UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Júlio Carlos Pezzi

Polimorfismos das DNA-metiltransferases em Doenças Neurodegenerativas

Porto Alegre
2016

Júlio Carlos Pezzi

Polimorfismos das DNA-metiltransferases em Doenças Neurodegenerativas

Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor

Orientadora: Dr. Analuiza Camozzato de Pádua

Porto Alegre
2016

Catalogação na Publicação

Pezzi, Julio Carlos
Polimorfismos das DNA-metiltransferases em Doenças Neurodegenerativas / Julio Carlos Pezzi. -- 2016.
102 p. : il., graf., tab. ; 30 cm.

Tese (doutorado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2016.

Orientador(a): Analuiza Camozzato.

1. Doença de Alzheimer. 2. Doença de Parkinson. 3. Epigenômica. 4. Polimorfismo de Nucleotídeo Único. 5. DNA-metiltransferases. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

A Saul Pedro Pezzi
in memoriam

Pai, sempre que juntos plantávamos uma árvore, tua frase seguinte era: “agora só falta escrever um livro”... Vamos considerar esse nosso livro?

Agradecimentos

Às agências de fomento CNPq e em especial a CAPES, que me concedeu uma bolsa de nove meses de doutorado sanduíche.

À toda equipe do Laboratório de Biologia Molecular da UFCSPA.

Ao Serviço de Neurologia do Hospital de Clínicas de Porto Alegre.

À Profa. Marilu Fiegenbaum, pela sempre disponível ajuda.

À Cintia Ens, pois dividimos bons momentos no laboratório.

À minha orientadora, Analuiza Camozzato. Um exemplo de profissionalismo, ética. Além de uma grande pesquisadora, possui um raciocínio clínico dos mais perspicazes. Muito obrigado por ter me dado essa grande oportunidade. Muito obrigado por sensibilidade com que tu tratas as pessoas, aprendi muito.

Às minhas colegas de trabalho Alessandra Gemelli e Gladis Stadtolber por todo absoluto auxílio. Nossa relação ultrapassou ao âmbito do trabalho diário e hoje somos bons amigos. Por falar nisso, cito também minhas amigas Fernanda Farina e Mônica Schneider, também sempre disponíveis para um abraço na hora que mais preciso.

À minha amiga Liliane Vidor. Nos conhecemos ainda durante um mestrado bastante conturbado, em outra instituição. Lutamos sempre.

À minha mãe Jonilda e minha irmã Samanta por serem presenças que me iluminam.

A Rodrigo Grassi. A melhor companhia para uma viagem, a melhor companhia para ir ao cinema, a melhor companhia para ficar em casa num domingo chuvoso. Inteligente, divertido, não há rotina. Tu és minha fonte de inspiração e motivação para tocar a diante. Sem teu sorriso minha vida não seria a mesma.

Considere a sutileza do mar; como as suas criaturas mais temidas deslizam sob as águas, invisíveis na maior parte, e traiçoeiramente ocultas sob os matizes mais encantadores do azul. Considere também o brilho e a beleza diabólica de muitas de suas tribos sem piedade, como a forma delicadamente adornada de muitas espécies de tubarões. Considere, uma vez mais, o canibalismo universal do mar; cujas criaturas todas se devoram umas às outras, continuando a guerra eternal desde o início do mundo.

Considere tudo isso; e então se volte para esta terra tão verde, suave e dócil; ambos considere, o mar e a terra; e você não acha que existe uma analogia estranha com algo dentro de você? Pois, tal como o oceano aterrador cerca a terra verdejante, também na alma do homem há um Taiti insular, cheio de paz e alegria, mas rodeado por todos os horrores da metade desconhecida da vida.

Herman Melville, Moby Dick (1851)

Resumo

Introdução: Doença de Alzheimer (DA) e Parkinson (DP) são as duas doenças neurodegenerativas mais prevalentes na população. Ambas podem ter um início precoce, em geral com fatores genéticos definidos, ou manifestarem-se com um início tardio, frequentemente sem influência genética. São consideradas doenças de etiopatologia complexa, tendo em vista que apresentam aspectos genéticos, somados a fatores ambientais. Nesse sentido, mecanismos epigenéticos envolvidos no desenvolvimento da DA e DP têm sido recentemente investigados. Dentre esses mecanismos, a metilação de DNA é um dos principais processos de modificação epigenética, realizado por enzimas específicas, tais como DNA (citosina-5-)-metiltransferase 1 (*DNMT1*) e DNA (citosina-5-)-metiltransferase 3 beta (*DNMT3B)*.

Objetivos: Investigar a associação de polimorfismos nos genes que codificam as enzimas *DNMT1* e *DNMT3B* com a doença de Alzheimer e Parkinson, em indivíduos com início tardio da doença, comparando com controles saudáveis.

Material e Métodos: Os dados foram coletados em um serviço universitário de atenção terciária. Foram investigados 102 pacientes com DA (seguindo os critérios da NINCDS/ARDA), comparando com 108 controles com envelhecimento saudável. Além disso, investigou-se 214 pacientes com DP (utilizando os critérios do Critérios *UK Brain Bank*) e 308 controles sem a patologia.

Para os dois estudos, o DNA foi obtido a partir de sangue total, e genótipos foram detectados por ensaio de discriminação alélica utilizando sondas MGB TaqMan® em PCR tempo real. Os polimorfismos foram estudados rs2162560 e rs759920 (DNMT1) e rs998382, rs2424913 e rs2424932 (DNMT3B).

Resultados: o haplótipo TGG no gene *DNMT3B* mostrou associação significativa com a doença de Alzheimer. Os indivíduos que carreiam o haplótipo TGG apresentam um risco aumentado de doença de Alzheimer (OR = 3.03 ; IC de 95% 1.63-5.63 ; $p < 0.001$). Na população de pacientes com DP, comparado a controles saudáveis, evidenciou-se que a presença do alelo T

(rs2424913) do gene *DNMT3B* aumenta em 1,8 vezes o risco de um paciente possuir o diagnóstico dessa patologia (indivíduos CT e TT: OR = 1.80 ; IC de 95% 1.16 - 2.81 ; p = 0.009). Em ambos os grupos de pacientes investigados, não houve relação significativa com os polimorfismos investigados para a enzima *DNMT1*.

Conclusão: até onde se tem conhecimento, esses são os primeiros achados correlacionando polimorfismos das DNA-metiltransferases e a ocorrência de duas das principais doenças neurodegenerativas. Estes polimorfismos da *DNMT3B* podem interagir com o impulso epigenético determinado pela idade, conferindo então um risco para DA e para DP.

Palavras-Chave:
Doença de Parkinson. Doença de Alzheimer. Epigenômica. Polimorfismo de Nucleotídeo Único.

Abstract

Background: Alzheimer 's disease (AD) and Parkinson's (PD) are the two most prevalent neurodegenerative diseases in the population. Both can have an early onset, usually with defined genetic factors, or express with a late onset, often without genetic influences. These disorders are considered to have a complex etiopathology having genetic and environmental factors acting. In this sense, epigenetic mechanisms, involved in the development of AD and PD, has recently been investigated. Among these mechanisms, DNA methylation is one of the main epigenetic modification process, mediated by specific enzymes such as DNA-(cytosine-5)-methyltransferase 1 (DNMT1) and DNA-(cytosine-5)-methyltransferase 3 beta (DNMT3B).

Objectives: the aim was to investigate the association between polymorphisms in genes encoding *DNMT1* and *DNMT3B* in AD and PD in individuals with late onset of the disease compared to healthy controls.

Methods: Data were collected in a university reference care service. Was investigated 102 patients (following NINCDS/ARDA criteria) compared to 108 elderly healthy controls. Furthermore, it was investigated 214 patients with PD (using Brain Bank UK criteria) and 308 controls without pathology.
For both studies, DNA was obtained from whole blood and genotypes were detected by allelic discrimination assay using TaqMan MGB probes in real time PCR. The polymorphisms studied were rs2162560 and rs759920 (*DNMT1*), and rs998382, rs2424913 and rs2424932 (*DNMT3B*).

Results: TGG haplotype in *DNMT3B* gene was significantly associated with Alzheimer's disease. Carriers of the *DNMT3B* TGG haplotype were associated with AD (OR = 3.03, 95% CI 1.63 to 5.63, $p < 0.001$). The population of PD patients, compared to healthy controls, showed that the presence of T allele (rs2424913) in DNMT3B gene increases at 1.8 times the risk of a patient has the diagnosis of this pathology (CT and TT Individuals: OR = 1.80; 95% CI 1.16 - 2.81, $p = 0.009$).

In both studies, there was no significant relationship with the polymorphisms investigated for enzyme *DNMT1*.

Conclusion: as far as is known, these are the first findings correlating polymorphisms of DNA methyltransferases and the occurrence of two major neurodegenerative diseases. These *DNMT3B* polymorphisms may interact with epigenetic impulse determined by age, conferring a risk for AD and PD.

Keywords:
Parkinson Disease. Alzheimer Disease. Epigenomics. Polymorphism, Single Nucleotide.

Lista de Tabelas

Revisão Bibliográfica

Artigo Científico 1

DNA methyltransferase haplotype is associated with Alzheimer's disease

Artigo Científico 2

Association between DNA methyltransferase gene polymorphism and Parkinson's disease

Anexo 2: artigo científico, outras produções

The additive risk effect of apolipoprotein ε4 and DNMT3B haplotype for Alzheimer's disease

Lista de Figuras

Revisão Bibliográfica

Lista De Abreviaturas

DA	Doença de Alzheimer
AD	*Alzheimer's disease*
DP	Doença de Parkinson
PD	*Parkinson's disease*
Aβ	Proteína β-amilóide
SNP	*Single Nucleotide Polymorphism* ou Polimorfismo de Nucleotídeo Único
BACE-1	Enzima de clivagem beta-secretase1 ou *Beta-site Amyloid Precursor Protein–Cleaving Enzyme 1*
APP	Proteína Precursora de Amiloide ou *Amyloid Precursor Protein*
PSEN1	Presenilina 1
PSEN2	Presenilina 2
APOE	Apolipoproteína E
ε2	alelo épsilon 2 do gene da APOE
ε3	alelo épsilon 3 do gene da APOE
ε4	alelo épsilon 4 do gene da APOE
SNCA	α-sinucleína
BDNF	Fator Neurotrófico Derivado do Cérebro
DNMT	DNA-metiltransferase ou *DNA-methyltransferase*
DNMT1	DNA (citosina-5-)-metiltransferase 1
DNMT3B	DNA (citosina-5-)-metiltransferase 3 beta
LINE-1	*Long interspersed nucleotide element-1*
SAM	S-adenosil-metionina
SHA	S-adenosilhomocisteina
MTHFR	Metilenotetrahidrofolato Redutase
MAPT	Gene da proteína tau associada a microtúbulos
NINCDS-ADRDA	*National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association*

DSM-IV-TR	Manual Diagnóstico e Estatístico de Transtornos Mentais, quarta edição revisada
CDR	*Clinical Dementia Rating*
MMSE	*Mini Mental State Examination*
ADL	*Activities of Daily Living*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Sumário

1 Introdução

As formas mais comuns das doenças de Alzheimer (DA) e Parkinson (DP), de início mais tardio, são consideradas complexas do ponto de vista etiológico e influenciadas por diversos fatores de riscos: genéticos e ambientais.

Nesse sentido os mecanismos etiopatogênicos dessas doenças ainda são largamente desconhecidos. Somam-se a este conhecimento limitado, o importante prejuízo individual e social, bem como os altos custos gerados por estas doenças neurodegenerativas. Dessa maneira, estratégias para identificar fatores de risco para estas doenças são cruciais para o avanço da área.

A epigenética tem contribuído para a compreensão de doenças com etiologia complexa, como nos transtornos psiquiátricos, neoplasias e patologias neurodegenerativas. Esta disciplina refere-se ao estudo de alterações na expressão gênica que ocorrem sem alteração na sequência do DNA, sendo os processos de metilação e a modificação de histonas importantes mecanismos epigenéticos (WEBER et al., 2007).

O grupo de enzimas metiltransferases são as principais catalisadoras da metilação do DNA. O envelhecimento está associado a alterações de padrões de metilação que podem estar subsequentemente relacionadas ao surgimento de patologias associadas a idade, como a doença de Alzheimer (RICHARDSON, 2003) e Parkinson (POULOPOULOS; LEVY; ALCALAY, 2012). Polimorfismos de nucleotídeo único em genes que codificam metiltransferases foram identificados por sequenciamento de DNA (HALASCHEK-WIENER et al., 2009). A avaliação de polimorfismos nestes genes cujos produtos catalisam o processo de metilação do DNA poderá contribuir para os estudos de fatores de risco para estas doenças e poderá ser um marcador de traço, pressupondo que indivíduos com variantes genéticas terão alterações nos padrões de metilação de DNA associadas à idade que poderão contribuir para neurodegeneração.

Esta tese inclui um capítulo inicial de revisão da literatura, seguido de dois artigos científicos e uma conclusão. O primeiro artigo, já publicado na revista *Neuroscience Letters* (PEZZI et al., 2014), versa sobre a avaliação de polimorfismos dos genes das DNA-metiltransferases (*DNMTs*) em 102 pacientes com DA e 108 controles com envelhecimento saudável. O segundo artigo possui o mesmo enfoque, porém a

investigação ocorreu em uma população diferente: 214 pacientes com DP comparados a 308 controles sem a patologia.

2 Revisão de Literatura

As doenças neurodegenerativas resultam da perda gradual e progressiva de células neurais, conduzindo a disfunções do sistema nervoso. Dependendo da área e/ou das sinapses acometidas, mudam os sintomas. Essas doenças possuem alguma similaridade com o câncer no que diz respeito ao seu principal fator de risco, a idade. Entretanto, embora ambos tendam a aumentar a prevalência com o envelhecimento, câncer e neurodegeneração representam extremos opostos de um espectro: uma neoplasia é uma proliferação descontrolada de células, neurodegeneração é o resultado da morte de células, quer devido à morte celular por necrose direta ou processo de apoptose. Além disso, assim como em relação ao câncer , atualmente, tem sido dada atenção para o potencial dos agentes ambientais para precipitar alterações no sistema nervoso, resultando em doenças neurodegenerativas (BROWN; LOCKWOOD; SONAWANE, 2005).

