\*disease interaction have significant implications not only for the understanding of the pathophysiological mechanisms of major depression but to

the development of therapeutic interventions tailored to different ages. Nonetheless, the cross-sectional design of our study does not allow to address these hypotheses properly. Longitudinal studies with a lifespan approach are, thereof, necessary to confirm or refute these hypotheses.

Our results should be viewed in light of some limitations. We evaluated the miRNA expression in plasma, and there is a long-lasting debate in the literature whether biomarkers measured in the periphery can reliably reflect changes in the central nervous system (CNS). An important characteristic of the aging brain is the blood-brain barrier (BBB) deterioration and its increased permeability to systemic factors (Montagne *et al.*, 2015; Cai *et al.*, 2017) that is potentiated in older adults with a major depressive episode (Najjar *et al.*, 2013). The BBB deterioration allows an influx and efflux of humoral factors, including microRNAs, from the CNS to the peripheral circulation (e.g., blood), and vice-versa. Also, the miR-184 is mostly expressed in the brain and not in peripheral tissues indicating that the miR-184 identified in the brain is mostly, if not all, from brain tissue. Our study included only older adults with major depression recruited at a specialty center. Our results, thus, may not be generalized to other age groups or the general elderly population. Finally, the cross-sectional design does not allow to make causal inferences about the role of miR-184 downregulation and major depression in the elderly. However, our findings that knockout flies for miR-184 also have depressive-like behaviors that are reversed with overexpression of this miRNA offers strong evidence that the downregulation of miR-184 is a contributory mechanism for the development of major depressive disorder in the elderly. Finally, the absence of neuroimaging evaluation does not allow to

evaluate the impact of changes in miR-184 expression on brain structure and function.

In conclusion, we found significant downregulation of miR-184 expression in LLD. The downregulation of miR-184 was significantly associated with the severity of depressive symptoms and cognitive dysfunction in these individuals. These findings were mirrored in ortholog of miR-184 knockout *Drosophila melanogaster* flies that showed a depressive-like behaviors. Our findings suggest the role of downregulation of miR-184 as a mechanism that contributes to the development of depression and cognitive impairment in older adults.

### **Acknowledgments**

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### **Disclosures**

The authors declare no conflict of interest.

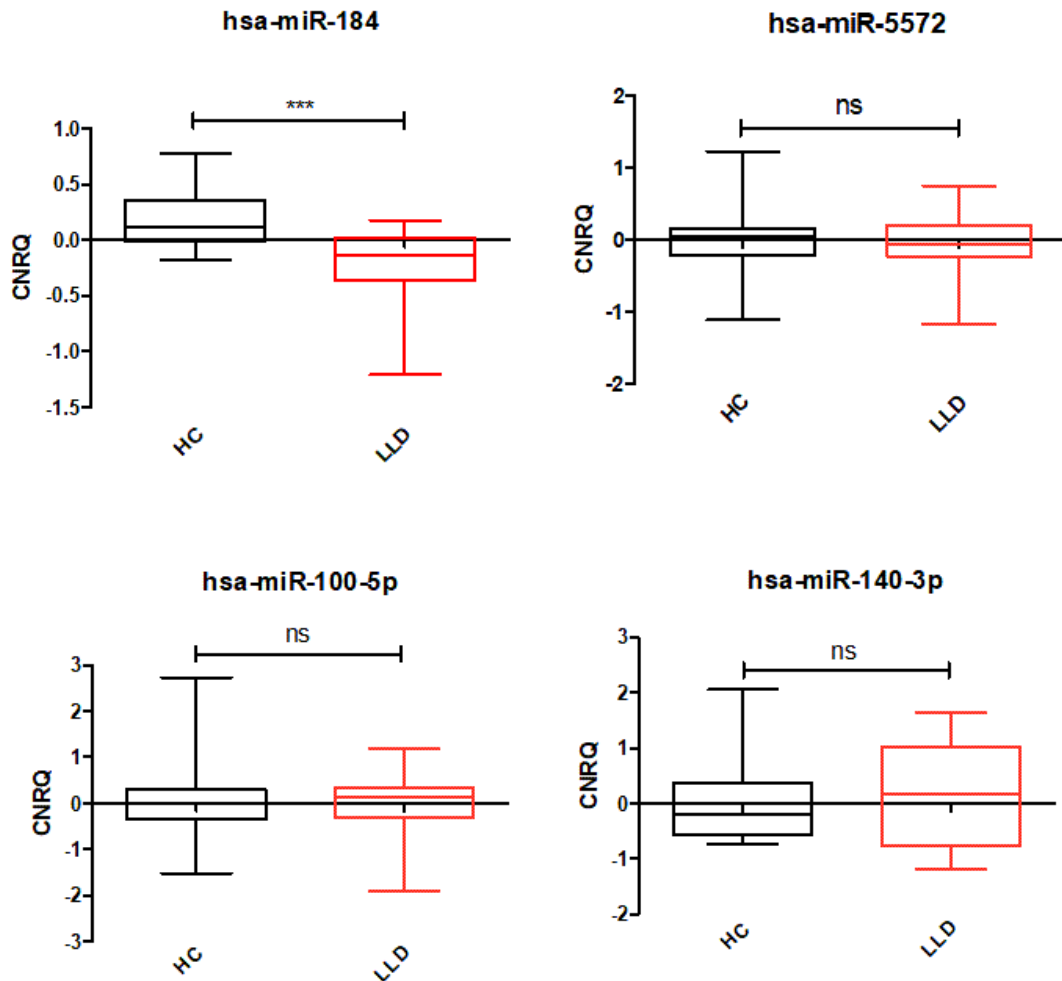
**Table 1** Demographics and clinical makeup of participants in the study

	HC	LLD		p-value
<b>NGS set</b>				
n	19	24		
Gender (male:female)	1:18	4:20	t=-1,149	0,257
Age(y)(mean±s.d.)	69,63 (±6,13)	72,25 (±7,87)	t=-1,19	0,135
Years of education	9,47 (±5,23)	4,63 (±2,43)	t=4,03	<b>&lt;0,001***</b>
<b>Age of onset</b>				
Late-onset depression		7 (29,2%)	X <sup>2</sup> =4,167	<b>0,041*</b>
Early-onset depression		17 (70,8%)		
HDRS17	2,63 (±5,06)	17,80 (±6,86)	t=-8,04	<b>0,012*</b>
HDRS21	2,68 (±5,28)	18,96 (±7,27)	t=-8,20	<b>0,009**</b>
MMSE (mean ± s.d.)	28,65 (±1,65)	24,80 (±3,89)	t=3,72	<b>0,001**</b>
DRS Attention score (mean±s.d.)	36,24 (±0,75)	34,64 (±3,41)	t=1,88	<b>0,021*</b>
DRS Initiation/Perseveration score (mean±s.d.)	36,12 (±1,76)	33,91 (±3,11)	t=2,40	0,058
DRS Construction score (mean±s.d.)	6,00 (±0,00)	5,18 (±1,33)	t=2,57	<b>&lt;0,0001***</b>
DRS Conceptualization score (mean±s.d.)	34,77 (±3,65)	33,00 (±7,95)	t=0,80	0,057
DRS Memory score (mean±s.d.)	23,18 (±1,81)	20,00 (±4,75)	t=2,51	<b>0,035*</b>
DRS Total score (mean±s.d.)	136,35 (±5,07)	126,73 (±19,40)	t=1,96	<b>0,014*</b>
<b>RT-qPCR-set</b>				
n	34	39		
Gender (male:female)	5:29	7:32	t=0,573	0,252
Age(y)(mean±s.d.)	70,88 (±8,09)	73,15(±8,12)	t=-1,05	0,670
Years of education	9,8 (±4,82)	4,51(±2,50)	t=5,39	<b>&lt;0,0001***</b>
<b>Age of onset</b>				
Late-onset depression		18 (46,2%)	X <sup>2</sup> =0,032	0,857
Early-onset depression		21 (53,8%)		
HDRS17	2,53 (±5,47)	18,64 (±6,21)	t=-11,4	<b>0,049*</b>
HDRS21	2,61 (±5,55)	19,56 (±6,82)	t=-11,25	<b>0,015*</b>
MMSE (mean ± s.d.)	28,04 (±2,30)	23,86 (±2,78)	t=5,71	0,268
DRS Attention score (mean±s.d.)	35,70 (±1,103)	34,89 (±1,40)	t=2,38	0,258
DRS Initiation/Perseveration score (mean±s.d.)	35,41 (±2,49)	31,93 (±3,96)	t=3,88	<b>0,011*</b>
DRS Construction score (mean±s.d.)	5,70 (±0,775)	5,31 (±1,34)	t=1,33	<b>0,006**</b>
DRS Conceptualization score (mean±s.d.)	34,00 (±4,82)	27,43 (±6,13)	t=4,41	0,121
DRS Memory score (mean±s.d.)	23,26 (±1,70)	19,11 (±4,03)	t=4,94	<b>&lt;0,0001***</b>
DRS Total score (mean±s.d.)	134,11 (±7,77)	118,79 (±12,35)	t=5,49	<b>0,029*</b>

Abbreviations: HC, healthy control; LLD, Late-Life Depression; HDRS17, 17-item Hamilton Depression Rating Scale; HDRS21, 21-item Hamilton Depression Rating Scale; MMSE, Mini-Mental State Examination; DRS, Dementia rating Scale.

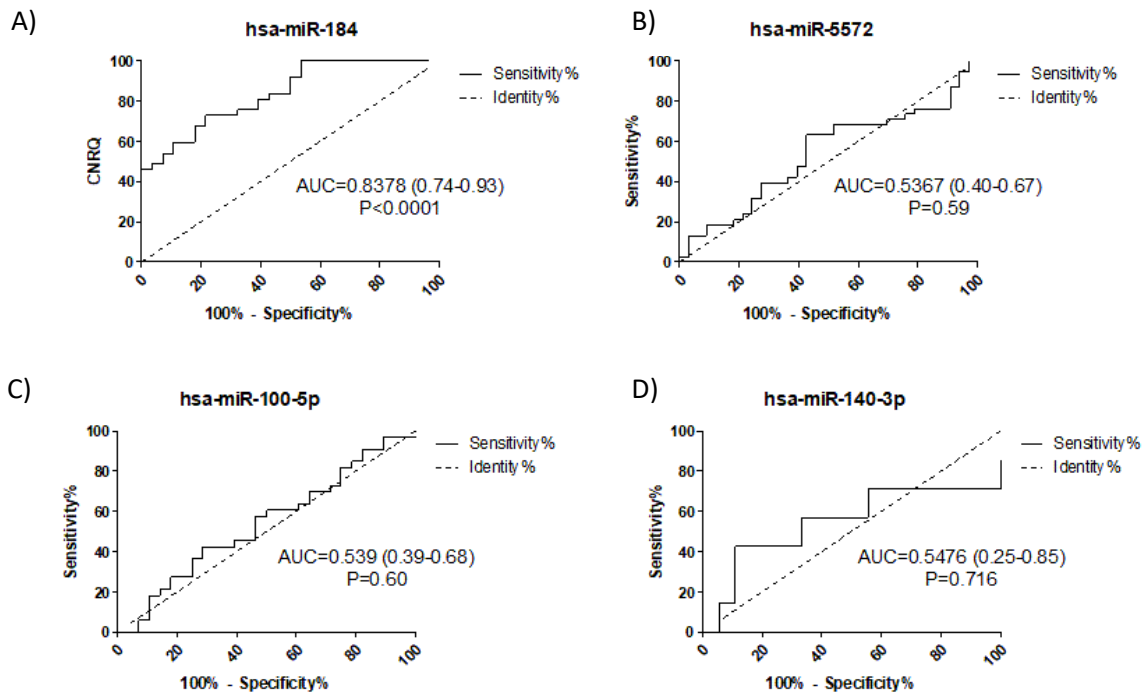


**Figure 2** Relative quantification of circulating miRNAs when comparing HC and LLD subjects determined by the Mann–Whitney U-test.



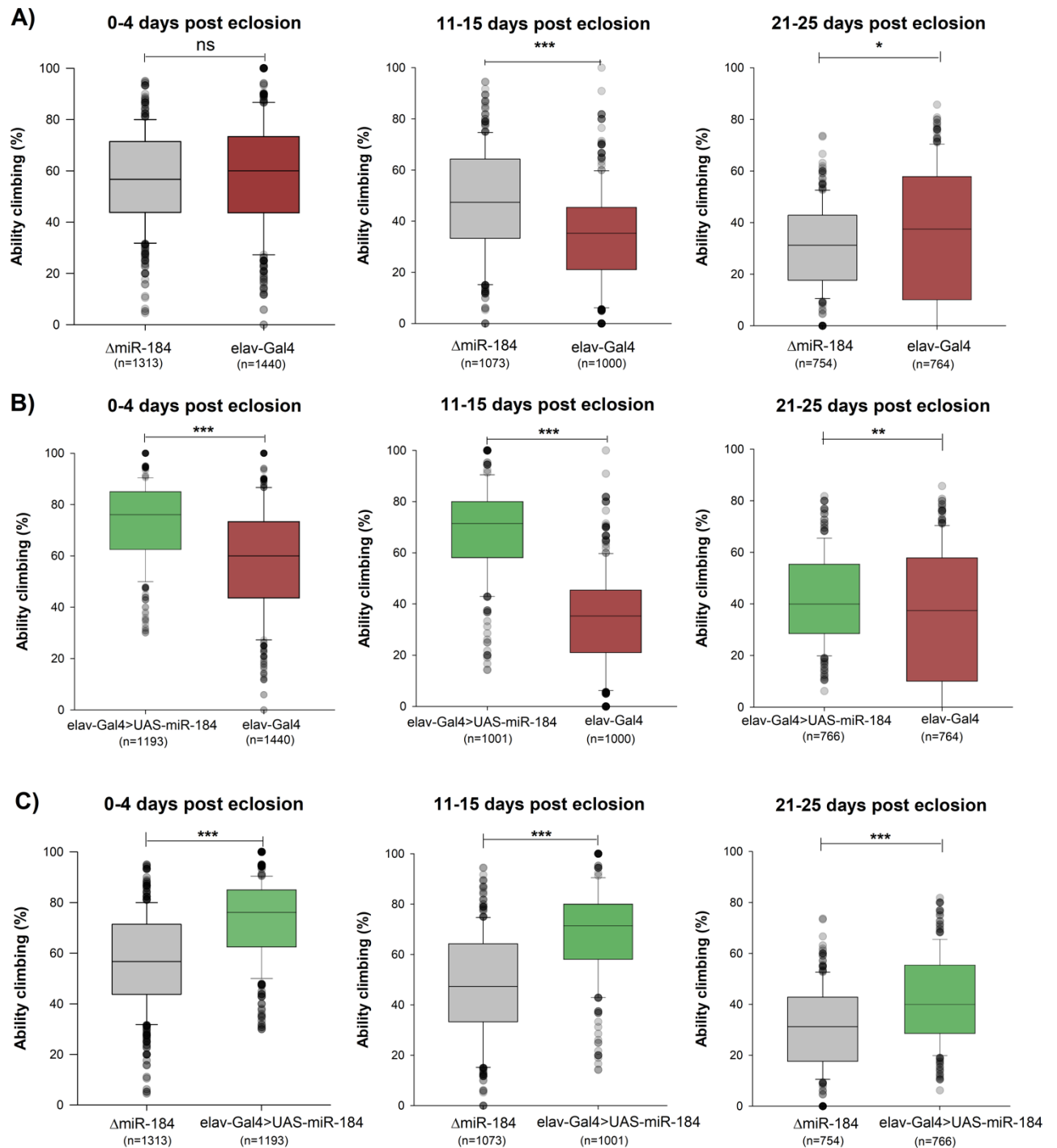
Abbreviations: HC, health control; LLD, late-life depression; ns, non-significant, \*\*\*, p-value<0.001.

**Figure 3** ROC curve analysis to discriminate late-life depression (LLD) cases from normal controls enrolled in the validation set of 4 miRNAs.



**A)** hsa-miR-184 ROC curve separates 39 LLD cases from 34 controls (AUC=0,8373 and p-value<0,0001), **B, C and D)** hsa-miR-5572, hsa-miR-100-5p and hsa-miR-140-3p ROC curves do not separate 39 LLD cases from 34 controls.