Dentre os processos neurodegenerativos, a doença de Alzheimer (DA) e a doenças de Parkinson (DP) são as mais emblemáticas, acometendo um número crescente de pessoas, sobretudo devido ao aumento da expectativa de vida global. A Organização Mundial de Saúde estima que, em 2025, três quartos das pessoas com mais de 60 anos de idade estará vivendo em países em desenvolvimento (HTTP://WWW.WHO.INT/HPR/AGEING/ACTIVEAGINGPOLICYFRAME.PDF.).

Atualmente, existem 900 milhões de pessoas com mais de 60 anos vivendo no mundo. Entre os anos de 2015 a 2050, o número de idosos habitando países desenvolvidos subirá em 56%, comparado a 138% em países em desenvolvimentos, e 239% em países com baixo poder aquisitivo.

A doença neurodegenerativa mais frequente na população geral é a doença de Alzheimer. É estimado que, em 2015, 46.8 milhões de pessoas vivam com demência. Esse número praticamente dobra a cada vinte anos, alcançando provavelmente 74.7 milhões em 2030, e 131 milhões em 2050 (HTTP://WWW.ALZ.CO.UK/RESEARCH/WORLDALZHEIMERREPORT2015.PDF).

A segunda mais prevalente é a doença de Parkinson, acometendo cerca de 4.6 milhões de pessoas ao redor do mundo. A estimativa é que dobre até 2030, chegando a 8.7 milhões de casos, principalmente em países em desenvolvimento (DORSEY et al., 2007).

Diante disso, é importante que novas estratégias para prevenir ou tratar esses transtornos relacionadas à idade sejam desenvolvidos, já que o impacto econômico e social torna-se cada vez mais preocupante. Neste contexto, insere-se a busca de biomarcadores para essas doenças.

O diagnóstico dessas doenças baseia-se principalmente nos aspectos semiológicos, que são de instalação insidiosa. Para a DP, o destaque é para a apresentação da síndrome extrapiramidal (característica da doença), manifestada por tremor de repouso, rigidez, perda do reflexo postural e hipocinesia. A marcha característica, consta de pequenos passos com velocidade crescente e também denuncia a presença do distúrbio. Alterações da mímica facial, do humor, da caligrafia e da voz também são marcos significativos da doença (HUGHES et al., 1992).

Na DA, o comprometimento da memória (alteração da capacidade de aprender informações novas ou de recordar informações antigas) é o achado mais importante, associado a um prejuízo em pelo menos uma das funções cognitivas (linguagem, gnosias, praxias ou funções executivas) e que interfere no desempenho social e/ou profissional do indivíduo e representa um declínio em relação ao nível de funcionamento anterior (APA, 2002; MCKHANN, G. et al., 1984).

Os critérios diagnósticos da Doença de Parkinson e Alzheimer são expostos na Tabela 1.

Tabela 1: Critérios Diagnósticos da Doença de Parkinson e Alzheimer

Parkinson	**Alzheimer**
I) Diagnóstico da Síndrome Parkinsoniana	Critérios principais para diagnóstico de Demência (de qualquer etiologia)
Bradicinesia e pelo menos um dos seguintes: 1. Rigidez muscular 2. Tremor em repouso (4–6 Hz) 3. Instabilidade postural não causada por alteração visual, vestibular, cerebelar ou disfunção proprioceptiva.	a) Demência é diagnosticada quando há sintomas cognitivos ou comportamentais (neuropsiquiátricos) que: interferem com a habilidade no trabalho ou em atividades usuais; representam declínio em relação a níveis prévios de funcionamento e desempenho; não são explicáveis por *delirium* (estado confusional agudo) ou doença psiquiátrica maior. b) O comprometimento cognitivo é detectado e diagnosticado mediante combinação de anamnese, avaliação cognitiva
II) Critérios de Exclusão	
História de: • Recorrentes isquemias cerebrais ou evolução em escada das características parkinsonianas • Trauma encefálico de repetição • Encefalite definida • Crises oculógiras	

• Tratamento com neurolépticos no início dos sintomas • Remissão sustentada • Mais de um familiar afetado • Sintomas estritamente unilaterais por mais de três anos • Paralisia supranuclear do olhar • Sinais cerebelares • Disautonomia grave precoce • Demência precoce com distúrbios de memória, linguagem e praxias • Sinal de Babinski • Tumor cerebral ou hidrocefalia em estudo de imagem • Exposição à tetra-hidropteridina (MPTP) • Resposta negativa à levodopa, a despeito de altas doses, na ausência de má-absorção	c) Os comprometimentos cognitivos ou comportamentais afetam no mínimo dois dos seguintes domínios: memória; funções executivas; habilidades visuais-espaciais; linguagem; personalidade ou comportamento.
III) Critérios de suporte prospectivos para diagnóstico de Doença de Parkinson	**Demência da doença de Alzheimer provável**
Três ou mais dos seguintes para o diagnóstico: • Início unilateral, acometimento assimétrico • Presença de tremor em repouso • Doença progressiva • Assimetria persistente afetando principalmente o lado de início da doença • Resposta excelente à levodopa (melhora de 70-100%) • Resposta à levodopa por cinco anos ou mais • Discinesia induzida pela terapia com levodopa • Evolução clinica de dez anos ou mais	Preenche critérios para demência e tem adicionalmente as seguintes características: • Início insidioso (meses ou anos). • História clara ou observação de piora cognitiva. • Déficits cognitivos iniciais e mais proeminentes em uma das seguintes categorias: • Apresentação amnéstica (deve haver outro domínio afetado). • Apresentação não-amnéstica (deve haver outro domínio afetado). • Linguagem (lembranças de palavras). • Visual-espacial (cognição espacial, agnosia para objetos ou faces, simultâneo agnosia e alexia). • Funções executivas (alteração do raciocínio, julgamento e solução de problemas). • Tomografia ou, preferencialmente, ressonância magnética do crânio deve ser realizada para excluir outras possibilidades diagnósticas ou comorbidades, principalmente a doença vascular cerebral.
Critérios UK Brain Bank (HUGHES et al., 1992)	Adaptado de (MCKHANN, G. M. et al., 2011) (FROTA, 2011)

2.1 Etiopatologia e Genética da doença de Alzheimer

Muitas lesões moleculares foram detectados na doença de Alzheimer, mas o mecanismo principal tende a focar na deposição de proteínas no cérebro, resultando em danos oxidativos e inflamatórios, que, por sua vez, levam à falha de energia e disfunção sináptica (QUERFURTH; LAFERLA, 2010).

Placas cerebrais de proteína β-amilóide (Aβ) e neurites distróficas em campos terminais neocortical, bem como emaranhados neurofibrilares de proteína tau proeminentes nas estruturas do lobo temporal medial são características patológicas importantes da doença de Alzheimer. Além disso, também estão presentes perda de neurônios e substância branca, angiopatia congofílica (amiloide), inflamação e dano oxidativo (QUERFURTH; LAFERLA, 2010).

Peptídeos Aβ são produtos naturais de metabolismo, sendo constituídos por cerca de 36 a 43 aminoácidos. Monômeros de $A\beta_{40}$ são muito mais prevalentes do que os da classe $A\beta_{42}$, esses potencialmente prejudiciais, amiloidogênicos. Os peptídeos β-amiloides são originários da quebra da proteína precursora amiloide pelas ações da enzima de clivagem beta-secretase1 (*beta-site amyloid precursor protein–cleaving enzyme 1: BACE-1*), da β-secretase, e da γ-secretase, um complexo protéico com presenilina 1 na sua ação catalítica. Um desequilíbrio entre a produção e recaptação, além da agregação de peptídeos, provoca a acumulação de Aβ, e este excesso torna-se o fator de início da doença de Alzheimer. Essa ideia, usualmente chamada de Hipótese Amiloide da DA, é baseada em estudos genéticos, incluindo participantes com Síndrome de Down, onde houve os primeiros relatos de que a $A\beta_{42}$ seria neurotóxica (SELKOE, 2001; SLEEGERS; VAN DUIJN, 2001).

Espontaneamente, Aβ se auto-agrega em várias formas físicas. Uma forma consiste em oligômeros (2 a 6 peptídeos), que se aglutinam em conjuntos intermediários. A proteína β-amiloide também pode crescer em fibras, que se organizam em folhas insolúveis de placas amiloides. Oligômeros solúveis são as formas mais neurotóxicas de Aβ (SLEEGERS; VAN DUIJN, 2001).

A protease neprilisina e a enzima degradadora de insulina regulam os níveis de estado estacionário de Aβ. Neprilisina, um endopeptídeo-zinco ancorado a membrana, agrupa monómeros de Aβ em oligômeros. Uma redução na neprilisina provoca acúmulo de Aβ cerebral. Por sua vez, a enzima degradadora de insulina,

uma tiol-metaloendopeptidase, degrada pequenos peptídeos tais como insulina e monômeros de Aβ (TANZI; BERTRAM, 2005).

Outro mecanismo fisiopatológico da DA é o acúmulo de emaranhados neurofibrilares, que são inclusões filamentosas em neurônios, marca da doença de Alzheimer e de outros distúrbios neurodegenerativos, denominado taupatia. O número de emaranhados neurofibrilares é um marcador da gravidade da doença de Alzheimer. O componente principal dos emaranhados é a forma hiperfosforilada e agregada da proteína tau (LEE; GOEDERT; TROJANOWSKI, 2001; QUERFURTH; LAFERLA, 2010). A proteína tau usualmente solúvel, abundante em axônios, promove a estabilidade e arquitetura dos microtúbulos e transporte vesicular. A tau hiperfosforilada é insolúvel, não tem afinidade pelos microtúbulos. Existem enzimas que adicionam ou removem resíduos de fosfato, regulando a fosforilação da tau. Como oligômeros de Aβ, agregados intermediários de moléculas tau anormais são citotóxicos e prejudicam a cognição (LEE et al., 2001).

A doença de Alzheimer pode também ser entendida primariamente como um distúrbio de falha sináptica. As sinapses hipocampais começam a diminuir em pacientes com comprometimento cognitivo leve devido a DA, e, nesse estágio, as sinapses restantes mostram aumentos compensatórios de tamanho. Na DA leve, há uma redução de cerca de 25% nas vesículas de proteínas pré-sinápticas. Com o avançar da doença, as sinapses vão sendo desproporcionalmente perdidas em comparação aos neurônios, sendo isso uma característica da DA, que ocorre principalmente na região do hipocampo (NITSCH, 1996).

A deficiência de projeções colinérgicos (que no início da doença acometem o Núcleo de Meynert) na doença de Alzheimer tem sido associada ao acúmulo de $A\beta_{42}$ e tau. Receptores nicotínicos pré-sinápticos alfa-7, essenciais para o processamento cognitivo, mostraram-se aumentados no início da doença de Alzheimer, antes de diminuir mais tarde. Estudos experimentais mostram relação entre esses receptores com o acúmulo que $A\beta_{42}$, prejudicando a liberação de acetilcolina e manutenção de potencialização de longo prazo. O nível de receptores de acetilcolina muscarínicos, ou o acoplamento do receptor, é reduzido em cérebros de pacientes com doença de Alzheimer (NITSCH, 1996).

No entanto, os mecanismos fisiopatológicos da DA são diversos, e não se sabe ao certo quais os fatores que desencadeiam o acúmulo de proteína $A\beta_{42}$ ou a taupatia. Alguns autores sugerem que o mecanismo da hipótese amiloide é apenas

um fator menor, da patogênese, e que algum processo relacionado a idade seria o gatilho para a doença (QUERFURTH; LAFERLA, 2010).

Com relação à genética da DA, os primeiros relatos de famílias com grande frequência de DA de início precoce foram na década de 30. Assim, famílias que claramente herdaram a doença tem características de herança Mendeliana autossômica dominante, ou seja, as crianças de um pai afetado apresentam 50% de risco para o desenvolvimento de DA de início precoce. Estes casos de herança autossômica dominante respondem a aproximadamente 1% da totalidade dos casos de DA (SCHELLENBERG; MONTINE, 2012). Na década de 80, foram identificados três genes com essa característica de herdabilidade: proteína precursora de amiloide (APP: *amyloid precursor protein*), Presenilina 1 (*PSEN1*) e Presenilina 2 (*PSEN2*) (SCHELLENBERG; MONTINE, 2012).

Até o momento, 59 mutações de nucleotídeo único no gene da APP conhecidamente causam AD (HTTP://WWW.MOLGEN.UA.AC.BE/ADMUTATIONS/DEFAULT.CFM?MT=1&ML=1&PAGE=MUTBYQUERY&QUERY=TBLCONTEXTS.ID=3&SELECTION=GENE%20=%20APP). Enquanto APP pode codificar múltiplas isoformas, sendo a maior possui 750 aminoácidos, todas as mutações para DA são agrupadas dentro de um segmento de 54 aminoácidos. (SCHELLENBERG; MONTINE, 2012).

Outras mutações da APP que causam DA, ocorrem no aminoácido Val717Ile. Essas mutações alteram a atividade de clivagem da γ-secretase. Quando a proteólise da APP normal é catalisada pela y-secretase, a espécie predominante formada é de 40 aminoácidos de comprimento ($A\beta_{40}$) com pequenas quantidades de Aβ de 42 aminoácidos de comprimento ($A\beta_{42}$). Mutações da APP na extremidade C-terminal da Aβ cursam com deslocamento da proteólise para produzir mais Aβ42 à custa de Aβ40, resultando num aumento da proporção $A\beta_{42}$ / $A\beta_{40}$ mas não necessariamente, uma mudança na quantidade total de peptídeos Aβ formados (BERGMANS; DE STROOPER, 2010).

Além disso, mais de 180 mutações no *PSEN1* são conhecidas por causar DA autossômica dominante com início precoce. A penetrância de mutações *PSEN1* é completa, ou seja, todos os portadores da mutação desenvolverão DA até a idade 60-65 anos. Menos de 15 mutações autossômica dominante conhecidas em *PSEN2*, que também podem cursar com início precoce AD, mas penetrância é mais variável (JAYADEV et al., 2010).

PSEN1 e *PSEN2* codificam proteínas intimamente relacionados, que são parte do complexo γ-secretase, sendo o mecanismo de ação dessas proteínas alteradas similar ao que ocorre com as alterações da APP, aumentando a relação $A\beta_{42}$ / $A\beta_{40}$ (SCHELLENBERG; MONTINE, 2012).