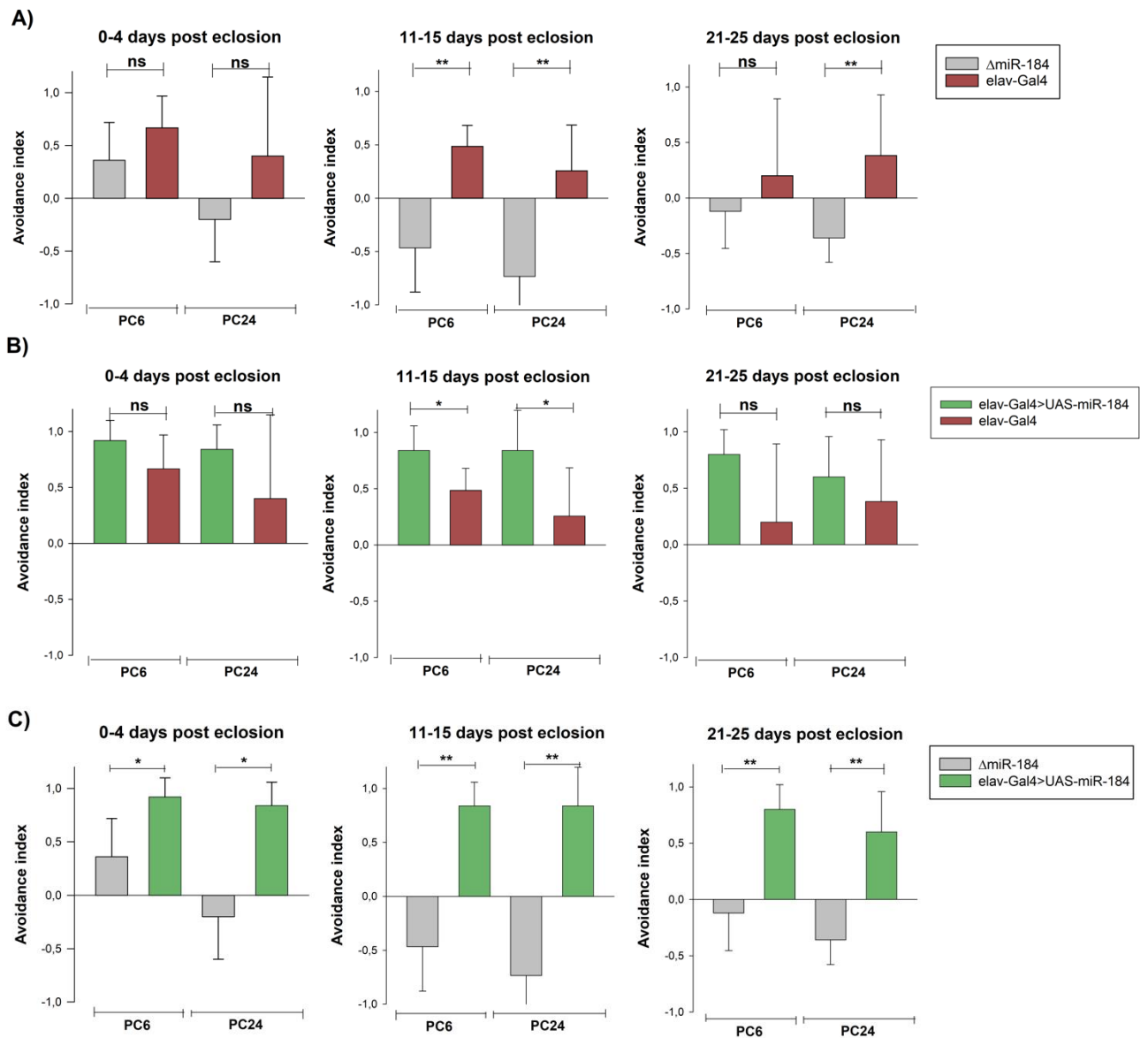
**Figure 4** Climbing ability in fruit flies



Results of the climbing ability (%) of fruit flies with different age ranges (measured in days post-eclosion). **A)** Comparison of climbing scores between  $\Delta$ miR-184 and control flies elav-Gal4. There was no significant difference found in 0-4 d.p.e, but  $\Delta$ miR-184 showed better climbing scores in flies with 11-15 d.p.e ( $p < 0.0001$ ) and

worst climbing scores in flies with 21-25 d.p.e ( $p=0.04$ ), **B**) Comparison of climbing scores between *elav-Gal4>UAS-mir-184* and control flies *elav-Gal4*. *Elav-Gal4>UAS-mir-184* showed better climbing scores at all age ranges (0-4 and 11-15,  $p<0,0001$ ; and 21-25 d.p.e,  $p=0,0061$ ). **C**) Knockout flies ( $\Delta$ miR-184) showed worst climbing scores when compared to *elav-Gal4>UAS-mir-184* at all age ranges (0-4, 11-15 and 21-25 d.p.e,  $p<0,0001$ ),

**Figure 5** Avoidance test in fruit flies.



Avoidance index 6 (PC6) and 24 (PC24) hours post conditioning at aversive odor stimulus calculated in three different ranges of days post eclosion (0-4, 11-15 and 21-25). **A)**  $\Delta$ miR-184 showed worst PC6 when compared to control elav-Gal4 (11-15 d.p.e,  $p$ -value=0.0029) and worst PC24 when compared to elav-Gal4 (11-21 with  $p$ -value=0.0048 and 21-25 d.p.e with  $p$ -value=0.0211). **B)** elav-Gal4>UAS-miR-184 showed best PC6 ( $p$ -value=0.0284) and PC24 ( $p$ -value=0.0484) when

compared to control elav-Gal4 in flies with 11-15 d.p.e and no significant differences were found in PC6 and PC24 between two groups in flies with 0-4 and 21-25 d.p.e **C**) Comparison between  $\Delta$ mir-184 and elav-Gal4>UAS-miR-184 showed worse avoidance index in knockout flies at 6 and 24 hours post conditioning to aversive odor stimulus in 0-4 d.p.e (PC6 with  $p$ -value=0.0236 and PC24 with  $p$ -value=0.0117), 11-15 d.p.e (PC6 with  $p$ -value=0.0066 and PC24 with  $p$ -value=0.0074) and 21-25 d.p.e (PC6 with  $p$ -value=0.0078 and PC24 with  $p$ -value=0.0074).

### 3.5 SUPPLEMENTARY MATERIAL

#### **Drosophila melanogaster methods Genetics and fly stocks**

All fly stocks were maintained at 25°C on a 12:12-h light: dark cycle at constant humidity on a standard sugar-yeast (SY) medium (15 g l<sup>-1</sup> agar, 50 g l<sup>-1</sup> sugar, 100 g l<sup>-1</sup> autolyzed yeast, 100 g l<sup>-1</sup> nipagin and 3 ml l<sup>-1</sup> propionic acid). The lines used for this study were obtained: P{GawB}elav<sup>C155</sup>/P{GawB}elav<sup>C155</sup>, P{UAS-mCD8::GFP.L}LL4, P{hsFLP}1, w<sup>\*</sup> (#5146, Bloomington Drosophila Stock Center), UAS-miR-184, UAS-miR-184/TM6B, Tb and  $\Delta mir-184$  (kindly provided by Dr. Ulrike Gaul at University of Munich, Munich, Germany) The flies P{GawB}elav<sup>C155</sup>, P{UAS-mCD8::GFP.L}LL4, P{hsFLP}1, w<sup>\*</sup> (#5146, Bloomington Drosophila Stock Center) and UAS-miR-184, UAS-miR-184/TM6B, Tb were crossed to obtain the overexpression of mir-184. The genotype obtained in cross was P{GawB}elav<sup>C155</sup>/ w<sup>\*</sup>; UAS-mir-184/w<sup>\*</sup>; w<sup>\*</sup> and from now on it will be called as elav-Gal4>UAS-mir184.

#### **Behavioral tests on Drosophila melanogaster**

##### **a. Negative Geotaxis Response**

The Drosophila negative geotaxis response is a commonly assayed behavior. It involves the mechanical stimulation of an innate escape response (Gargano *et al.*, 2005). We designed a Rapid Iterative Negative Geotaxis (RING) apparatus illustrated in Supplementary Figure 1, which holds 1–8 vials (2.85cm diameter x 9.5 cm height). Groups of 10-25 flies were transferred into clean and empty vials (FlyStuff cat # 32-121) and allowed to climb upward. Four distinct groups of flies were evaluated: elav-Gal4 (control strain), UAS-miR-184 (control strain),  $\Delta mir-184$  and elav-Gal4>UAS-mir-184. Each genotype was tested in three different

ranges of days post-eclosion (d.p.e), 0-4 d.p.e, 11-15 d.p.e and 21-25 d.p.e. Flies were acclimated for 20 minutes' prior the assay. The test consisted of three consecutive beats of the vials containing the flies and observation of climbing. All assays were recorded by means of filming. Fly cohorts are allowed to rest for 1 minute between 6 replicate runs At the end, flies that reached  $\geq 5$  centimeters after 4 seconds were accounted for QuickTime software was used for video analysis, with frame marking used in the count and when needed to confirm fly motion or if there were flies close to each other (Chakraborty *et al.*, 2011).

Scores recorded were the number of flies that reached  $\geq 5$  centimeters after 4 seconds ( $n \geq 5$ ) and the total number of flies assessed ( $n_{tot}$ ). A climbing index (CI) defined as  $n > 5 / n_{tot}$  was calculated and t-tests were performed using Graph Prism 5 software.

### **b. Learning and Memory Aversion Test**

Adapted from Le Bourg and Buecher (Le Bourg e Buecher, 2002), this assay exploits the positive phototactic behavior in flies to train them to associate light with aversive stimuli (in this case, aversive smell of citronella). After a training/conditioning phase, flies will be able to associate the lighted area with aversive smell and avoid it. Flies with compromised learning capacities will fail to make this association. Furthermore, the aversive phototaxis suppression (APS) assay can also be used to measure short term memory function of flies (Fresquet e Médioni, 1993; Le Bourg, 2004) by subjecting already trained flies to the same test 6 hours and 24 hours post conditioning to test their ability in remembering the learned task.

## **I. Flies**

Adult male flies of the four groups (6 to 11 flies per group) were individual trained and tested according to three different ranges of days post eclosion (0-4, 11-15 and 21-25 d.p.e) to evaluate the memory across aging.

## **II. General Procedures**

All experiments were performed in a room  $25 \pm 1$  ° C of temperature; only lit with the optical fiber above the maze under a dim red light (ie, red LED), which allows the researcher to see but prevents the fly from seeing, thus allowing the flies to concentrate on olfaction as opposed to visual inputs. Individual flies had to make successive choices between a lighted and a darkened alley in a T-maze. The lighted vial is internally covered with white filter paper, except at its opening and at the opposite end. The darkened vial, free of filter paper, is inserted into another opaque gray vial that impedes the access of light into the darkened vial (Supplementary figure 2). Only flies that responded positively to the stimulus of light were choose. First, each fly was introduced into the training tube and then attach to the T-maze and allow them to adapt to the darkened tube for 180 sec. After the adapt phase each fly was exposure to 1 time to a lighted vial with filter paper wetted with water and a darkened alley in a T-maze; and right after 10 successive times to a lighted vial filter paper wetted with 30mM solution of citronella (Du *et al.*, 2015) and a darkened alley in a T-maze. The fly was transferred without anesthesia to the darkened vial. Slowly open the trap door and turn on the light. Allow the fly walk into the quinine coated lighted chamber. After one minute, tap the fly back to the dark chamber and repeat more 10 times

## **III. Assessing Short-Term and Long-Term Memory Functions in Flies**

After training, each fly was placed back into its original food vial and kept aside for six hours. Six hours post training, each fly was subject to 5 trials again in the

same way as before, and the number of times the fly avoided or went into the lighted vial was recorded. This avoided rate is recorded as 6 hours post conditioning (PC6), which is an indicator of short-term memory. After the short-term memory test each fly was placed back into its original food vial and kept aside for more eighteen hours. Twenty-four hours after training, each fly was subject to 5 trials again in the same way as before, and the number of times the fly avoided or went into the lighted vial was recorded. This avoided rate is recorded as 24 hours post conditioning (PC24), which is an indicator of long-term memory.

#### **IV. Avoidance Index and Statistical Analysis**

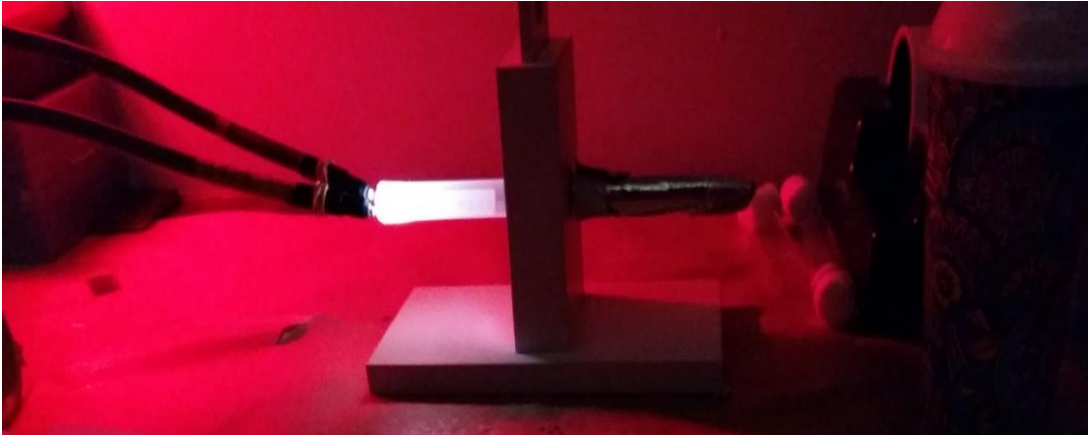
Avoidance Index was calculated for short-term and long-term memory to each group as the percentage number of flies that avoided the lighted vial with citronella over the total trial (five trials). Data was graphically represented as average pass rate PC6 and PC24 with respective SEM. Memory indices are calculated and compared between four groups and t-tests were performed using Graph Prism 5.

**Supplementary figure 1** The *Drosophila* RING apparatus



An apparatus was developed and used to measure and quantify the negative geotaxis response of flies. The average climbing index (CI) is the distance that individual flies climbed in 4 seconds after beats and was determined from 6 replicate trials of fly groups representing a particular age or genetic background.

**Supplementary figure 2** T-Maze setup for aversive phototaxis suppression assay



The overall experimental setup for the APS experiment with the light source connected to the "lighted" falcon tube, lined with filter paper and the "dark" tube on the right (covered in foil), separated by a trap door. During the training and testing phase, the fly is in the dark chamber and the trap door opened after the light is turned on in the lighted chamber.

**Supplementary table 1** MiRNA profile to each group

	<b>HC</b>	<b>LLD</b>
<b>1</b>	hsa-miR-30c-5p	hsa-miR-16-2-3p
<b>2</b>	hsa-miR-380-5p	hsa-miR-223-3p
<b>3</b>	hsa-miR-155-5p	hsa-let-7b-3p
<b>4</b>	hsa-miR-1-3p	hsa-miR-144-5p
<b>5</b>	hsa-miR-451a	hsa-miR-199a-3p
<b>6</b>	hsa-miR-7-5p	hsa-miR-199b-3p
<b>7</b>	hsa-miR-8485	hsa-miR-15b-3p
<b>8</b>	hsa-miR-100-5p	hsa-miR-15b-5p
<b>9</b>	hsa-miR-184	hsa-miR-145-5p
<b>10</b>	hsa-miR-103a-3p	hsa-miR-20b-5p
<b>11</b>	hsa-miR-30b-5p	hsa-miR-221-3p
<b>12</b>	hsa-miR-16-5p	hsa-miR-23b-3p
<b>13</b>	hsa-miR-21-5p	hsa-miR-106a-5p
<b>14</b>	hsa-miR-191-5p	hsa-miR-140-3p
<b>15</b>	hsa-miR-26a-5p	hsa-miR-27a-3p
<b>16</b>	hsa-miR-126-5p	hsa-miR-22-3p
<b>17</b>	hsa-miR-126-3p	hsa-miR-30e-3p
<b>18</b>	hsa-miR-181a-5p	hsa-miR-27b-3p
<b>19</b>	hsa-let-7b-5p	hsa-miR-29b-3p
<b>20</b>	hsa-miR-181b-5p	hsa-miR-130a-3p
<b>21</b>	hsa-miR-192-5p	hsa-miR-9-3p
<b>22</b>	hsa-miR-3591-3p	hsa-miR-590-5p
<b>23</b>	hsa-miR-486-5p	hsa-miR-124-5p
<b>24</b>	hsa-miR-92a-3p	hsa-miR-378a-3p
<b>25</b>	hsa-miR-151a-5p	hsa-miR-619-3p
<b>26</b>	hsa-miR-151b	hsa-miR-18b-5p
<b>27</b>	hsa-let-7a-5p	hsa-miR-505-3p
<b>28</b>	hsa-miR-222-3p	hsa-miR-374b-5p
<b>29</b>	hsa-miR-335-5p	hsa-miR-204-5p
<b>30</b>	hsa-miR-19b-3p	hsa-miR-142-5p