Entretanto, a maior parte dos casos de DA são esporádicos, apresentando uma genética complexa, envolvendo a interação entre múltiplos fatores de risco genéticos e ambientais (BALLARD et al., 2011; JAYADEV et al., 2010). O principal fator de risco genético no casos esporádicos para o desenvolvimento das placas amiloides na DA de início tardio tem sido relacionado à Apolipoproteína E (APOE) (BALLARD et al., 2011). A APOE é uma molécula transportada com o colesterol que está envolvida na depuração de Aβ. As isoformas de APOE possuem uma diferença na eficiência de transporte dos lipídios. Durante a evolução ocorreram mutações no gene codificante da *APOE*, resultando em genes com pequenas diferenças ou alelos, que ocasionaram alterações na sequência da proteína. Em humanos, são três os alelos principais do gene APOE, resultantes de duas alterações do DNA, sendo denominados de ε2, ε3 e ε4. Uma pessoa com um genótipo de ε4 / ε4 apresenta maior risco do que alguém com genótipo ε3 / ε4 ou ε2 / ε4 . Da mesma forma, o genótipo ε2 / ε2 é mais protetor do que genótipos onde apenas um alelo ε2 é herdado. O alelo ε4 está associado à carga amiloide e à disfunção colinérgica aumentada. Estima-se que esse alelo apresente um efeito de risco de 3-10 vezes maior para o desenvolvimento da doença (BALLARD et al., 2011). A associação do polimorfismo da APOE com a DA de início tardio é um dos fatores de risco genético mais forte e robusto para doença. Em comparação com o alelo ε3, ε4 aumenta o risco e diminui a idade de início da DA de uma forma dose-dependente, enquanto o alelo ε2 confere uma vantagem de proteção (JUN et al., 2012).

Uma metanálise de *genome-wide association studies* (GWAS) com 17.000 pacientes com DA, comparando com 37.154 controles foi conduzida com mais de 7 milhões de SNPs. Desses, 80 loci associaram-se significativamente com DA de início tardio, sendo os principais correlacionados a genes da *sortilina-1*, *clusterina*, *PICALM* (LAMBERT et al., 2013).

2.2 Etiopatologia e Genética da doença de Parkinson

Com relação à DP, as principais características fisiopatológicas são a perda dos neurônios dopaminérgicos nigrostriatais e a presença de inclusões de proteínas (corpúsculos de Lewy) citoplasmáticas intraneuronais, que nos neurônios nigrostriatais do SNC, projetando-se principalmente para o putâmen (DAUER; PRZEDBORSKI, 2003). A perda desses neurônios, que normalmente contêm quantidades importantes de neuro-melanina, produzem um achado neuropatológico clássico: despigmentação. O padrão de perda de células do SNC aparece em paralelo ao nível de expressão do RNAm transportador de dopamina e isso é consistente com a descoberta de que a depleção de dopamina é mais pronunciada no putâmen dorsolateral (UHL et al., 1994), a principal região a qual se projetam neurônios. No início dos sintomas, a dopamina no putâmen está depletada em aproximadamente 80%, e cerca de 60% dos neurônios dopaminérgicos no SNC já estão comprometidos. Os neurônios dopaminérgicos mesolímbicos e os corpúsculos celulares adjacente à área ventral tegmentar, são muito menos afetados na DP. Por conseguinte, existe significativamente menos depleção de DA no caudado, o principal local de projeção desses neurônios (DAUER; PRZEDBORSKI, 2003).

Além disso, sabe-se que a agregação de proteínas tem emergido como um aspecto comum em doenças neurodegenerativas. Cada doença neurodegenerativa é categorizada de acordo com a proteína mais abundante nas inclusões celulares no SNC (KALIA; LANG, 2015). Na DP, esta proteína foi identificada como α-sinucleína, seguindo da descoberta de que as mutações no seu gene (*SNCA*) poderiam causar uma forma monogênica da doença. Em situação propícia, a α-sinucleína torna-se insolúvel e agrega-se para formar inclusões intracelulares dentro do corpo celular (corpúsculos de Lewy) e processos patogênicos (neurite de Lewy) (SPILLANTINI et al., 1997). Na doença de Lewy a patologia não se restringe ao cérebro, mas também pode ser encontrada na medula espinhal e no sistema nervoso periférico, incluindo o nervo vago, gânglios simpáticos, plexo cardíaco, do sistema nervoso entérico, dentre outros (KALIA; LANG, 2015).

Entretanto, sugere-se que a patologia da DP é mais complexa do que a neurodegeneração devido à patologia de Lewy isoladamente. Em primeiro lugar, α-sinucleína é conhecida por formar uma variedade de diferentes tipos de agregados, incluindo estruturas muito pequenas que se acumulam em neurônios pré-sinápticos,

e oligômeros solúveis constituídos por monómeros 2-100 α-sinucleína (SAITO et al., 2003). Essas formas alternativas de agregados dessa proteína podem desempenhar um papel importante na neurodegeneração na DP, inclusive algumas formas oligoméricas sendo tóxicas a neurônios. Em segundo lugar, patologias distintas de agregados α-sinucleína, tais como inclusões de outros compostos proteicos, são frequentemente vistas no cérebro de pacientes com DP. Por exemplo, as placas β-amilóide e emaranhados neurofibrilares de proteína tau (características da DA), podem ser encontrados nos cérebros dos pacientes com Parkinson em quantidades e distribuição comparáveis aos cérebros de pacientes com doença de Alzheimer. Concomitantemente, a patologia da doença de Alzheimer está associada com uma maior carga de patologia de Lewy, a uma menor latência até ao início da demência na doença de Parkinson, e ocorre em até 50% dos pacientes com doença de Parkinson e demência. Assim, inclusões de outras proteínas, além da α-sinucleína, podem atuar de forma sinérgica com patologia de Lewy e contribuir para a expressão clínica da DP. Além disso, estudos neuropatológicos têm documentado a ausência de patologia de Lewy na maioria dos pacientes com doença de Parkinson que têm doença relacionada com o gene *Parkin* e, em menor proporção, com mutações *LRRK2 (POULOPOULOS et al., 2012)*. Isso novamente sugere que há outras explicações para a etiopatogenia dessa doença.

Neuroinflamação é outra característica de patologia da DP. A presença de resposta inflamatória no cérebro, mediado principalmente por astrócitos e microglia têm sido desde há muito reconhecido. Os astrócitos e a microglia estão ambos envolvidos na depuração dos detritos extracelulares, o que pode ajudar na sobrevivência de neurônios. A ativação da microglia pode liberar fatores tróficos, tais como o fator neurotrófico derivado do cérebro (BDNF: *brain derived neurotrophic fator*; traduzido por fator neurotrófico derivado do cérebro) e o fator neurotrófico derivado das células gliais, mas também variantes nocivas de oxigênio e citoquinas pró-inflamatórias. Se o saldo dessas ações é benéfico ou prejudicial aos neurônios ainda não está estabelecido (PHANI; LOIKE; PRZEDBORSKI, 2012).

Em relação à genética da DP, evoluiu-se muito com as descobertas dos últimos 15 anos. Embora os casos de herança familiar sejam menos comuns, as primeiras investigações utilizaram a ligação em famílias com numerosos casos da doença, tentando identificar algum gene de ligação. O primeiro identificado foi o *SNCA*, e associação de mutações autossômicas dominantes relacionadas com parkinsonismo

(POULOPOULOS et al., 2012). Alterações causadoras de doenças incluem mutações *missense*, que resultam em substituições de aminoácidos, e multiplicações do locus do gene. Essas alterações tornariam a α-sinucleína propensa a agregar-se (DEVINE et al., 2011).

Seis genes têm sido propostos para mediar formas autossômicas dominantes da DP: *SNCA*, *LRRK2*, *VPS35*, *EIF4G1*, *DNAJC13* e *CHCHD2 (KALIA; LANG, 2015)*.

O gene da *LRRK2* codifica a leucina quinase-2, uma proteína envolvida em vários processos celulares, incluindo morfogênese sináptica, o tráfico de membrana, autofagia, e síntese de proteínas. Pelo menos oito mutações causadoras de doenças em *LRRK2* foram identificadas, todas agrupadas principalmente dentro dos domínios catalíticos da proteína. Mutações nesse gene são a causa mais frequente de DP genética, sendo encontrados em cerca de 4% da doença familiar de Parkinson, e representando 1% de doença de Parkinson esporádica. A mutação mais comum resulta na substituição de aminoácidos Gly2019Ser, o que aumenta a atividade quinase da proteína.

VPS35, EIF4G1, DNAJC13, e *CHCHD2* são os genes mais recentemente associados com a doença autossômica dominante na DP (DEVINE et al., 2011; KALIA; LANG, 2015).

Parkin, *PINK1* e *DJ-1* estão associados às formas recessivas autossômicas da DP. Ao contrário da doença autossômica dominante, que tende a ter uma idade de início semelhante à doença esporádica de Parkinson, essas formas hereditárias recessivas estão mais frequentemente associadas com início precoce (idade inferior a 40 anos). As mutações em *Parkin* são a causa mais comum de doença autossômica recessiva de Parkinson. Em pacientes com doença de Parkinson início antes da idade de 45 anos, mutações em *Parkin* estão presentes em até 50% dos casos familiares e cerca de 15% dos casos esporádicos. Mutações nos genes *PINK1* e *DJ-1* são menos frequentes (1-8% nos casos de início precoce). As proteínas codificadas pelos *Parkin*, *PINK1* e *DJ-1* estão todos implicados na saúde mitocondrial (KALIA; LANG, 2015; PERIQUET et al., 2003).

Com relação a GWAS, uma metanálise conduzida com 13.780 pacientes do DP comparando com 95.282 controles, em mais de 7 milhões de SNPs identificou-se 26 *loci* como tendo associação significante com a forma esporádica da doença. Os principais genes associados forma da *SNCA* e *LRRK2*. Desse banco, foi estudada uma sub-amostra de tecido cerebral, correlacionando níveis de metilação a

proteínas de ligação do DNA. Um achado interessante foi que no SNP rs199347 do cromossomo 7, o alelo de risco (A) foi associado a maior expressão do gene da *Nucleoporin-like 2*, além de uma diminuição da metilação do gene da *Glicoproteina-nmb* (NALLS et al., 2014).

2.3 Considerações sobre o papel da epigenética nos casos esporádicos de doença de Alzheimer e Parkinson

Considerando o exposto acima, de forma concisa, do ponto de vista genético a maioria dos casos de doenças de Alzheimer e Parkinson são esporádicos, sendo sua patogênese pensada a partir da combinação de fatores ambientais e características genéticas, ambos ainda não sendo totalmente compreendidos. Além disso, nessas patologias, existe uma forte dicotomia entre as formas familiares (mais raras, de início precoce) e não-familiares (comuns, de início após as 60 anos) (BERTRAM; TANZI, 2005; MARQUES et al., 2011). Diante disso, essas patologias são consideradas de etiologia altamente complexas, de uma maneira similar ao que é percebido em outras doenças, como câncer, diabetes, cardiopatias e outros transtornos neuropsiquiátricos (MARQUES et al., 2011). Fica evidente a necessidade de entender como a interação gene-ambiente faz com que o processo de doença seja desencadeado.

Uma forma pela qual o ambiente pode influenciar o risco a doenças é através de modificações epigenéticas do DNA. A questão de como as células (apesar de carregarem a mesma informação genética) em um organismo se diferenciam em tipos distintos, cumprindo diferentes funções intriga cientistas há décadas (GAPP et al., 2014). Assim como faz-se ainda necessário compreender como as condições ambientais podem permitir que os sistemas celulares adquiram características específicas, tais como a resistência ao estresse, e mantenham esses recursos ao longo da vida (VILLENEUVE; REDDY; NATARAJAN, 2011). Na tentativa de explicar esses fenômenos, Conrad Waddington, em 1956 (WADDINGTON, 1956), introduziu o conceito de epigenética, e propôs a teoria de que fatores ambientais poderiam modificar o genótipo, alterar os processos de desenvolvimento e, assim, conferir propriedades específicas para as células.

Epigenética refere-se a mudanças na expressão gênica que ocorrem independentemente de mudanças na sequência do DNA e são adquiridas ao longo da vida, podendo decorrer de pistas ambientais, tais como estilo de vida, dieta e exposição a toxinas (SCHUMACHER, 2011).

Diversas pesquisas na área da oncologia tem focado seus estudos na epigenética, sendo a área pioneira na publicação de estudos nessa vertente (BAO et al., 2011; RUBIN, 1992). Mais recentemente, pensando nessa hipótese da etiologia multifatorial para as doenças, a epigenética tornou-se um campo de investigação em doenças neuropsiquiátricas. Uma vez que DA e DP são patologias complexas relacionadas com a idade, a epigenética pode ser o elo de ligação entre os fatores de risco ambientais e a forma esporádica das doenças neurodegenerativas (MARQUES et al., 2011).

2.4 Mecanismos Epigenéticos

Os mecanismos epigenéticos são a metilação do DNA, a modificações em histonas e os RNAs não-codificantes.

Os RNAs não-codificantes foram descritos no final da década de noventa (FIRE et al., 1998). Embora tenham esta denominação, possuem papel na regulação da expressão gênica. Algumas formas de ação têm sido propostas para esses elementos, dentre as quais destacam-se o recrutamento de marcas epigenéticas ligadas ao silenciamento transcricional, o bloqueio da transcrição por impedimento da ligação da RNA polimerase II e a diminuição da quantidade de RNA mensageiro (LOCHMATTER; MULLIS, 2011; MORRIS, 2009). Primeiramente, foram descritos como responsáveis pelo silenciamento gênico, no entanto, alguns estudos sugerem também sua ação na ativação de alguns genes (TURNER; MORRIS, 2010).

O outro mecanismo epigenético, a modificação pós-traducional de histonas, é mediada pelas enzimas acetiltransferases, que catalisam acetilação das histonas, e a histona-desacetilase, sendo que sua ação resulta na remoção do grupo acetil das histonas. Essas modificações podem ocorrer em toda a extensão da fita de DNA, no entanto são mais comuns na região terminal das proteínas. Fundamentalmente, o aumento da acetilação de histonas é associado com maior atividade gênica e vice versa (FUKS et al., 2000).

O processo epigenético melhor estudado é a metilação do DNA. Em mamíferos, citosinas podem ser quimicamente modificadas, em uma ou ambas as cadeias de DNA, pela ligação covalente de um grupo metil ao quinto carbono do anel pirimidínico. Essa modificação ocorre principalmente no contexto de dinucleótidos CpG e tem sido tradicionalmente associado com a redução da expressão gênica (CEDAR, 1988). Durante o desenvolvimento do organismo humano, a metilação do DNA é cuidadosamente orquestrada, e padrões de metilação do DNA são reprogramados em células germinativas e em embriões de pré-implantação de todo o genoma. A metilação do DNA tem um importante papel no silenciamento de genes durante a embriogênese (WEBER et al., 2007). No entanto, observou-se que o processo segue acontecendo durante o desenvolvimento e ao longo da vida (WAGNER; FERNANDEZ-REBOLLO; FROBEL, 2015).