	<b>HC</b>	<b>LLD</b>
<b>31</b>	hsa-miR-320a	hsa-miR-30e-5p
<b>32</b>	hsa-let-7i-5p	hsa-miR-454-3p
<b>33</b>	hsa-miR-107	hsa-miR-125a-5p
<b>34</b>	hsa-miR-30d-5p	hsa-miR-3613-3p
<b>35</b>	hsa-miR-24-3p	hsa-miR-30c-5p
<b>36</b>	hsa-miR-5572	hsa-miR-380-5p
<b>37</b>	hsa-miR-15a-5p	hsa-miR-155-5p
<b>38</b>	hsa-miR-93-5p	hsa-miR-1-3p
<b>39</b>	hsa-miR-185-5p	hsa-miR-451a
<b>40</b>	hsa-let-7f-5p	hsa-miR-7-5p
<b>41</b>	hsa-miR-146a-5p	hsa-miR-100-5p
<b>42</b>	hsa-miR-144-3p	hsa-miR-184
<b>43</b>	hsa-miR-484	hsa-miR-103a-3p
<b>44</b>	hsa-miR-23a-3p	hsa-miR-30b-5p
<b>45</b>	hsa-miR-142-3p	hsa-miR-16-5p
<b>46</b>	hsa-miR-106b-5p	hsa-miR-21-5p
<b>47</b>	hsa-miR-106b-3p	hsa-miR-191-5p
<b>48</b>	hsa-miR-449a	hsa-miR-26a-5p
<b>49</b>	hsa-miR-449b-5p	hsa-miR-126-5p
<b>50</b>	hsa-miR-18a-5p	hsa-miR-126-3p
<b>51</b>	hsa-miR-34a-5p	hsa-miR-181a-5p
<b>52</b>	hsa-miR-26b-5p	hsa-let-7b-5p
<b>53</b>	hsa-miR-20a-5p	hsa-miR-181b-5p
<b>54</b>	hsa-let-7g-5p	hsa-miR-192-5p
<b>55</b>	hsa-miR-526b-5p	hsa-miR-3591-3p
<b>56</b>	hsa-miR-423-5p	hsa-miR-486-5p
<b>57</b>	hsa-miR-589-5p	hsa-miR-92a-3p
<b>58</b>	hsa-miR-19a-3p	hsa-miR-151a-5p
<b>59</b>	hsa-miR-200a-3p	hsa-miR-151b
<b>60</b>	hsa-miR-628-5p	hsa-let-7a-5p
<b>61</b>	hsa-miR-125b-5p	hsa-miR-222-3p

	<b>HC</b>	<b>LLD</b>
<b>62</b>	hsa-miR-17-5p	hsa-miR-335-5p
<b>63</b>	hsa-let-7d-5p	hsa-miR-19b-3p
<b>64</b>	hsa-miR-342-3p	hsa-miR-320a
<b>65</b>	hsa-miR-101-3p	hsa-let-7i-5p
<b>66</b>	hsa-miR-10a-5p	hsa-miR-107
<b>67</b>	hsa-miR-643	hsa-miR-30d-5p
<b>68</b>	hsa-miR-130b-3p	hsa-miR-24-3p
<b>69</b>	hsa-miR-150-5p	hsa-miR-15a-5p
<b>70</b>	hsa-miR-31-5p	hsa-miR-93-5p
<b>71</b>	hsa-miR-425-5p	hsa-miR-185-5p
<b>72</b>	-	hsa-let-7f-5p
<b>73</b>	-	hsa-miR-146a-5p
<b>74</b>	-	hsa-miR-144-3p
<b>75</b>	-	hsa-miR-484
<b>76</b>	-	hsa-miR-23a-3p
<b>77</b>	-	hsa-miR-142-3p
<b>78</b>	-	hsa-miR-106b-5p
<b>79</b>	-	hsa-miR-106b-3p
<b>80</b>	-	hsa-miR-449a
<b>81</b>	-	hsa-miR-449b-5p
<b>82</b>	-	hsa-miR-18a-5p
<b>83</b>	-	hsa-miR-34a-5p
<b>84</b>	-	hsa-miR-26b-5p
<b>85</b>	-	hsa-miR-20a-5p
<b>86</b>	-	hsa-let-7g-5p
<b>87</b>	-	hsa-miR-526b-5p
<b>88</b>	-	hsa-miR-423-5p
<b>89</b>	-	hsa-miR-589-5p
<b>90</b>	-	hsa-miR-19a-3p
<b>91</b>	-	hsa-miR-628-5p
<b>92</b>	-	hsa-miR-125b-5p

	<b>HC</b>	<b>LLD</b>
<b>93</b>	-	hsa-miR-17-5p
<b>94</b>		hsa-let-7d-5p
<b>95</b>		hsa-miR-342-3p
<b>96</b>		hsa-miR-101-3p
<b>97</b>		hsa-miR-130b-3p
<b>98</b>		hsa-miR-150-5p
<b>99</b>		hsa-miR-31-5p
<b>100</b>		hsa-miR-425-5p

**Supplementary table 2** List of primer sequences used in the present study

<b>Primer</b>	<b>Sequence</b>
<b>hsa-miR-100-5p</b>	5'-AACCCGTAGATCCGAACTTGTG-3'
<b>hsa-miR-184</b>	5'-TGGACGGAGAACTGATAAGGGT-3'
<b>hsa-miR-125b-5p</b>	5'-TCCCTGAGACCCTAACTTGTGA-3'
<b>hsa-miR-5572</b>	5'-GTTGGGGTGCAGGGTCTGCT-3'
<b>hsa-miR-1-3p</b>	5'-TGGAATGTAAAGAAGTATGTAT-3'
<b>hsa-miR-140-3p</b>	5'-TACCACAGGGTAGAACCACGG-3'
<b>hsa-let-7a-5p</b>	5'-TGAGGTAGTAGGTTGTATAGTT-3'
<b>U6</b>	5'GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGA AGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGC GTTCCATATTTT-3'
<b>cel-miR-39</b>	5'-TCACCGGGTGTAATCAGCTTG-3'

**Supplementary table 3** Significant KEEG pathways on putative target genes  
(3'UTR region) of input miRNA(s)

<b>PathName</b>	<b>PathFg</b>	<b>PathBg</b>	<b>GenomeFG</b>	<b>GenomeBG</b>	<b>pval</b>	<b>BH</b>
<b>Pathways in cancer</b>	162	330	6308	19747	5,11271E-11	9,81640 E-9
<b>Chronic myeloid leukemia</b>	47	75	6308	19747	4,30106E-8	8,21503 E-6
<b>Non small cell lung cancer</b>	36	54	6308	19747	1,69188E-7	3,18074 E-5
<b>Glioma</b>	41	65	6308	19747	2,39724E-7	4,50682 E-5
<b>Phosphatidylinositol signaling system</b>	46	76	6308	19747	2,66816E-7	4,98945 E-5
<b>ErbB signaling pathway</b>	51	89	6308	19747	6,81196E-7	1,26702E-3
<b>Axon guidance</b>	68	129	6308	19747	7,76909E-7	1,44505E-3
<b>Prostate cancer</b>	50	89	6308	19747	1,95192E-6	3,59154E-3
<b>Calcium signaling pathway</b>	86	178	6308	19747	3,65437E-6	6,65096E-3
<b>Endocytosis</b>	89	187	6308	19747	5,41641E-6	9,80371E-4
<b>Acute myeloid leukemia</b>	35	58	6308	19747	7,76844E-6	1,39832E-3
<b>Inositol phosphate metabolism</b>	33	54	6308	19747	9,61198E-6	1,72055E-3
<b>Oocyte meiosis</b>	57	112	6308	19747	2,32329E-5	4,089E-3
<b>Focal adhesion</b>	93	203	6308	19747	2,32368E-5	4,08968E-3
<b>Small cell lung cancer</b>	45	84	6308	19747	3,22259E-5	5,63954-3
<b>Pancreatic cancer</b>	41	75	6308	19747	3,82743E-5	6,65974E-3
<b>Neurotrophin signaling pathway</b>	63	129	6308	19747	4,59450E-5	7,9485E-3
<b>Wnt signaling pathway</b>	72	152	6308	19747	4,96255E-5	8,58521E-3
<b>Insulin signaling pathway</b>	66	139	6308	19747	9,14368E-5	1,56357E-2
<b>Adherens junction</b>	40	76	6308	19747	1,44812E-4	2,44732E-2