A metilação do DNA é mediada por uma família de enzimas chamadas DNA-metiltransferases (DNMT). As metilações de manutenção, que são necessárias a cada replicação do DNA, são mediadas pela DNMT1, que é também a mais abundante. A acurácia da transmissão da informação epigenética para a próxima geração de células é dependente da ação da DNMT1 (KRIAUCIONIS; HEINTZ, 2009).

Por outro lado, DNMT3A e DNMT3B são responsáveis pela metilação *de novo* do DNA não metilado. Dessa forma, essas enzimas influenciam diversos processos biológicos mediados pela repressão gênica (TAHILIANI et al., 2009). Além disso, outro importante papel desempenhado pela DNMT3B seria a de corrigir erros deixados pelas DNMT1 (JONES; LIANG, 2009).

2.5 Dinâmica da Metilação do DNA durante o Envelhecimento

Os padrões de metilação variam durante o ciclo celular, com os níveis de metilação de DNA diminuindo durante a fase G1 e aumentando durante a fase S da divisão celular. Adicionalmente , a metilação é também observada em células pós-mitóticas diferenciadas ocorrendo ao longo da vida (MARTINOWICH et al., 2003).

Em geral, um significante número de erros na metilação que ocorrem durante o envelhecimento são devidos a erros estocásticos durante a replicação, e nem sempre chegarão a ter relevância clinica (GOYAL; REINHARDT; JELTSCH, 2006).

Entretanto um outro aspecto a ser salientado é que a metilação de manutenção é um processo eficiente, que acontece com a DNMT1 se movendo ao longo do DNA de maneira randômica, metilando substratos hemimetilados com alta processividade. No entanto, ao contrário da sequência de DNA, padrões de metilação mudam notavelmente com o envelhecimento, sugerindo que taxas de erros na replicação epigenética podem ser significantemente maiores do que taxas de erros na replicação gênica (CHU et al., 2007).

Com o envelhecimento, mudanças no padrão da expressão do gene das *DNMTs* são observadas, como o RNAm das *DNMT1* e *DNMT3A* tornando-se reduzido, enquanto a produção da *DNMT3B* se mantém de forma constante. Esses estudos indicam que essas mudanças no controle das *DNMTs* podem ser uma explicação para alterações do padrão da metilação do DNA em idosos (CASILLAS et al., 2003; SCHUMACHER, 2011).

Além disso, estudos apontam que alguns loci mostraram alterações bidirecionais em DNA de células neuronais durante a transição da infância para a idade avançada, como consequência do aumento ou diminuição da metilação em locais específicos. Essas mudanças no padrão de metilação indicam que mecanismos de reparo epigenéticos podem não estar restritos somente à manutenção da metilação, mas também envolvem ativação da desmetilação (MA et al., 2009).

Recentemente foram sequenciados 24 genes candidatos a envelhecimento saudável, ou seja, genes que estiveram relacionados à possibilidade de o indivíduo viver até os 85 anos ou mais sem desenvolver doenças associadas ao envelhecimento, como a doença de Alzheimer. Estes pesquisadores detectaram vários SNPs nestes genes. Entre os genes sequenciados estavam os genes das metiltransferases envolvidos na metilação do DNA (*DNMT1, DNMT3A, DNMT3B*) (HALASCHEK-WIENER et al., 2009), as quais são codificadas por genes independentes (HENDRICH; BIRD, 2000).

Adicionalmente, dados do *Boston Normative Aging Study* indicam uma progressiva perda da metilação do DNA ao longo do genoma em regiões *Alu* e LINE-1 (*Long interspersed nucleotide element-1*). A metilação do DNA em sangue de 1097 pessoas entre 55 a 92 anos de idade, que participaram de uma coorte por 8 anos, foi avaliada e observou-se um declínio longitudinal linear da metilação nas regiões *Alu*, altamente correlacionado com o tempo. Não foi observada alteração de metilação nas regiões LINE-1. No entanto, os autores discutiram sobre a dificuldade de

relacionar essa hipometilação global do DNA com alguma alteração clínica, devido à variabilidade do padrão de metilação interindivíduos (BOLLATI et al., 2009).

Ainda não há consenso sobre um padrão global de hipo ou hipermetilação do DNA em idosos. Tanto aumento como diminuição da metilação do DNA têm sido associados ao processo de envelhecimento e tem se acumulado evidências de que as alterações de metilação idade-dependentes estão envolvidas no desenvolvimento de doenças neurológicas, autoimunes e câncer em pessoas acima de 60 anos de idade (RICHARDSON, 2003).

2.6 Alterações na Metilação do DNA nas doenças de Alzheimer e Parkinson

Mudanças no padrão de metilação global e gene-específicas têm sido reportadas como contribuinte de doenças neuropsiquiátricas como esquizofrenia (CHEN et al., 2015), depressão (MURPHY et al., 2013), autismo (GRAYSON; GUIDOTTI, 2016) e doenças neurodegenerativas como DA e DP (JOWAED et al., 2010; LANDGRAVE-GOMEZ; MERCADO-GOMEZ; GUEVARA-GUZMAN, 2015; MASTROENI et al., 2009; MATTSON; SHEA, 2003; TOHGI; UTSUGISAWA; NAGANE; YOSHIMURA; UKITSU; et al., 1999; XU; LI; JIN, 2012).

Na Doença de Alzheimer, há relatos de que algumas citosinas na região promotora do gene da APP são frequentemente metiladas nos casos de início precoce, contrastando com o que ocorre nos casos de início tardio, onde foi observada desmetilação. Essas modificações na metilação do DNA relacionadas à idade alterariam a expressão da APP e, consequentemente, afetariam a deposição de proteína beta-amilóide no cérebro em envelhecimento (TOHGI; UTSUGISAWA; NAGANE; YOSHIMURA; GENDA; et al., 1999).

Outro aspecto envolvendo alterações na metilação do DNA na DA refere-se ao efeito da redução dos níveis de ácido fólico, vitamina B12, e S-adenosil-metionina (SAM) encontrados em indivíduos com DA (HO et al., 2002). A SAM é um cofactor enzimático envolvido na transferência de grupos metilo. É formada a partir de adenosina tri-fostato (ATP) e metionina pela ação da metionina-S-adenosil-transferase (METIVIER et al., 2008). Ambos, folato e SAM, são mediadores importantes nos processos de metilação do DNA. Os níveis de folato no líquido cefalo-raquidiado e no soro foram significativamente menores em indivíduos com DA, indicando uma forte associação com atrofia do córtex cerebral (MATTSON;

SHEA, 2003). Níveis de SAM, bem como a atividade de metionina-S-adenosil-transferase, também são reduzidos no líquor e em cérebro de indivíduos com DA em comparação com controles da mesma idade (Bottiglieri et al., 1990). Além disso, hiperhomocisteinemia pode aumentar os níveis de S-adenosil-homocisteina (SHA), um inibidor potente das metiltransferases. A literatura discute que níveis elevados de SAH no cérebro de pacientes com DA inibe as metiltransferases e está relacionada com marcadores de progressão da doença e prejuízo cognitivo (KENNEDY et al., 2004; MARQUES et al., 2011). Essas alterações nutricionais têm sido associados à modulação epigenética de alguns genes relacionados com a DA e parece que possuem algum papel na fisiopatologia dessa doença. A administração de SAM, em culturas de células de neuroblastoma humano, ocasionou *downregulation* no gene da *PSEN1* e na produção de proteína beta-amiloide (SCARPA et al., 2003).

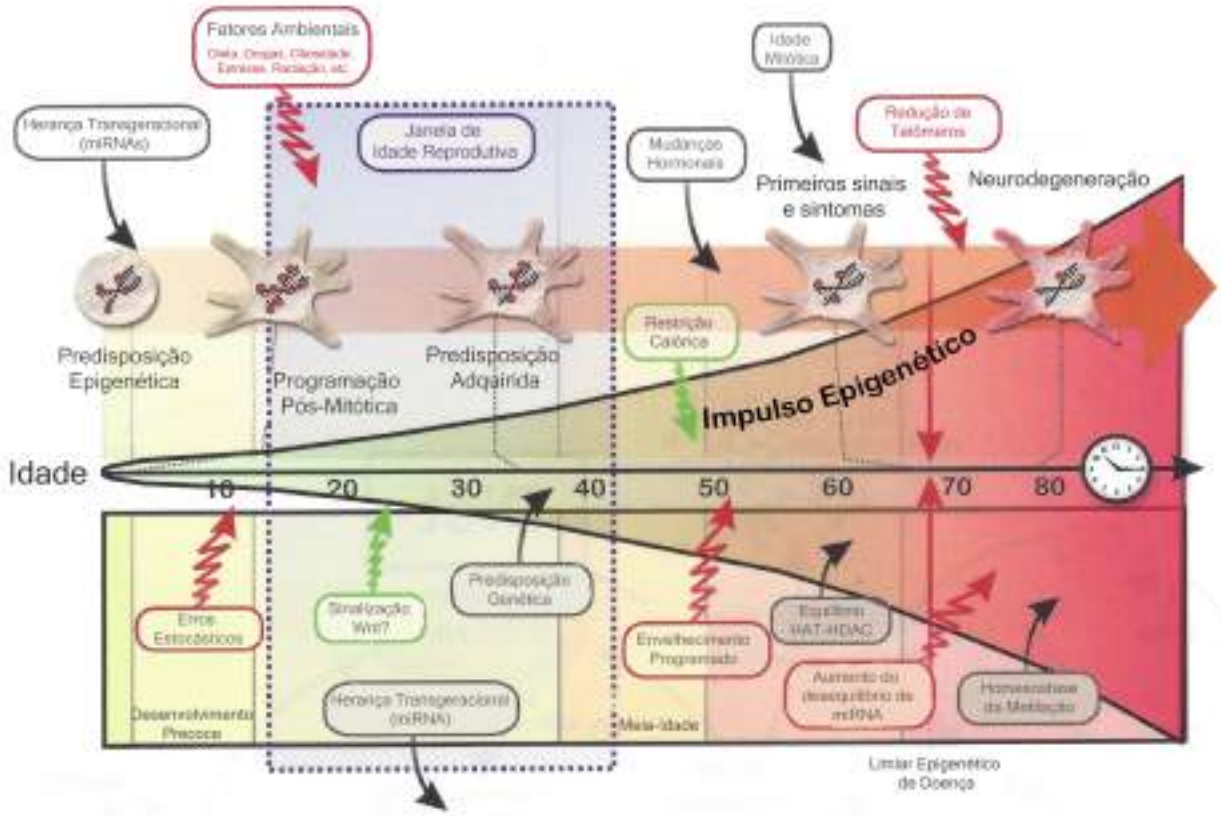
Um estudo em humanos, com amostra do córtex entorrinal, utilizando técnica de imuno-histoquímica, mostrou que a enzima DNA metiltransferase (*DNMT1*) e o fator de ligação MBD2 (proteínas que mantém a metilação ao longo do tempo), interagiram com o RNA ribossômico, alteraram a síntese proteica de MBD2, por fim gerando diminuição global do DNA (MASTROENI et al., 2010). Adicionalmente, outro estudo do mesmo autor avaliando gêmeos monozigóticos discordantes para DA demonstrou que os níveis globais de metilação do DNA estavam reduzidos no neocórtex temporal dos indivíduos com DA. Este resultado foi consistente com a hipótese de que a epigenética possa mediar as diferenças de risco para determinadas doenças, apesar das semelhanças genéticas (MASTROENI et al., 2009).

O modelo do impulso epigenético (tradução adaptada do termo em inglês *epigenetic drift*) idade-dependente oferece um aporte teórico para etiopatologia de doenças neurodegenerativas, que são difíceis de serem explicadas pelas hipóteses de envelhecimento sozinhas. Essa teoria (esquematizada na Figura 1) sugere que o envelhecimento resulta de uma acumulação progressiva de dano epigenético. O impulso epigenético seria um fenômeno natural, que estaria presente em humanos saudáveis, mas pode se tornar danoso, cursando como papel central em patologias neurodegenerativas. Com o envelhecimento, o fenótipo do indivíduo mostra-se como um interação de predisposição genética com fatores de risco, tais como, ambientais, nutricionais, flutuações estocásticas, que agem no maquinário epigenômico, aumentando a variabilidade epigenética com a idade. Enquanto mutações genéticas

variam de forma linear durante a vida, as epimutações aumentariam de forma exponencial. As células do organismo suportariam essas variações até um certo limiar de desregulação. Uma vez ultrapassado esse limiar, um efeito cascata, influenciando diversos outros processos genéticos e epigenéticos, consequentemente levariam a apresentações de relevância clínica (SCHUMACHER, 2011). Estudos que exemplificam este hipótese são os achados de que mesmo em gêmeos idênticos o início da DA pode diferir em mais de 20 anos (NEE; LIPPA, 1999), e que gêmeos idênticos jovens são essencialmente indistinguíveis em seus perfis epigenéticos , enquanto que pares de gêmeos mais velhos mostram diferenças substanciais em suas marcas epigenéticas (MARTIN, 2005). Essas variações podem ser explicadas pelo impulso epigenético, causada pela interação gene-ambiente.

Outro aspecto a ser discutido refere-se ao BDNF. Essa proteína regula a plasticidade sináptica e desempenha um papel fundamental na formação e armazenamento da memória (HELLWEG; JOCKERS-SCHERUBL, 1994), tornando-se alvo de investigações na demência (DINIZ; TEIXEIRA, 2011; FUKUMOTO et al., 2010), bem como em doenças neuropsiquiátricas (BARBOSA et al., 2010; KAPCZINSKI et al., 2008) e nas neurodegenerativas em geral (NOWAK et al., 2015). Em modelos animais, o BDNF parece regular a plasticidade hipocampal e os processos de aprendizagem dependentes desta região (GOTTSCHALK et al., 1998). A regulação da transcrição do BDNF, determinadas por mudanças no padrão de metilação do DNA do gene que o codifica, geraram alterações de prejuízo em memória em estudo com animais (LUBIN; ROTH; SWEATT, 2008). Um recente estudo em humanos, investigou o padrão de metilação da região promotora do gene do BNDF, através de linfócitos. Participaram desse estudo 20 pacientes com DA, pareados a 20 controles saudáveis. Como resultado, foi obtido que a razão total de metilação (em porcentagem) dos 20 locais CpG foi significativamente maior nos pacientes com DA (5,08 ± 5,52%) comparando aos controles (2,09 ± 0,81% ; $p < 0,05$). Além disso, o nível de metilação foi significativa e negativamente correlacionada com escores em testes neuropsicológicos (NAGATA et al., 2015).

Figura 1: Modelo Esquemático da Teoria do Impulso Epigenético



Reproduzido e traduzido com autorização da Editora (SCHUMACHER, 2011).
https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=fc0fe493-2250-443e-996e-d74b91fa962c

Com relação à doença de Parkinson, a maioria dos estudos que avaliam o papel da epigenética na patogênese dessa doença centra-se na análise da metilação do promotor de genes causadores de DP (principalmente *SNCA*, *Parkin*, *PINK1* e *DJ-1*), em cérebros *post-mortem* ou em sangue periférico (COPPEDE, 2012). Estudos recentes têm mostrado que a metilação do gene *SNCA* pode estar envolvida na doença através de alterações estruturais ou a superexpressão da proteína, conduzindo a agregação dessa ou através de expressão prejudicada do gene (AMMAL KAIDERY; TARANNUM; THOMAS, 2013).

O aumento da expressão de α-sinucleína em neurônios dopaminérgicos é associado com DP esporádica. Um estudo mostrou que a metilação do íntron no gene *SNCA-1* correlaciona-se com uma diminuição da expressão do gene, enquanto que a inibição de metilação do DNA ativa expressão SNCA. A metilação do íntron no gene *SNCA-1* foi reduzida em pacientes com DP esporádica em substância negra,

putâmen e córtex, apontando para uma regulação epigenética da expressão do gene SNCA na DP, como consequência da hipometilação do gene em questão (JOWAED et al., 2010).

Além disso, foi demonstrado que α-sinucleína sequestra DNMT1 do citoplasma, levando a hipometilação global do DNA na DP e Demência por corpos de Lewy. Isso foi tanto observado em estudos de tecido cerebral post-mortem, como em modelos animal (DESPLATS et al., 2011).

Essas investigações exploraram possíveis alvos terapêuticos para o tratamento de doenças neurodegenerativas. Inibidores de HDAC e DNMTs são atualmente aprovados e disponíveis para investigação clínica (XU et al., 2012). A esse respeito, o alvo de regulação negativa SIRT2 mostrou reduzir a toxicidade α-sinucleína e perda dopaminérgica em moscas e em cultura de células de mesencéfalo (LANDGRAVE-GOMEZ et al., 2015). Diante disso, salienta-se a importância de estudos nessa área do conhecimento.

Um estudo focou no papel do tabagismo sobre a metilação do promotor LINE-1 e risco para DP. Foram estudados 292 casos de DP, comparando com 401 controles, através de DNA de sangue periférico. O fumo de nicotina levaria à redução da metilação do promotor LINE-1. Curiosamente, a relação inversa entre tabagismo e risco de DP foi mais acentuada nos pacientes com o menor metilação LINE-1, diminuindo significativamente com hipermetilação em LINE-1 (SEARLES NIELSEN et al., 2012).

Outro estudo investigou o gene da proteína tau associada a microtúbulos (*MAPT*), que confere suscetibilidade a DP idiopática. Foram investigados DNA de leucócitos de 358 participantes com DP, comparando-os com 1084 controles), além de amostras de tecido cerebral de 82 pacientes, comparando com 52 controles. Nos participantes com DP, a idade de início foi positivamente associada com a metilação de *MAPT* em leucócitos, aumentando ao longo do tempo. Além disso, houve hipermetilação no cerebelo e hipometilação no putâmen de pacientes com DP comparados com os controles. Finalmente, *in vitro*, por método de cultura de leucócitos, o estado de metilação dessas células associou-se positivamente com os níveis de vitamina E no sangue. Os efeitos significativos sugerem que a hipermetilação do gene *MAPT* é neuroprotetor através da redução de sua expressão. É discutido ainda que o efeito da vitamina E sobre o gene da *MAPT* representa uma possível interação gene-ambiente (COUPLAND et al., 2014).

2.7 Associação de polimorfismos de nucleotídeo único das DNA-metiltransferases com as doenças de Alzheimer e Parkinson

O sequenciamento já mostrou que, como esperado, as sequências genômicas dos indivíduos em uma espécie são muito idênticas. Por exemplo, as comparações de sequências de pessoas diferentes revelam que 99.9% do DNA é idêntico entre os indivíduos. Quase toda a diferença de 0.1% é baseada em diferenças em um único nucleotídeo. Tais diferenças entre indivíduos são chamadas de SNP (*single nucleotide polymorphism* traduzido por polimorfismo de nucleotídeo único). Um SNP ou mutação puntiforme é igual a uma troca de par de base em relação a um genoma fixo de comparação. A quantidade de SNPs no genoma fornece o grau da variabilidade da espécie humana. Podemos dizer que os SNPs são mutações que se apresentam na população em taxas maiores ou iguais a 1%. Acredita-se que existam 3 milhões de SNPs, com frequência de 1 em cada 300 a 1000 bases, dando um conjunto útil de marcadores para mapeamento em escala fina (GRIFFITHS et al., 2009).

Polimorfismos de nucleotídeo único em genes que codificam metiltransferases foram identificados por sequenciamento de DNA (HALASCHEK-WIENER et al., 2009)

Em um estudo em população italiana de 376 pacientes com DA e 308 controles saudáveis não foi encontrada relação significativa nas frequências genotípica e alélicas nos polimorfismos da *DNMT3B* investigados (rs2424913 e rs1569686) (COPPEDE et al., 2012). Uma investigação mais recente avaliou o efeito de polimorfismos das DNA-metiltransferases em uma coorte de pacientes com declínio cognitivo leve. O principal achado dessa publicação foi a associação do genótipo CT+TT no polimorfismo da *DNMT3A* (rs1187120) com piora mais intensa da função cognitiva ao longo do tempo, sendo discutido que essa enzima poderia moderar o declínio cognitivo em sujeitos propensos a desenvolver DA (CHOULIARAS et al., 2015).

Outro estudo investigou a metilação do DNA em 79 indivíduos com tentativa de suicídio comparando-os com 80 que não haviam tentado (ambos os grupos eram de pacientes com algum transtorno psiquiátrico). Neste estudo, o alelo menor A, do polimorfismo do gene da *DNMT3B* (rs2424932), esteve associado aos pacientes que

haviam feito tentativa de suicídio. Além disso, nesses pacientes que apresentavam o polimorfismo, observou-se um aumento da metilação do DNA global (MURPHY et al., 2013).

Considerando o que foi publicado até o presente momento, ainda há poucos estudos investigando uma possível associação entre polimorfismos das metiltransferases e doenças neuropsiquiátricas, incluindo doenças neurodegenerativas. Além disso, os resultados em direções opostas podem refletir o fato desta associação ter sido avaliada em distintas populações. Uma explicação para isso seria justamente a variabilidade (epi)genética das diferentes partes do mundo, sendo até mesmo influenciados por diferentes fatores de risco. Até o momento da presente revisão, não foram encontrados estudos publicados investigando polimorfismos de metiltransferases e DP.

Tendo em vista que: (a) o envelhecimento, principal fator de risco pra DA e DP, está associado a alterações do padrão de metilação do DNA, (b) alterações do padrão de metilação global e gene-especificas têm sido relatadas na DA e DP, (c) as metiltransferases são as principais catalisadoras do processo de metilação e (d) existem polimorfismos de nucleotídeo único identificados para estas enzimas, decidiu-se investigar se polimorfismos de nucleotídeo único em genes que codificam estas enzimas podem estar associados a estas doenças.

A complexidade das doenças neurodegenerativas, seu impacto econômico social e individual nos indivíduos afetados em suas famílias, bem como o promissor campo de investigação sobre a epigenética dessas doenças, a escassez de literatura e originalidade e de estudar especificamente o papel dos polimorfismos das metiltransferases como um fator de risco nestas doenças justificam a presente investigação.

2.8 Bibliografia

AMMAL KAIDERY, N.; TARANNUM, S.; THOMAS, B. Epigenetic landscape of Parkinson's disease: emerging role in disease mechanisms and therapeutic modalities. **Neurotherapeutics,** v. 10, n. 4, p. 698-708, Oct 2013.

APA - AMERICAN PSYCHIATRIC ASSOCIATION. **Manual diagnóstico e estatístico de transtornos mentais: texto revisado (DSM-IV-TR)**. Artmed, 2002.

BALLARD, C. et al. Alzheimer's disease. **Lancet,** v. 377, n. 9770, p. 1019-31, Mar 19 2011.

BAO, Q. et al. Genetic variation in the promoter of DNMT3B is associated with the risk of colorectal cancer. **Int J Colorectal Dis,** v. 26, n. 9, p. 1107-12, Sep 2011.

BARBOSA, I. G. et al. Increased plasma levels of brain-derived neurotrophic factor in patients with long-term bipolar disorder. **Neurosci Lett,** v. 475, n. 2, p. 95-8, May 14 2010.

BERGMANS, B. A.; DE STROOPER, B. gamma-secretases: from cell biology to therapeutic strategies. **Lancet Neurol,** v. 9, n. 2, p. 215-26, Feb 2010.

BERTRAM, L.; TANZI, R. E. The genetic epidemiology of neurodegenerative disease. **J Clin Invest,** v. 115, n. 6, p. 1449-57, Jun 2005.

BOLLATI, V. et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. **Mech Ageing Dev,** v. 130, n. 4, p. 234-9, Apr 2009.

BROWN, R. C.; LOCKWOOD, A. H.; SONAWANE, B. R. Neurodegenerative diseases: an overview of environmental risk factors. **Environ Health Perspect,** v. 113, n. 9, p. 1250-6, Sep 2005.

CASILLAS, M. A., JR. et al. Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically-transformed human fibroblasts. **Mol Cell Biochem,** v. 252, n. 1-2, p. 33-43, Oct 2003.

CEDAR, H. DNA methylation and gene activity. **Cell,** v. 53, n. 1, p. 3-4, Apr 8 1988.

CHEN, X. S. et al. Advancements in the Underlying Pathogenesis of Schizophrenia: Implications of DNA Methylation in Glial Cells. **Front Cell Neurosci,** v. 9, p. 451, 2015.

CHOULIARAS, L. et al. DNMT3A moderates cognitive decline in subjects with mild cognitive impairment: replicated evidence from two mild cognitive impairment cohorts. **Epigenomics,** v. 7, n. 4, p. 533-7, 2015.

CHU, M. W. et al. Lack of increases in methylation at three CpG-rich genomic loci in non-mitotic adult tissues during aging. **BMC Med Genet,** v. 8, p. 50, 2007.

COPPEDE, F. Genetics and epigenetics of Parkinson's disease. **ScientificWorldJournal,** v. 2012, p. 489830, 2012.

COPPEDE, F. et al. DNMT3B promoter polymorphisms and risk of late onset Alzheimer's disease. **Curr Alzheimer Res,** v. 9, n. 5, p. 550-4, Jun 2012.

COUPLAND, K. G. et al. DNA methylation of the MAPT gene in Parkinson's disease cohorts and modulation by vitamin E in vitro. **Mov Disord,** v. 29, n. 13, p. 1606-14, Nov 2014.

DAUER, W.; PRZEDBORSKI, S. Parkinson's disease: mechanisms and models. **Neuron,** v. 39, n. 6, p. 889-909, Sep 11 2003.

DESPLATS, P. et al. Alpha-synuclein sequesters Dnmt1 from the nucleus: a novel mechanism for epigenetic alterations in Lewy body diseases. **J Biol Chem,** v. 286, n. 11, p. 9031-7, Mar 18 2011.

DEVINE, M. J. et al. Parkinson's disease and alpha-synuclein expression. **Mov Disord,** v. 26, n. 12, p. 2160-8, Oct 2011.

DINIZ, B. S.; TEIXEIRA, A. L. Brain-derived neurotrophic factor and Alzheimer's disease: physiopathology and beyond. **Neuromolecular Med,** v. 13, n. 4, p. 217-22, Dec 2011.

DORSEY, E. R. et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. **Neurology,** v. 68, n. 5, p. 384-6, Jan 30 2007.

FIRE, A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. **Nature,** v. 391, n. 6669, p. 806-11, Feb 19 1998.

FROTA, N. A. F. E. A. Critérios para o diagnóstico de doença de Alzheimer. **Dement. neuropsychol,** v. 5, n. n. supl 1, 2011.

FUKS, F. et al. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. **Nat Genet,** v. 24, n. 1, p. 88-91, Jan 2000.

FUKUMOTO, N. et al. Sexually dimorphic effect of the Val66Met polymorphism of BDNF on susceptibility to Alzheimer's disease: New data and meta-analysis. **Am J Med Genet B Neuropsychiatr Genet,** v. 153B, n. 1, p. 235-42, Jan 5 2010.

GAPP, K. et al. Epigenetic regulation in neurodevelopment and neurodegenerative diseases. **Neuroscience,** v. 264, p. 99-111, Apr 4 2014.

GOTTSCHALK, W. et al. Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. **J Neurosci,** v. 18, n. 17, p. 6830-9, Sep 1 1998.

GOYAL, R.; REINHARDT, R.; JELTSCH, A. Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. **Nucleic Acids Res,** v. 34, n. 4, p. 1182-8, 2006.

GRAYSON, D. R.; GUIDOTTI, A. Merging data from genetic and epigenetic approaches to better understand autistic spectrum disorder. **Epigenomics,** v. 8, n. 1, p. 85-104, Jan 2016.

GRIFFITHS, A. J. F. et al. **Mapeamento de Cromossomos Eucarióticos por Recombinação In. Introdução a Genética**. Rio de Janeiro, RJ: Guanabara Koogan, 2009.

HALASCHEK-WIENER, J. et al. Genetic variation in healthy oldest-old. **PLoS One,** v. 4, n. 8, p. e6641, 2009.

HELLWEG, R.; JOCKERS-SCHERUBL, M. Neurotrophic factors in memory disorders. **Life Sci,** v. 55, n. 25-26, p. 2165-9, 1994.

HENDRICH, B.; BIRD, A. Mammalian methyltransferases and methyl-CpG-binding domains: proteins involved in DNA methylation. **Curr Top Microbiol Immunol,** v. 249, p. 55-74, 2000.

HO, P. I. et al. Multiple aspects of homocysteine neurotoxicity: glutamate excitotoxicity, kinase hyperactivation and DNA damage. **J Neurosci Res,** v. 70, n. 5, p. 694-702, Dec 1 2002.