<b>PathName</b>	<b>PathFg</b>	<b>PathBg</b>	<b>GenomeFG</b>	<b>GenomeBG</b>	<b>pval</b>	<b>BH</b>
<b>Colorectal cancer</b>	44	86	6308	19747	1,62824E-4	2,73545E-2
<b>mTOR signaling pathway</b>	30	53	6308	19747	1,78591E-4	3,00033E-2
<b>MAPK signaling pathway</b>	115	272	6308	19747	1,99595E-4	3,33323E-2
<b>Endometrial cancer</b>	29	52	6308	19747	3,22185E-4	5,31605E-2
<b>Cell cycle</b>	58	124	6308	19747	3,85741E-4	6,32615E-2
<b>Ubiquitin mediated proteolysis</b>	61	134	6308	19747	6,70757E-4	1,08663E-1
<b>VEGF signaling pathway</b>	39	78	6308	19747	6,76966E-4	1,09668E-1
<b>Regulation of actin cytoskeleton</b>	90	212	6308	19747	7,91616E-4	1,26659E-1
<b>Circadian rhythm mammal</b>	10	13	6308	19747	1,13135E-3	1,77622E-1
<b>Progesterone mediated oocyte maturation</b>	42	88	6308	19747	1,42305E-3	2,20573E-1
<b>Chemokine signaling pathway</b>	80	189	6308	19747	1,651E-3	2,54253E-1
<b>Melanoma</b>	35	71	6308	19747	1,71425E-3	2,63995E-1
<b>B cell receptor signaling pathway</b>	36	75	6308	19747	2,66721E-3	4,02748E-1
<b>Hedgehog signaling pathway</b>	28	56	6308	19747	3,69334E-3	5,42921E-1
<b>Type II diabetes mellitus</b>	25	49	6308	19747	4,22422E-3	6,08288E-1
<b>Notch signaling pathway</b>	24	47	6308	19747	4,94401E-3	6,97105E-1
<b>T cell receptor signaling pathway</b>	48	110	6308	19747	6,52293E-3	8,61027E-1
<b>Renal cell carcinoma</b>	33	71	6308	19747	7,26812E-3	9,37588E-1
<b>Chondroitin sulfate biosynthesis</b>	13	22	6308	19747	7,81083E-3	9,91975E-1
<b>Basal cell carcinoma</b>	26	55	6308	19747	1,24816E-2	1
<b>Thyroid cancer</b>	14	29	6308	19747	4,88243E-2	1

<b>PathName</b>	<b>PathFg</b>	<b>PathBg</b>	<b>GenomeFG</b>	<b>GenomeBG</b>	<b>pval</b>	<b>BH</b>
<b>Cell adhesion molecules CAMs</b>	56	133	6308	19747	8,55576E-3	1
<b>Long term potentiation</b>	31	71	6308	19747	2,51057E-2	1
<b>SNARE interactions in vesicular transport</b>	20	39	6308	19747	9,33595E-3	1
<b>Heparan sulfate biosynthesis</b>	13	26	6308	19747	4,20076E-2	1
<b>Bladder cancer</b>	20	43	6308	19747	3,21811E-2	1
<b>Viral myocarditis</b>	33	72	6308	19747	9,36929E-3	1
<b>Hypertrophic cardiomyopathy HCM</b>	35	86	6308	19747	5,37028E-2	1
<b>Jak STAT signaling pathway</b>	61	156	6308	19747	3,45258E-2	1
<b>Adipocytokine signaling pathway</b>	31	70	6308	19747	2,01936E-2	1
<b>Dorso ventral axis formation</b>	12	24	6308	19747	4,99178E-2	1
<b>Melanogenesis</b>	41	102	6308	19747	4,78559E-2	1
<b>Lysine degradation</b>	20	45	6308	19747	5,3114E-2	1
<b>Fc gamma R mediated phagocytosis</b>	42	97	6308	19747	1,21904E-2	1
<b>Glycerolipid metabolism</b>	21	46	6308	19747	3,56341E-2	1
<b>Apoptosis</b>	39	87	6308	19747	7,89663E-3	1

'PathFg', 'PathBg', 'GenomeFG' and 'GenomeBG' stand for 'number of genes predicted as putative targets in a given pathway', 'number of genes in a given pathway', 'total number of target genes within genome' and 'total number of genes within genome'

**Supplementary table 4** Significant KEEG pathways on putative target genes  
(5'UTR region) of input miRNA(s)

<b>PathName</b>	<b>PathFg</b>	<b>PathBg</b>	<b>GenomeFG</b>	<b>GenomeBG</b>	<b>pval</b>	<b>BH</b>
<b>Regulation of actin cytoskeleton</b>	65	212	3484	19747	2,36049E-6	4,53216E-4
<b>Neurotrophin signaling pathway</b>	41	129	3484	19747	6,60743E-5	1,26202E-2
<b>Oocyte meiosis</b>	35	112	3484	19747	3,13586E-4	5,89542E-1
<b>Adherens junction</b>	26	76	3484	19747	3,81473E-4	7,1717E-1
<b>Metabolic pathways</b>	235	1091	3484	19747	3,94724E-4	7,42081E-1
<b>MAPK signaling pathway</b>	69	272	3484	19747	8,20697E-4	1,5265E-1
<b>Wnt signaling pathway</b>	42	152	3484	19747	1,45457E-3	2,69096E-1
<b>Axon guidance</b>	36	129	3484	19747	2,53764E-3	4,517E-1
<b>Long term potentiation</b>	22	71	3484	19747	4,28158E-3	6,93616E-1
<b>Glycosaminoglycan degradation</b>	9	21	3484	19747	6,24744E-3	9,24856E-1
<b>Heparan sulfate biosynthesis</b>	9	26	3484	19747	2,88706E-2	0.9999999
<b>Arginine and proline metabolism</b>	15	55	3484	19747	5,01634E-2	0.9999999
<b>Fatty acid biosynthesis</b>	4	6	3484	19747	1,07212E-2	0.9999999
<b>Glioma</b>	18	65	3484	19747	2,95261E-2	0.9999999
<b>Arrhythmogenic right ventricular cardiomyopathy ARVC</b>	20	74	3484	19747	2,90886E-2	0.9999999
<b>Glycine serine and threonine metabolism</b>	11	31	3484	19747	1,35097E-2	0.9999999
<b>Pathways in cancer</b>	75	330	3484	19747	1,04702E-2	0.9999999
<b>Calcium signaling pathway</b>	40	178	3484	19747	5,81177E-2	0.9999999
<b>Glycerolipid metabolism</b>	13	46	3484	19747	5,06899E-2	0.9999999

<b>PathName</b>	<b>PathFg</b>	<b>PathBg</b>	<b>GenomeFG</b>	<b>GenomeBG</b>	<b>pval</b>	<b>BH</b>
<b>Endometrial cancer</b>	15	52	3484	19747	3,16128E-2	0.9999999
<b>Focal adhesion</b>	47	203	3484	19747	2,69077E-2	0.9999999
<b>Pathogenic Escherichia coli infection</b>	15	54	3484	19747	4,32984E-2	0.9999999
<b>Fc gamma R mediated phagocytosis</b>	25	97	3484	19747	2,83767E-2	0.9999999
<b>Apoptosis</b>	22	87	3484	19747	4,60467E-2	0.9999999
<b>Sphingolipid metabolism</b>	12	39	3484	19747	3,214E-2	0.9999999
<b>Nitrogen metabolism</b>	9	23	3484	19747	1,24282E-2	0.9999999
<b>Prostate cancer</b>	24	89	3484	19747	1,85432E-2	0.9999999
<b>Basal cell carcinoma</b>	15	55	3484	19747	5,01634E-2	0.9999999
<b>Melanogenesis</b>	26	102	3484	19747	2,93708E-2	0.9999999
<b>Amyotrophic lateral sclerosis ALS</b>	16	55	3484	19747	2,49919E-2	0.9999999
<b>Other glycan degradation</b>	6	16	3484	19747	4,83398E-2	0.9999999
<b>Leukocyte transendothelial migration</b>	31	116	3484	19747	9,48069E-3	0.9999999
<b>Alanine aspartate and glutamate metabolism</b>	10	31	3484	19747	3,54571E-2	0.9999999
<b>Purine metabolism</b>	40	158	3484	19747	9,51777E-3	0.9999999
<b>Propanoate metabolism</b>	10	33	3484	19747	5,30947E-2	0.9999999

'PathFg', 'PathBg', 'GenomeFG' and 'GenomeBG' stand for 'number of genes predicted as putative targets in a given pathway', 'number of genes in a given pathway', 'total number of target genes within genome' and 'total number of genes within genome'

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## ANEXOS

### ANEXO 1 - PARECER DO CONSELHO DE ÉTICA EM PESQUISA (COEP)

Universidade Federal de Minas Gerais  
Comitê de Ética em Pesquisa da UFMG - COEP

Parecer nº. ETIC 334/06

Interessado: Prof. Marco Aurélio Romano Silva  
Departamento de Farmacologia  
Instituto de Ciências Biológicas-UFMG

#### DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP, aprovou, *ad referendum*, no dia 29 de novembro de 2006, depois de atendidas as solicitações de diligência, o projeto de pesquisa intitulado “Abordagem multidisciplinar do binômio depressão-demência no idoso” bem como o Termo de Consentimento Livre e Esclarecido do referido projeto.

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

  
Profa. Dra. Maria Elena de Lima Perez Garcia  
Presidente do COEP/UFMG

## **ANEXO 2 - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Nº Registro COEP: ETIC334/06\_\_\_\_\_

### **Título do Projeto: Abordagem multidisciplinar do binômio depressão-demência no idoso**

O senhor (a) está sendo convidado (a) a participar, como voluntário(a), em uma pesquisa de título: " Abordagem multidisciplinar do binômio depressão-demência no idoso"

O documento abaixo contém todas as informações necessárias sobre a pesquisa que está sendo realizada. Sua colaboração neste estudo é muito importante, mas a decisão de participar deve ser sua. Para tanto, leia atentamente as informações abaixo e não se apresse em decidir. Se você não concordar em participar ou quiser desistir em qualquer momento, isso não causará nenhum prejuízo a você. Se você concordar em participar basta preencher os seus dados e assinar a declaração concordando com a pesquisa. Se você tiver alguma dúvida pode esclarecê-la com o responsável da pesquisa. Obrigado(a).

#### **Objetivo do estudo**

Este projeto está sendo proposto porque há pouco conhecimento sobre os mecanismos fisiológicos e genéticos envolvidos no processo do envelhecimento. O objetivo desse trabalho é o de estudar, em uma população brasileira, as interações clínicas, genéticas, ambientais e neurobiológicas como fatores de risco ou proteção no desenvolvimento de doenças neuropsiquiátricas do idoso.

A partir desta compreensão, esperamos, no futuro, desenvolver terapias que possam melhor atender aos pacientes idosos.

#### **Procedimentos**

Todos os(as) participantes pessoas com mais de 60 anos de idade, que assinarão termo de consentimento esclarecido serão considerados sujeito de pesquisa e irão seguir os procedimentos dessa pesquisa. Os únicos inconvenientes aos quais os pacientes serão submetidos serão a coleta de sangue total e o tempo gasto na avaliação do idoso. Esses procedimentos serão realização de testes psicológicos realizados por pessoas qualificadas. O desconforto da coleta de sangue é o habitual de um exame de sangue de rotina e é realizada com material estéril, descartável e por pessoas treinadas. O material colhido (50 ml de sangue) será devidamente etiquetado e utilizado somente para os propósitos dessa pesquisa, não havendo quaisquer custos para a paciente. As informações obtidas serão objeto de estrita confidencialidade e não envolvem custos ou pagamentos de qualquer espécie.

#### **Pesquisadores**

A equipe de pesquisadores é composta pelos seguintes profissionais: Dr. Marco Aurélio Romano-Silva (coordenador da pesquisa), Dr. Edgar Nunes de Moraes, Dr. Humberto Correa, Dra. Maria Aparecida Bicalho, Dr Marco Túlio G Cintra, Dr Edgar Nunes de Moraes, Dr. Luiz Armando De Marco.

### **Confidencialidade**

Todos os dados gerados nessa pesquisa serão mantidos em sigilo e apenas a equipe de pesquisadores terá acesso a eles. Os dados de cada participante receberão um código e não terão nenhuma identificação que permita associá-lo a um participante em particular. Caso o senhor/senhora queira poderá ter acesso aos resultados individuais.

### **Benefícios e riscos**

Esta pesquisa não oferece qualquer risco ao participante, uma vez que consiste apenas de coleta de sangue total. Esta pesquisa é sem benefício individual direto, mas as informações obtidas nos auxiliarão a melhor compreender os mecanismos envolvidos no envelhecimento.

Não haverá qualquer tipo de despesa ao paciente, no que tange a materiais ou testes. Ao assinar esse termo de consentimento o senhor/senhora não está abrindo mão de seus direitos legais.

### **Desligamento**

**A sua participação neste estudo é voluntária e sua recusa em participar ou seu desligamento do estudo não envolverá penalidades ou perda de benefícios aos quais você tem direito. Você poderá cessar sua participação a qualquer momento sem afetar seu acompanhamento médico em andamento.**

Através deste documento fica assegurado o direito ao Sr(a) \_\_\_\_\_ que terá todos os esclarecimentos relativos à pesquisa, garantidos, incluindo os métodos utilizados. A partir do momento que o paciente participante da pesquisa não desejar mais fazer parte da pesquisa, reserve-lhe o direito de retirar o seu consentimento, livre de sofrer qualquer penalidade ou danos, quaisquer que sejam. Se no transcorrer da pesquisa tiver alguma dúvida, ou por qualquer outro motivo necessitar de orientações, poderá procurar um dos pesquisadores, Dr. Marco Aurélio Romano-Silva, no tel. 3409-9135 (UFMG), ou Dra. Maria Aparecida Camargos Bicalho, no telefone 3241-1848. Caso seja necessário, você pode entrar em contato com o COEP/UFMG, sito à Av. Antonio Carlos 6627, Unidade Administrativa II, 2º andar, Belo Horizonte – 31270-901, telefone 3409-4592, fax 3409-4027, e-mail [coep@prpq.ufmg.br](mailto:coep@prpq.ufmg.br), onde maiores esclarecimentos poderão ser obtidos.

Eu, \_\_\_\_\_, paciente voluntário, dou consentimento livre e esclarecido, autorizo a retirada de 50 mL de sangue para que se façam os testes necessários a esta pesquisa e posterior uso e publicação dos dados nos relatórios finais e conclusivos, a fim de que estes sirvam para beneficiar a ciência e a humanidade.

Declaro, ainda, que recebi cópia do presente Termo de Consentimento.

Belo Horizonte, \_\_\_\_ de \_\_\_\_\_ de 20\_\_

Assinatura do participante

\_\_\_\_\_

Assinatura do responsável

\_\_\_\_\_

Assinatura do pesquisador:

\_\_\_\_\_

Nome do pesquisador:

\_\_\_\_\_

## ANEXO 3 - PROTOCOLO DE TRIAGEM DOS PACIENTES

### PROJETO BINOMIO DEPRESSÃO DEMÊNCIA

Sr(a) \_\_\_\_\_, meu nome é (*Avaliador*) e gostaria de fazer algumas perguntas sobre seu estado de saúde e seu humor para um projeto sobre envelhecimento, depressão e problemas de memória. Você se importaria se fizesse estas perguntas?

Data da triagem: \_\_\_\_/\_\_\_\_/\_\_\_\_

Nome: \_\_\_\_\_

Data de Nascimento: \_\_\_\_/\_\_\_\_/\_\_\_\_ Idade: \_\_\_\_\_

Escolaridade: \_\_\_\_\_

Contato: \_\_\_\_\_

Acompanhante: \_\_\_\_\_

#### Perguntas de triagem:

Você já foi diagnosticado ou está fazendo tratamento ou tomando remédios para estes problemas de saúde:

	Não	Sim
Depressão ou Ansiedade		
Doença de Alzheimer		
Outros problemas de memória		
Demência		
Transtorno Bipolar		

	Não	Sim
Esquizofrenia		
AVC		
Doença de Parkinson		
Epilepsia		

Você está tomando alguns destes remédios

	Não	Sim
Sertralina, Citalopram, fluoxetina, paroxetina		
Nortriptilina, Amitriptilina, Imipramina		
Mirtazapina, Venlafaxina, Lítio		
Donepezil, Rivastigmina, Galantamina (Reminyl)		
Risperidona, Quetiapina, Olanzapina, Ziprazidona		
Haloperidol, clorpromazina, ampictil, haldol		
Levodopa, Pramipexol, amantadina		

#### Lista de Fatores de risco para Depressão

1	Gênero Feminino	<b>NÃO</b>	<b>SIM</b>	10	Insônia	<b>NÃO</b>	<b>SIM</b>
2	Luto recente	<b>NÃO</b>	<b>SIM</b>	11	Quadro doloroso	<b>NÃO</b>	<b>SIM</b>

3	Situação estressora grave	<b>NÃO</b>	<b>SIM</b>	12	Obesidade	<b>NÃO</b>	<b>SIM</b>
4	Incapacidade física / funcional	<b>NÃO</b>	<b>SIM</b>	13	AVC	<b>NÃO</b>	<b>SIM</b>
5	Mora sozinho	<b>NÃO</b>	<b>SIM</b>	14	IAM	<b>NÃO</b>	<b>SIM</b>
6	Viúvo / Divorciado	<b>NÃO</b>	<b>SIM</b>	15	Cuidador	<b>NÃO</b>	<b>SIM</b>
7	Baixa condição socioeconômica	<b>NÃO</b>	<b>SIM</b>	16	Institucionalizado	<b>NÃO</b>	<b>SIM</b>
8	Diabetes Melito	<b>NÃO</b>	<b>SIM</b>	17	HF Depressão	<b>NÃO</b>	<b>SIM</b>
9	Depressão no passado	<b>NÃO</b>	<b>SIM</b>				

### PHQ-9

Durante as últimas 2 semanas, com que frequência você foi incomodado/a por qualquer um dos problemas abaixo?