HTTP://WWW.ALZ.CO.UK/RESEARCH/WORLDALZHEIMERREPORT2015.PDF

HTTP://WWW.MOLGEN.UA.AC.BE/ADMUTATIONS/DEFAULT.CFM?MT=1&ML=1&PAGE=MUTBYQUERY&QUERY=TBLCONTEXTS.ID=3&SELECTION=GENE = APP

HTTP://WWW.WHO.INT/HPR/AGEING/ACTIVEAGINGPOLICYFRAME.PDF.

HUGHES, A. J. et al. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. **J Neurol Neurosurg Psychiatry,** v. 55, n. 3, p. 181-4, Mar 1992.

JAYADEV, S. et al. Alzheimer's disease phenotypes and genotypes associated with mutations in presenilin 2. **Brain,** v. 133, n. Pt 4, p. 1143-54, Apr 2010.

JONES, P. A.; LIANG, G. Rethinking how DNA methylation patterns are maintained. **Nat Rev Genet,** v. 10, n. 11, p. 805-11, Nov 2009.

JOWAED, A. et al. Methylation regulates alpha-synuclein expression and is decreased in Parkinson's disease patients' brains. **J Neurosci,** v. 30, n. 18, p. 6355-9, May 5 2010.

JUN, G. et al. Comprehensive search for Alzheimer disease susceptibility loci in the APOE region. **Arch Neurol,** v. 69, n. 10, p. 1270-9, Oct 2012.

KALIA, L. V.; LANG, A. E. Parkinson's disease. **Lancet,** v. 386, n. 9996, p. 896-912, Aug 29 2015.

KAPCZINSKI, F. et al. Brain-derived neurotrophic factor and neuroplasticity in bipolar disorder. **Expert Rev Neurother,** v. 8, n. 7, p. 1101-13, Jul 2008.

KENNEDY, B. P. et al. Elevated S-adenosylhomocysteine in Alzheimer brain: influence on methyltransferases and cognitive function. **J Neural Transm (Vienna),** v. 111, n. 4, p. 547-67, Apr 2004.

KRIAUCIONIS, S.; HEINTZ, N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. **Science,** v. 324, n. 5929, p. 929-30, May 15 2009.

LAMBERT, J. C. et al. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. **Nat Genet,** v. 45, n. 12, p. 1452-8, Dec 2013.

LANDGRAVE-GOMEZ, J.; MERCADO-GOMEZ, O.; GUEVARA-GUZMAN, R. Epigenetic mechanisms in neurological and neurodegenerative diseases. **Front Cell Neurosci,** v. 9, p. 58, 2015.

LEE, V. M.; GOEDERT, M.; TROJANOWSKI, J. Q. Neurodegenerative tauopathies. **Annu Rev Neurosci,** v. 24, p. 1121-59, 2001.

LOCHMATTER, D.; MULLIS, P. E. RNA interference in mammalian cell systems. **Horm Res Paediatr,** v. 75, n. 1, p. 63-9, 2011.

LUBIN, F. D.; ROTH, T. L.; SWEATT, J. D. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. **J Neurosci,** v. 28, n. 42, p. 10576-86, Oct 15 2008.

MA, D. K. et al. Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. **Science,** v. 323, n. 5917, p. 1074-7, Feb 20 2009.

MARQUES, S. C. et al. Epigenetics in neurodegeneration: a new layer of complexity. **Prog Neuropsychopharmacol Biol Psychiatry,** v. 35, n. 2, p. 348-55, Mar 30 2011.

MARTIN, G. M. Epigenetic drift in aging identical twins. **Proc Natl Acad Sci U S A,** v. 102, n. 30, p. 10413-4, Jul 26 2005.

MARTINOWICH, K. et al. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. **Science,** v. 302, n. 5646, p. 890-3, Oct 31 2003.

MASTROENI, D. et al. Epigenetic changes in Alzheimer's disease: decrements in DNA methylation. **Neurobiol Aging,** v. 31, n. 12, p. 2025-37, Dec 2010.

MASTROENI, D. et al. Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. **PLoS One,** v. 4, n. 8, p. e6617, 2009.

MATTSON, M. P.; SHEA, T. B. Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. **Trends Neurosci,** v. 26, n. 3, p. 137-46, Mar 2003.

MCKHANN, G. et al. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. **Neurology,** v. 34, n. 7, p. 939-44, Jul 1984.

MCKHANN, G. M. et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. **Alzheimers Dement,** v. 7, n. 3, p. 263-9, May 2011.

METIVIER, R. et al. Cyclical DNA methylation of a transcriptionally active promoter. **Nature,** v. 452, n. 7183, p. 45-50, Mar 6 2008.

MORRIS, K. V. RNA-directed transcriptional gene silencing and activation in human cells. **Oligonucleotides,** v. 19, n. 4, p. 299-306, Dec 2009.

MURPHY, T. M. et al. Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients. **Genes Brain Behav,** v. 12, n. 1, p. 125-32, Feb 2013.

NAGATA, T. et al. Association between DNA Methylation of the BDNF Promoter Region and Clinical Presentation in Alzheimer's Disease. **Dement Geriatr Cogn Dis Extra,** v. 5, n. 1, p. 64-73, Jan-Apr 2015.

NALLS, M. A. et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. **Nat Genet,** v. 46, n. 9, p. 989-93, Sep 2014.

NEE, L. E.; LIPPA, C. F. Alzheimer's disease in 22 twin pairs--13-year follow-up: hormonal, infectious and traumatic factors. **Dement Geriatr Cogn Disord,** v. 10, n. 2, p. 148-51, Mar-Apr 1999.

NITSCH, R. M. From acetylcholine to amyloid: neurotransmitters and the pathology of Alzheimer's disease. **Neurodegeneration,** v. 5, n. 4, p. 477-82, Dec 1996.

NOWAK, A. et al. Analysis of the expression and polymorphism of APOE, HSP, BDNF, and GRIN2B genes associated with the neurodegeneration process in the pathogenesis of primary open angle glaucoma. **Biomed Res Int,** v. 2015, p. 258281, 2015.

PERIQUET, M. et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. **Brain,** v. 126, n. Pt 6, p. 1271-8, Jun 2003.

PEZZI, J. C. et al. DNA methyltransferase haplotype is associated with Alzheimer's disease. **Neurosci Lett,** v. 579, p. 70-4, Sep 5 2014.

PHANI, S.; LOIKE, J. D.; PRZEDBORSKI, S. Neurodegeneration and inflammation in Parkinson's disease. **Parkinsonism Relat Disord,** v. 18 Suppl 1, p. S207-9, Jan 2012.

POULOPOULOS, M.; LEVY, O. A.; ALCALAY, R. N. The neuropathology of genetic Parkinson's disease. **Mov Disord,** v. 27, n. 7, p. 831-42, Jun 2012.

QUERFURTH, H. W.; LAFERLA, F. M. Alzheimer's disease. **N Engl J Med,** v. 362, n. 4, p. 329-44, Jan 28 2010.

RICHARDSON, B. Impact of aging on DNA methylation. **Ageing Res Rev,** v. 2, n. 3, p. 245-61, Jul 2003.

RUBIN, H. Cancer development: the rise of epigenetics. **Eur J Cancer,** v. 28, n. 1, p. 1-2, 1992.

SAITO, Y. et al. Accumulation of phosphorylated alpha-synuclein in aging human brain. **J Neuropathol Exp Neurol,** v. 62, n. 6, p. 644-54, Jun 2003.

SCARPA, S. et al. Presenilin 1 gene silencing by S-adenosylmethionine: a treatment for Alzheimer disease? **FEBS Lett,** v. 541, n. 1-3, p. 145-8, Apr 24 2003.

SCHELLENBERG, G. D.; MONTINE, T. J. The genetics and neuropathology of Alzheimer's disease. **Acta Neuropathol,** v. 124, n. 3, p. 305-23, Sep 2012.

SCHUMACHER, A. Aging Epigenetics. In: TOLLEFSBOL, T. (Ed.). **Handbook of Epigenetics**. 1. San Francisco, USA: Elsevier, v.1, 2011. cap. 24, p.405-19. ISBN 978-0-12-375709-8.

SEARLES NIELSEN, S. et al. LINE-1 DNA methylation, smoking and risk of Parkinson's disease. **J Parkinsons Dis,** v. 2, n. 4, p. 303-8, 2012.

SELKOE, D. J. Alzheimer's disease: genes, proteins, and therapy. **Physiol Rev,** v. 81, n. 2, p. 741-66, Apr 2001.

SLEEGERS, K.; VAN DUIJN, C. M. Alzheimer's Disease: Genes, Pathogenesis and Risk Prediction. **Community Genet,** v. 4, n. 4, p. 197-203, Jun 2001.

SPILLANTINI, M. G. et al. Alpha-synuclein in Lewy bodies. **Nature,** v. 388, n. 6645, p. 839-40, Aug 28 1997.

TAHILIANI, M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. **Science,** v. 324, n. 5929, p. 930-5, May 15 2009.

TANZI, R. E.; BERTRAM, L. Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. **Cell,** v. 120, n. 4, p. 545-55, Feb 25 2005.

TOHGI, H. et al. Reduction with age in methylcytosine in the promoter region -224 approximately -101 of the amyloid precursor protein gene in autopsy human cortex. **Brain Res Mol Brain Res,** v. 70, n. 2, p. 288-92, Jul 5 1999.

TOHGI, H. et al. The methylation status of cytosines in a tau gene promoter region alters with age to downregulate transcriptional activity in human cerebral cortex. **Neurosci Lett,** v. 275, n. 2, p. 89-92, Nov 12 1999.

TURNER, A. M.; MORRIS, K. V. Controlling transcription with noncoding RNAs in mammalian cells. **Biotechniques,** v. 48, n. 6, p. ix-xvi, Jun 2010.

UHL, G. R. et al. Dopamine transporter messenger RNA in Parkinson's disease and control substantia nigra neurons. **Ann Neurol,** v. 35, n. 4, p. 494-8, Apr 1994.

VILLENEUVE, L. M.; REDDY, M. A.; NATARAJAN, R. Epigenetics: deciphering its role in diabetes and its chronic complications. **Clin Exp Pharmacol Physiol,** v. 38, n. 7, p. 451-9, Jul 2011.

WADDINGTON, C. H. Genetic assimilation of the bithorax phenotype. **Evolution**, p. 1-13, 1956.

WAGNER, W.; FERNANDEZ-REBOLLO, E.; FROBEL, J. DNA-methylation changes in replicative senescence and aging: two sides of the same coin? **Epigenomics**, Dec 23 2015.

WEBER, M. et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. **Nat Genet,** v. 39, n. 4, p. 457-66, Apr 2007.

XU, Z.; LI, H.; JIN, P. Epigenetics-Based Therapeutics for Neurodegenerative Disorders. **Curr Transl Geriatr Exp Gerontol Rep,** v. 1, n. 4, p. 229-236, Dec 1 2012.

3 Objetivos

- Investigar a associação de polimorfismos nos genes que codificam as enzimas DNA (citosina-5-)-metiltransferase 1 (*DNMT1*) e DNA (citosina-5-)-metiltransferase 3 beta (*DNMT3B)* com o doença de Alzheimer, em indivíduos com mais de 60 anos, comparando com controles saudáveis.

- Investigar a associação de polimorfismos nos genes que codificam as enzimas DNA (citosina-5-)-metiltransferase 1 (*DNMT1*) e DNA (citosina-5-)-metiltransferase 3 beta (*DNMT3B)* com o doença de Parkinson, comparando com controles saudáveis.

4 Artigo Científico 1

Title

DNA methyltransferase haplotype is associated with Alzheimer's disease

Authors

Julio Carlos Pezzi[1]*, Cintia Monique Boschmann Ens[1], Ericksen Mielle Borba[2], Artur F Schumacher-Schuh[2], Fabiana Michelsen de Andrade[3], Marcia Lorena Fagundes Chaves[2], Marilu Fiegenbaum[1], Ana Luiza Camozzato[1]

[1] Graduate Program in Health Science. Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre/RS, Brazil.

[2] Dementia Clinic, Neurology Service, Hospital de Clinicas de Porto Alegre, Porto Alegre/RS, Brazil.

[3] Graduate Program in Biomedicine, Universidade Feevale, Novo Hamburgo/RS, Brazil.

Running title

DNMT3B haplotype and Alzheimer's disease

*** Corresponding Author**

Rua Prof. Carvalho Monteiro, 234/303. Porto Alegre/RS, Brazil.

E-mail: pezzi.julio@gmail.com.br

Phone-Number +55 51 92826876

Citation

Pezzi JC, Ens CM, Borba EM, Schumacher-Schuh AF, de Andrade FM, Chaves ML, Fiegenbaum M, Camozzato AL. DNA methyltransferase haplotype is associated with Alzheimer's disease. Neurosci Lett. 2014 Sep 5;579:70-4. doi: 10.1016/j.neulet.2014.07.013. Epub 2014 Jul 17.

ABSTRACT

Epigenetic mechanisms have been implicated in syndromes associated with neuropsychiatric disorders, but little is known about the role of epigenetics in Alzheimer's disease (AD). DNA methylation, one of the main epigenetic mechanisms, is a complex process carried out by specific enzymes, such as *DNMT1* and *DNMT3B*. This study aimed to investigate the association between *DNMT1* and *DNMT3B* polymorphisms and AD. Two hundred and ten elderly subjects (108 healthy controls and 102 with AD-NINCDS/ARDA, DSM-IV-TR criteria) were assessed. DNA was obtained from whole blood, and genotypes were detected by an allelic discrimination assay using TaqMan® MGB probes on a real-time PCR system. The polymorphisms studied were rs2162560 and rs759920 (*DNMT1*) and rs998382, rs2424913 and rs2424932 (*DNMT3B*). For both genes, the polymorphisms were in strong linkage disequilibrium. Carriers of the *DNMT3B* TGG haplotype were associated with AD (OR = 3.03, 95% CI 1.63 to 5.63, $p < 0.001$). No significant difference between AD and the control group were observed for *DNMT1* polymorphisms. This study is one of the first describing a significant association between *DNMT3B* polymorphisms and AD. This enzyme, which is responsible for methylation in a general way, may be involved in AD.

KEYWORDS

Alzheimer's Disease. Single Nucleotide Polymorphism. Methyltransferases. Epigenomics.

HIGHLIGHTS

- Minor alleles of two SNPs (rs998382, rs2424913) in the *DNMT3B* gene were associated with Alzheimer's disease (AD) when compared to healthy controls.
- The *DNMT3B* TGG haplotype was associated with AD.
- This difference was not observed for *DNMT1* polymorphisms.

INTRODUCTION

Epigenetic mechanisms have been implicated in syndromes associated with neuropsychiatric disorders, but little is known about the role of epigenetics in sporadic Alzheimer's disease (AD), a complex multifactorial neurodegenerative disorder and the most common cause of dementia [3]. In this sense, epigenetic processes may have a role in the gene–environment interaction process, the most accepted model linked with neurodegeneration in sporadic AD. DNA methylation is the most stable epigenetic modification, modulating the transcriptional plasticity of mammalian genomes. It is linked to gene expression, with an inverse correlation between the degree of promoter DNA methylation and the level of expression [15, 30]. This process is mediated by a family of conserved enzymes, DNA methyltransferases (DNMT), responsible for adding a methyl group to position 5 of the cytosine pyrimidine ring in the CpG dinucleotide [9]. The DNMTs (mainly *DNMT1* and *DNMT3B*) are enzymes responsible for establishing and maintaining DNA methylation patterns. *DNMT1* is a maintenance enzyme, which binds methyl groups to hemi-methylated DNA during DNA replication. *DNMT3B* are de novo methyltransferase, which establish methylation patterns during embryonic development [8, 29].

DNA methyltransferases were first investigated in some varieties of cancer [1, 4], but interest in the neuropsychiatric field has been increasing [13, 14, 36], with most studies considering the *DNMT3B* enzyme. A study conducted with psychiatric patients with a history of suicide attempts showed significantly higher levels of global DNA methylation compared with controls, and this finding was associated with *DNMT3B* polymorphisms [29]. A post-mortem brain regions study revealed a marked reduction in DNA methylation in cortical neurons of AD subjects when compared to controls, and it was associated with increased *PSEN1* gene expression [32]. It is still not clear whether *DNMTs* polymorphisms imply hyper or hypo DNA methylation in Alzheimer's disease.

In addition, some genes that participate in amyloid-beta processing (*PSEN1, APOE*) and methylation homeostasis (*MTHFR, DNMT1*) show significant inter-individual epigenetic variability in AD brain samples, showing a notably age-specific epigenetic drift, which supports the potential role of epigenetic effects in developing the disease [35].

The complexity of AD degeneration, as well as the attractive hypothesis of epigenetic mechanisms contributing to AD pathogenesis and the influence of environmental factors on phenotypic constitution, justifies further studies on epigenetics. This study aims to evaluate the association of AD and five known *DNMT* gene polymorphisms (*DNMT1:* rs2162560, rs759920; *DNMT3B:* rs998382, rs2424932 and rs2424913).

Methods and materials

Study design

This is a case-control study comparing a group of Alzheimer's disease patients to healthy control subjects.

The study was approved by the bioethics committees of the participating institutions and was performed in compliance with the Declaration of Helsinki. All participants or their proxies in AD cases provided written informed consent.

Participants

All participants (AD patients and healthy controls) were Caucasian and were from a similar geographic region, matched for the same low-income economic status.

One hundred and two sporadic AD patients were recruited by convenience from two academic outpatient neuropsychiatric services located in a southern Brazilian city. All of them fulfilled probable NINCDS-ADRDA [25] and DSM-IV-TR [2] AD criteria. This diagnosis was ascertained by a psychiatrist or neurologist from the research team with expertise in the dementia field. Brain tomography or magnetic resonance imaging and complete medical and laboratory evaluations were performed to exclude other causes of dementia. Other exclusion criteria were history of cancer, family history of dementia and any other neurological or psychiatric disorders.

A control group of 108 age and sex-matched cognitively healthy and independent community-dwelling elderly individuals were recruited from the catchment areas of the same academic services. The inclusion criteria were age greater than 65 years, Clinical Dementia Rating (CDR) of 0 [27], Mini Mental State Examination (MMSE) score higher than 26 [12] and independence for activities of daily living (ADL) [17, 20]. Controls were excluded if they presented chronic renal disease, history of significant head injury or stroke, history of cancer, family history of dementia, other psychiatric conditions such as major affective disorder or evidence of

current depression, uncorrectable vision or hearing loss or other conditions such as substance abuse or use of medications that could impair cognitive function.

Genotyping

The DNA was extracted from 500 µL of EDTA-treated whole blood using the salting out method [19]. After extraction, the DNA was quantified on a UV visible spectrophotometer (Biospec® Nano). The final concentration of DNA used was from 10 ng/mL. The single nucleotide polymorphism (SNP) selection investigated in this study was performed using the HapMap (HapMap Genome Browser release #24) (Phases 1 and 2 — full dataset) using the following settings for the tool “annotate TagSNP Picker”: European population (CEU), minimum frequency of the rarer allele of 20% and a coefficient of determination (R2) of 80%. The five polymorphisms were genotyped with the use of TaqMan Genotyping Master Mix and TaqMan SNP Genotyping assays (Applied Biosystems).

For each reaction plate, genomic control DNA samples and non-template controls (water) were included. A control on the TaqMan SNP genotyping assay was also performed (25% of randomly chosen samples from both groups) to check for genotyping accuracy, and identical genotypes were identified in all repeated samples. The researchers who performed the genotyping were blinded to the patients’ diagnostic status.

Statistical Analysis

The results were entered into a database, and SPSS statistical package SPSS® version 18.0 was used to perform the analyses.

A non-parametric Mann–Whitney test was used to calculate the differences in age and education between cases and controls. For sex comparisons, a chi-squared test of association was used. The Student *t* test was used to compare economic income between the AD group and the control group.

Frequencies were described as proportions for categorical variables and as mean plus standard deviations for quantitative variables. Allelic frequencies were obtained by direct counting throughout the genotype frequency.

Chi-square testing was carried out to verify whether the genotypic frequencies were in agreement with Hardy-Weinberger equilibrium. The linkage disequilibrium between the polymorphisms in each genomic region was estimated with MLocus 3.0

[22], and haplotypes were imputed with PHASE 2.1 [33, 34]. Haplotypes with frequencies less than 3% were pooled.

Univariate analyses to verify the associations between the polymorphisms in the genes encoding the enzymes *DNMT1* and *DNMT3B* and Alzheimer's disease were carried out by chi-square association tests with a dominant model. The Bonferroni test was performed for multiple testing corrections.

Multivariate logistic regression analysis was performed for the outcome AD, with polymorphisms or haplotypes as independent variables. The confounders entered in the model were age and education, based on the literature review [3, 7].

A two-tailed $p < 0.05$ was considered significant for all analyses.

RESULTS

The sample is depicted in Table 1. The AD and control groups were comparable by age and sex. Education and MMSE scores were significantly lower in the AD group than in the control group. Family income (US$/month) was not significantly different between the AD group (M = 587.32; SD = 491.21) and healthy controls (M = 640.13; SD = 501.12) (t = 0.96, P = 0.31).

Table 1: Sample Description

Variable	Total Sample	Control Group	AD Group	P^a
N	210	108	102	
Age (years)	75.83 (7.60)	74.91 (7.75)	76.80 (7.34)	0.072*
Sex (female)	67.6	72.2	62.7	0.184**
Education (years)	6.55 (3.97)	7.98 (4.18)	5.03 (3.09)	<0.001*
MMSE	20.36 (8.50)	27.55 (2.02)	12.74 (5.60)	<0.001*

Note: AD: Alzheimer's disease. MMSE: Mini Mental State Examination score. Variables are described as % or mean (standard deviation). [a] Comparison between the control group and AD Group. * Mann-Whitney Test; ** Chi-square Test.

The genotypic frequencies of *DNMT1* and *DNMT3B* polymorphisms were consistent with Hardy-Weinberg equilibrium (P > 0.05). For both genes, the

polymorphisms were in strong linkage disequilibrium (D' > 0.8 and *P* < 0.001 in all comparisons).

The genotypic and allelic frequencies related to each gene polymorphism are described in Table 2 for both groups.

Table 2: Genotype and Allelic Frequencies of *DNMT3B* gene polymorphisms rs998382, rs2424913, rs2424932 and *DNMT1* rs2162560, rs759920 in AD and control groups: descriptive and univariate analyses.

		Genotype Frequency			Allelic Frequency	
		%	%	%	%	%
DNMT3B	rs2424913	CC	CT	TT	C	T
	Control	38.9	48.1	13.0	62.95	37.05
	Alzheimer	24.5	48.0	27.5	48.50	51.50
	P		0.036*		0.046*	
	rs998382	AA	AG	GG	A	G
	Control	50.9	36.1	13.0	68.95	31.05
	Alzheimer	25.5	47.0	27.5	49.0	51.0
	P		0.015*		0.005*	
	rs2424932	AA	AG	GG	A	G
	Control	17.6	44.4	38.0	39.8	60.2
	Alzheimer	15.7	37.2	47.1	34.25	65.75
	P		*NS*		*NS*	
DNMT1	rs2162560	AA	AG	GG	A	G
	Control	17.6	46.3	36.1	40.75	59.25
	Alzheimer	15.7	48.0	36.3	39.7	60.3
	P		*NS*		*NS*	
	rs759920	AA	AG	GG	A	G
	Control	20.5	53.6	25.9	47.3	52.7
	Alzheimer	22.5	53.9	23.6	49.45	50.55
	P		*NS*		*NS*	

Note: Control: Control Group. Alzheimer: Alzheimer's disease Group. *Bonferroni adjusted *P*-values. *NS:* Not Significant.

Univariate analyses showed that the G allele of *DNMT3B* polymorphism rs998382 and the T allele of *DNMT3B* polymorphism rs2424913 were associated with AD (*P* = 0.005 and *P* = 0.046, respectively). The frequency of *DNMT3B* haplotypes in the whole sample is shown in Table 3. Taken into account the haplotype distribution, the TGG haplotype was defined as risk variant since it carries both alleles associated to AD in univariate analysis. No other haplotype had both alleles.

Table 3: Haplotype distribution

Gene	Haplotype*	Patients (*n*)	Frequency
DNMT3B	TGG	113	0.35
	CAA	108	0.31
	CAG	81	0.25
	TAG	22	0.05
	TAA	9.6	0.03
	Others	3.2	0.01

*DNMT3B: rs2424913/rs998382/rs2424932

In order to verify whether the effect of the haplotype was independent of age and education, a multivariate logistic regression analysis was performed. The AD group was associated with the presence of the TGG haplotype (OR = 3.03, 95% CI 1.63 to 5.63, p < 0.0001). These results are shown in Table 4.

Table 4. Multiple logistic regression analysis for outcome AD

Variables in the Model	B	OR	95%CI	*P*
DNMT3B TGG haplotype	1.11	3.03	1.63 – 5.63	<0.001
Education (years)	0.22	1.25	1.14 – 1.36	<0.001
Age (years)	-0.029	0.97	0.93 – 1.01	0.166

Note: DNMT3B TGG haplotype: rs2424913/rs998382/rs2424932; OR: Odds Ratio; B: estimated coefficient; 95%CI: Confidence Interval 95%.

DISCUSSION

This study evaluated the association between Alzheimer's disease and polymorphisms in genes encoding the enzymes DNA methyltransferase *1* and *3B*. The TGG haplotype in the *DNMT3B* gene showed significant association with Alzheimer's disease. Individuals carrying the TGG haplotype have an increased risk of Alzheimer's disease (OR = 3.03, 95% CI 1.63 to 5.63, $p < 0.001$). The same was not observed in the SNPs investigated for gene *DNMT1*. To the best of our knowledge, this is the first study describing a significant association.

We have studied *DNMT3B* rs998382, rs2424932 and rs2424913, while a previous study conducted on an Italian sample focused on *DNMT3B* rs2424932 and rs1569686 [10]. In contrast to our results, no difference in allelic or genotypic frequencies was found for either polymorphism between AD subjects and the healthy control group in this previous investigation [10]. Despite these conflicting results, it should be noted that AD has a complex inheritance pattern, which means that the individual is the product of the interaction between genetic inheritances and the environment they live in, so the analyzed populations may differ in their genetic background and exposure to environmental factors. Accordingly, it should be noted that, for example, the onset of AD in identical twins can differ by more than 20 years [21], and it is also known that young pairs of identical twins are essentially indistinguishable in their epigenetic profiles, while twin pairs show substantial differences in their epigenetic marks with advancing age [24]. These variations can be precisely explained by epigenetic drift mediated by *DNMTs*, caused, for example, by environmental exposure, lifestyle, diet, drug use or simply stochastic fluctuations.

Moreover, according to the epigenetic perspective, some genes may not predispose one to disease *per se*, but rather act through interaction with specific environmental triggers. This interaction between genes and environmental risk may explain why putative association studies of complex diseases sometimes cannot be replicated in different geographic areas [31].

Furthermore, it is known that *DNMT3B* functions in *de novo* methylations, which occur during embryonic development [11]. However, another important role of DNMT3B is to maintain DNA methylation patterns, correcting errors left by DNMT1 [16]. According to this theory, many studies have shown that epigenetic changes occur more frequently than gene mutations and could thus be particularly important

in age-related phenotypes [5]. The high frequency of epimutations catalyzed by DNMT3B suggests that epigenetic alterations accumulate during aging. Small epimutations in essential genes could be tolerated to a certain extent and reflect only the range of inter-individual variation. However, once a critical threshold of epigenetic deregulation is reached, the cerebral apparatus goes into deregulation, justifying the relevance of this enzyme in neurodegenerative diseases [5]. In addition, it was found that, in aging cells, changes in the gene expression of DNMTs were observed, with the mRNA of DNMT1 and DNMTa becoming reduced, while the production of DNMT3B increased progressively [6]. Other issue to be discussed is that DNMT3B SNPs (rs998382 and rs2424913) are located in noncoding regions. Although, they do not translate protein, these regions are receiving attention due their predictive role in transcription regulation, DNA replication, chromosome pairing, and chromosome condensation [23, 26]. Also, polymorphisms in the 3'UTR region may have effects on gene expression regulation. For example, analyzing the prediction of its functionality through bioinformatics tools, the rs2424932 (DNMT3B) seems to create a binding site for transcription factors [29].

Our study didn't find an association between the AD and control groups for the rs759920 and rs2162560 gene polymorphisms in DNMT1. This enzyme is a key maintenance methyltransferase enzyme responsible for copying pre-existing methylation patterns onto newly replicated DNA strands during cell divisions [18]. Aberrant expression of the DNMT1 gene has previously been associated with schizophrenia [37]. However, polymorphisms in *DNMT1* have been investigated to assess their influence on conditions such as deafness, dementia and other neuropathies. Some studies have been conducted to analyze the polymorphisms described above, but so far there are no data that associate them with a particular condition [13, 28].