(Marque sua resposta com "✓")

	Nenhuma vez	Vários dias	Mais da metade dos dias	Quase todos os dias
1. Pouco interesse ou pouco prazer em fazer as coisas	0	1	2	3
2. Se sentir "para baixo", deprimido/a ou sem perspectiva	0	1	2	3
3. Dificuldade para pegar no sono ou permanecer dormindo, ou dormir mais do que de costume	0	1	2	3
4. Se sentir cansado/a ou com pouca energia	0	1	2	3
5. Falta de apetite ou comendo demais	0	1	2	3
6. Se sentir mal consigo mesmo/a — ou achar que você é um fracasso ou que decepcionou sua família ou você mesmo/a	0	1	2	3
7. Dificuldade para se concentrar nas coisas, como ler o jornal ou ver televisão	0	1	2	3
8. Lentidão para se movimentar ou falar, a ponto das outras pessoas perceberem? Ou o oposto – estar tão agitado/a ou irrequieto/a que você fica andando de um lado para o outro muito mais do que de costume	0	1	2	3
9. Pensar em se ferir de alguma maneira ou que seria melhor estar morto/a	0	1	2	3

Total: \_\_\_\_\_

## Escala GAD - 7

Durante as <u>últimas 2 semanas</u> , com que frequência você foi incomodado/a pelos problemas abaixo?	Nenhuma vez	Vários dias	Mais da metade dos dias	Quase todos os dias
<i>(Marque sua resposta com "✓")</i>				
1. Sentir-se nervoso/a, ansioso/a ou muito tenso/a	0	1	2	3
2. Não ser capaz de impedir ou de controlar as preocupações	0	1	2	3
3. Preocupar-se muito com diversas coisas	0	1	2	3
4. Dificuldade para relaxar	0	1	2	3
5. Ficar tão agitado/a que se torna difícil permanecer sentado/a	0	1	2	3
6. Ficar facilmente aborrecido/a ou irritado/a	0	1	2	3
7. Sentir medo como se algo horrível fosse acontecer	0	1	2	3

**Total:** \_\_\_\_\_

- 0= normal / ou nunca o fez, mas poderia fazê-lo agora;  
 1= faz com dificuldade/ ou nunca o fez e agora teria dificuldade  
 2= necessita de ajuda  
 3= não é capaz

1) Ele (Ela) manuseia seu próprio dinheiro?	0	1	2	3
2) Ele (Ela) é capaz de comprar roupas, comida, coisas para casa sozinho (a)?	0	1	2	3
3) Ele (Ela) é capaz de esquentar a água para o café e apagar o fogo?	0	1	2	3
4) Ele (Ela) é capaz de preparar uma comida?	0	1	2	3
5) Ele (Ela) é capaz de manter-se em dia com atualidades, acontecimentos da comunidade ou da vizinhança?	0	1	2	3
6) Ele (Ela) é capaz de prestar atenção, entender e discutir programa de rádio, televisão, um jornal ou uma revista?	0	1	2	3
7) Ele (Ela) é capaz de lembrar-se de compromissos, acontecimentos familiares, feriados?	0	1	2	3
8) Ele (Ela) é capaz de manusear seus próprios remédios?	0	1	2	3
9) Ele (Ela) é capaz de passear pela vizinhança e encontrar o caminho de volta para casa?	0	1	2	3
10) Ele (Ela) pode ser deixado (a) sozinho (a) de forma segura?	0	1	2	3
<b>SUBTOTAL:</b>				
<b>TOTAL:</b>				

## Questionário Funcional de Pfeffer

**Escala de Satisfação com a Vida (Diener et al. Journal of Personality Assessment, 49: 71-5, 1985)**

**Instruções:** Eu vou lhe falar cinco afirmações com as quais pode ou não concordar. Você vai me dizer o quanto concorda ou discorda com cada uma.

7 = Concordo totalmente

6 = Concordo

5 = Concordo ligeiramente

4 = Nem concordo nem discordo

3 = Discordo ligeiramente

2 = Discordo

1 = Discordo totalmente

### Questões

1. \_\_\_\_ Na maioria dos aspectos, minha vida é próxima ao meu ideal.
2. \_\_\_\_ As condições da minha vida são excelentes.
3. \_\_\_\_ Estou satisfeito(a) com minha vida.
4. \_\_\_\_ Dentro do possível, tenho conseguido as coisas importantes que quero da vida.
5. \_\_\_\_ Se pudesse viver uma segunda vez, não mudaria quase nada na minha vida.

Total: \_\_\_\_\_

### Índice de Religiosidade da Universidade Duke

(1) Com que frequência você vai a uma igreja, templo ou outro encontro religioso?

1. Mais do que uma vez por semana
2. Uma vez por semana
3. Duas a três vezes por mês
4. Algumas vezes por ano
5. Uma vez por ano ou menos
6. Nunca

(2) Com que frequência você dedica o seu tempo a atividades religiosas individuais, como preces, rezas, meditações, leitura da bíblia ou de outros textos religiosos?

1. Mais do que uma vez ao dia
2. Diariamente
3. Duas ou mais vezes por semana
4. Uma vez por semana
5. Poucas vezes por mês
6. Raramente ou nunca

*A seção seguinte contém três frases a respeito de crenças ou experiências religiosas. Por favor, anote o quanto cada frase se aplica a você.*

(3) Em minha vida, eu sinto a presença de Deus (ou do Espírito Santo).

1. Totalmente verdade para mim
2. Em geral é verdade
3. Não estou certo
4. Em geral não é verdade
5. Não é verdade

(4) As minhas crenças religiosas estão realmente por trás de toda a minha maneira de viver.

1. Totalmente verdade para mim
2. Em geral é verdade
3. Não estou certo
4. Em geral não é verdade
5. Não é verdade

(5) Eu me esforço muito para viver a minha religião em todos os aspectos da vida.

1. Totalmente verdade para mim
2. Em geral é verdade
3. Não estou certo
4. Em geral não é verdade
5. Não é verdade

Discutido em: \_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_

Data de agendamento: \_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_

horário: \_\_\_\_\_

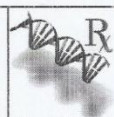
Avaliador: \_\_\_\_\_

## ANEXO 4 – FOLHA DE APROVAÇÃO DA TESE



UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR



### FOLHA DE APROVAÇÃO

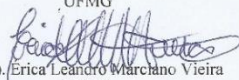
**Expressão do microRNA 184 em idosos com depressão maior: uma perspectiva translacional**

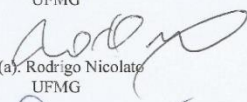
**ANA PAULA MENDES SILVA**

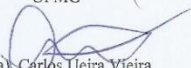
Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em MEDICINA MOLECULAR, como requisito para obtenção do grau de Doutor em MEDICINA MOLECULAR, área de concentração MEDICINA MOLECULAR.

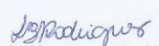
Aprovada em 08 de março de 2018, pela banca constituída pelos membros:

Prof(a). Breno Satler de Oliveira Diniz - videoconferência - Orientador  
UFMG

  
Prof(a). Erica Leandro Marciano Vieira  
UFMG

  
Prof(a). Rodrigo Nicolato  
UFMG

  
Prof(a). Carlos Ueira Vieira  
Universidade Federal de Uberlândia

  
Prof(a). Luciana Bastos Rodrigues  
UFUF

Belo Horizonte, 8 de março de 2018.