Some limitations should be pointed out. (a) The difference in education between the control and AD groups was expected, taking into account the well-known role of cognitive reserve as a protective factor in the development of Alzheimer's disease – a concept that encompasses education, occupation and mental activities [7]. However, in the multiple logistic regression model adjusted for age and education, the risk variant TGG haplotype maintained its significance, suggesting that the mutation has an independent effect. (b) Considering the exploratory nature of this study, our results should be replicated in larger and

separate samples in order to confirm our findings.

In conclusion, our findings highlight the hypotheses of epigenetic mechanisms related to AD, stressing the role of DNA methyltransferase SNPs as risk factors for this complex neurodegenerative disease. Further studies investigating the effects of these polymorphisms in the DNA methylation should the addressed.

ACKNOWLEDGMENTS

We are thankful to our source of support, the National Council for Scientific and Technological Development (CNPq).

REFERENCES

[1] S. Agarwal, K.S. Amin, S. Jagadeesh, G. Baishay, P.G. Rao, N.C. Barua, S. Bhattacharya, P.P. Banerjee, Mahanine restores RASSF1A expression by down-regulating DNMT1 and DNMT3B in prostate cancer cells, Molecular cancer 12 (2013) 99.

[2] A.P.A. APA, Diagnostic and Statistical Manual of MentalDisorders - Text Revision (DSM-IV-TR), 2000.

[3] C. Ballard, S. Gauthier, A. Corbett, C. Brayne, D. Aarsland, E. Jones, Alzheimer's disease, Lancet 377 (2011) 1019-1031.

[4] Q. Bao, B. He, Y. Pan, Z. Tang, Y. Zhang, L. Qu, Y. Xu, C. Zhu, F. Tian, S. Wang, Genetic variation in the promoter of DNMT3B is associated with the risk of colorectal cancer, International journal of colorectal disease 26 (2011) 1107-1112.

[5] P.E. Bennett-Baker, J. Wilkowski, D.T. Burke, Age-associated activation of epigenetically repressed genes in the mouse, Genetics 165 (2003) 2055-2062.

[6] M. Casillas, N. Lopatina, L. Andrews, T. Tollefsbol, Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically-transformed human fibroblasts, Mol Cell Biochem 252 (2003) 33-43.

[7] R.J. Castellani, R.K. Rolston, M.A. Smith, Alzheimer disease, Dis Mon 56 (2010) 484-546.

[8] F. Chedin, The DNMT3 family of mammalian de novo DNA methyltransferases, Progress in molecular biology and translational science 101 (2011) 255-285.

[9] T. Chen, E. Li, Establishment and maintenance of DNA methylation patterns in mammals, Curr Top Microbiol Immunol 301 (2006) 179-201.

[10] F. Coppede, M.T. Zitarosa, F. Migheli, A. Lo Gerfo, S. Bagnoli, A. Dardano, B. Nacmias, M. Mancuso, F. Monzani, G. Siciliano, S. Sorbi, L. Migliore, DNMT3B promoter polymorphisms and risk of late onset Alzheimer's disease, Curr Alzheimer Res 9 (2012) 550-554.

[11] J. Feng, H. Chang, E. Li, G. Fan, Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system, J Neurosci Res 79 (2005) 734-746.

[12] M.F. Folstein, S.E. Folstein, P.R. McHugh, "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician, Journal of psychiatric research 12 (1975) 189-198.

[13] P. Haggarty, G. Hoad, S.E. Harris, J.M. Starr, H.C. Fox, I.J. Deary, L.J. Whalley, Human intelligence and polymorphisms in the DNA methyltransferase genes involved in epigenetic marking, PLoS One 5 (2010) e11329.

[14] F. Higuchi, S. Uchida, H. Yamagata, K. Otsuki, T. Hobara, N. Abe, T. Shibata, Y. Watanabe, State-dependent changes in the expression of DNA methyltransferases in mood disorder patients, Journal of psychiatric research 45 (2011) 1295-1300.

[15] R. Jaenisch, A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals, Nat Genet 33 Suppl (2003) 245-254.

[16] P.A. Jones, G. Liang, Rethinking how DNA methylation patterns are maintained, Nat Rev Genet 10 (2009) 805-811.

[17] S. Katz, C.A. Akpom, A measure of primary sociobiological functions, Int J Health Serv 6 (1976) 493-508.

[18] S.R. Kinney, S. Pradhan, Regulation of expression and activity of DNA (cytosine-5) methyltransferases in mammalian cells, Progress in molecular biology and translational science 101 (2011) 311-333.

[19] D.K. Lahiri, J.I. Nurnberger, Jr., A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies, Nucleic Acids Res 19 (1991) 5444.

[20] V.T.S.P. Lino, S.R.M.; CamachoI, L.A.B.; FilhoII, S.T.R.; BuksmanII, S., Adaptação transcultural da Escala de Independência em Atividades da Vida Diária (Escala de Katz), Cad. Saúde Pública 24 (2008).

[21] C.F. Lippa, Familial Alzheimer's disease: genetic influences on the disease process (Review), Int J Mol Med 4 (1999) 529-536.

[22] J.C. Long, R.C. Williams, M. Urbanek, An E-M algorithm and testing strategy for multiple-locus haplotypes, Am J Hum Genet 56 (1995) 799-810.

[23] M.Z. Ludwig, Functional evolution of noncoding DNA, Current opinion in genetics & development 12 (2002) 634-639.

[24] G.M. Martin, Epigenetic drift in aging identical twins, Proc Natl Acad Sci U S A 102 (2005) 10413-10414.

[25] G. McKhann, D. Drachman, M. Folstein, R. Katzman, D. Price, E.M. Stadlan, Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease, Neurology 34 (1984) 939-944.

[26] M.H. Meisler, Evolutionarily conserved noncoding DNA in the human genome: how much and what for?, Genome research 11 (2001) 1617-1618.

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

[28] T.M. Murphy, N. Mullins, M. Ryan, T. Foster, C. Kelly, R. McClelland, J. O'Grady, E. Corcoran, J. Brady, M. Reilly, A. Jeffers, K. Brown, A. Maher, N. Bannan, A. Casement, D. Lynch, S. Bolger, A. Buckley, L. Quinlivan, L. Daly, C. Kelleher, K.M. Malone, Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients, Genes, brain, and behavior (2012).

[29] T.M. Murphy, N. Mullins, M. Ryan, T. Foster, C. Kelly, R. McClelland, J. O'Grady, E. Corcoran, J. Brady, M. Reilly, A. Jeffers, K. Brown, A. Maher, N. Bannan, A. Casement, D. Lynch, S. Bolger, A. Buckley, L. Quinlivan, L. Daly, C. Kelleher, K.M. Malone, Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients, Genes, brain, and behavior 12 (2013) 125-132.

[30] B.P. Rutten, J. Mill, Epigenetic mediation of environmental influences in major psychotic disorders, Schizophr Bull 35 (2009) 1045-1056.

[31] D.K. Shumaker, T. Dechat, A. Kohlmaier, S.A. Adam, M.R. Bozovsky, M.R. Erdos, M. Eriksson, A.E. Goldman, S. Khuon, F.S. Collins, T. Jenuwein, R.D. Goldman, Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging, Proc Natl Acad Sci U S A 103 (2006) 8703-8708.

[32] K.D. Siegmund, C.M. Connor, M. Campan, T.I. Long, D.J. Weisenberger, D. Biniszkiewicz, R. Jaenisch, P.W. Laird, S. Akbarian, DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons, PLoS One 2 (2007) e895.

[33] M. Stephens, P. Scheet, Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation, Am J Hum Genet 76 (2005) 449-462.

[34] M. Stephens, N.J. Smith, P. Donnelly, A new statistical method for haplotype reconstruction from population data, Am J Hum Genet 68 (2001) 978-989.

[35] S.C. Wang, B. Oelze, A. Schumacher, Age-specific epigenetic drift in late-onset Alzheimer's disease, PLoS One 3 (2008) e2698.

[36] C. Zhang, Y. Fang, B. Xie, Y.S. Du, W.H. Cheng, D.X. Wang, S.Y. Yu, [Association of DNA methyltransferase 3B gene polymorphism with early-onset schizophrenia], Zhonghua yi xue yi chuan xue za zhi = Zhonghua yixue yichuanxue zazhi = Chinese journal of medical genetics 27 (2010) 697-699.

[37] A. Zhubi, M. Veldic, N.V. Puri, B. Kadriu, H. Caruncho, I. Loza, H. Sershen, A. Lajtha, R.C. Smith, A. Guidotti, J.M. Davis, E. Costa, An upregulation of DNA-methyltransferase 1 and 3a expressed in telencephalic GABAergic neurons of schizophrenia patients is also detected in peripheral blood lymphocytes, Schizophr Res 111 (2009) 115-122.

5 Artigo Científico 2

Title

Association between DNA methyltransferase gene polymorphism and Parkinson's disease.

Authors

Julio Carlos Pezzi[1*], Cintia Monique Boschmann Ens[1], Artur F Schumacher-Schuh[2,3], Marcia Lorena Fagundes Chaves[2], Mara H Hutz[3] , Marilu Fiegenbaum[1], Ana Luiza Camozzato[1]

[1] Graduate Program in Health Science. Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre/RS, Brazil.

[2] Neurology Service, Hospital de Clinicas de Porto Alegre, Porto Alegre/RS, Brazil.

[3] Genetics Department, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Running title

DNMT3B single nucleotide polymorphism in Parkinson's disease.

*** Corresponding Author:**

Rua Prof. Carvalho Monteiro, 234/303. Porto Alegre/RS, Brazil.

E-mail: julio@pezzi.com.br

Phone-Number +55 51 92826876

Paper Submited to Neuroscience Letters

Submition date: Feb, 25th 2016.

ABSTRACT

Parkinson's disease (PD) is a common and complex neurodegenerative disorder, the second most prevalent only behind Alzheimer's disease. Recent studies suggest that environmental factors may contribute for neurodegeneration through induction of epigenetic modifications, such as DNA methylation, that is carried out by enzymes, such as DNMT1 and DNMT3B.

This present study targeted to investigate the association between DNMT1 and DNMT3B polymorphisms and PD.

Five hundred and twenty-two (214 PD patients following UK Brain Bank criteria and 308 healthy individuals) were evaluated. DNA was obtained from whole blood, and genotypes were detected by an allelic discrimination assay using TaqMan® MGB probes on a real-time PCR system. The polymorphisms studied were rs2162560 and rs759920 (DNMT1) and rs998382, rs2424913 and rs2424932 (DNMT3B).

We found association between DNMT3B rs2424913 in allele T carriers (individuals with genotypes TT and CT) with PD. The PD group was associated with the presence of the T (TT+CT) (OR = 1.80, 95% CI 1.16 to 2.81, p = 0.009). No significant difference was observed in the others SNPs. Also, no association between PD and the control group were observed for DNMT1 polymorphisms.

This is the first study addressing an association between DNMT3B polymorphism and PD. The polymorphism may play a role in the pathogenesis of PD.

KEYWORDS

Pakinson's Disease. Single Nucleotide Polymorphism. Methyltransferases. Epigenomics.

INTRODUCTION

In the last years, it has arisen a bunch of studies regarding epigenetics in neuropsychiatric disorders, such as depression with suicide risk [1], schizophrenia [2], and Alzheimer's disease (AD) [3], all conditions which environmental factors can affect disease risk. Additionally, an age- specific epigenetic drift was supposed as a contributor to AD [4]. Parkinson's disease (PD) is a progressive neurodegenerative disorder as AD, being the second most prevalent after Alzheimer's disease (AD), and it is also an age-related disease [5]. Most of the cases of PD are sporadic [6] and, like AD, PD is understood as a multifactorial disorder, where environmental and genetic factors are complexly linked [7].

Epigenetic mechanisms may have a role mediating gene-environment interaction mechanisms in PD. DNA methylation is one of the most important epigenetic modification and modulates gene expression [2]. An increased global methylation was suggested as an inducing factor to parkinsonism in animal model [8], as well as, changes in the DNA methylation of human α-synuclein (SNCA) in brain [9, 10] and in leukocytes [11] of Parkinson's disease patients have been reported. A tendency for hypomethylation of the tumor necrosis factor alpha (TNF-alpha), a key inflammatory cytokine associated to dopaminergic cell death in PD, was also described [12].

This process of DNA methylation is driven by DNA methyltransferases enzymes (DNMT) which add a methyl group to position 5 of the cytosine pyrimidine ring in the CpG dinucleotide [13]. DNMT1 is a maintenance enzyme and DNMT3B is responsible to establish *de novo* methylation patterns during embryonic development. Mutations in *DNMT1* gene were associated with a myriad of neurodegenerative disorders, probably mediated through its role in protein homeostasis and autophagy [14]. Polymorphisms in *DNMT3B* gene were associated with an increased DNA methylation in *post-mortem* brain tissue in patients with psychiatric diseases and suicide attempt [1].

Recently, our group found an association between polymorphisms in gene encoding the enzyme *DNMT3B* and AD. Individuals carrying the *DNMT3B* TGG haplotype presented an increased risk of Alzheimer's disease (OR = 3.03, 95% CI 1.63 to 5.63, $p < 0.001$) [3]. A possible association between *DNMT3B* gene polymorphisms and PD has not yet been studied. Taking into account that epigenetic

process may be enrolled either in AD and PD, this study aims to evaluate if our previous finding was specific for AD or if it is also present in another neurodegenerative disorders, like PD.

METHODS AND MATERIALS

Participants

A case-control study was conducted, including a group of Parkinson's disease patients and healthy control subjects. All participants were of European ancestry from southern Brazil, matched for the same low-income economic status.

Two hundred and fourteen PD patients were recruited from an outpatient Movement Disorder clinic and underwent a structured interview for collecting clinical and demographic data. The diagnosis of PD was determined based on UK Brain Bank criteria [15].

A control group of 308 age-matched healthy individuals were recruited from the same catchment areas. The inclusion criteria were absence of any Parkinson symptom and independence for activities of daily living (ADL) [16, 17]. Controls were excluded if they presented cognitive deficit, chronic renal disease, history of significant head injury or stroke, history of cancer, family history of dementia, psychiatric conditions such as major mood disorder, evidence of current depression or substance abuse, and finally uncorrectable vision or hearing loss.

The study was performed in compliance with the Declaration of Helsinki. All participants provided written informed consent.

Genotyping

Genomic DNA was extracted from 500 µL of EDTA-treated whole blood using the salting out method [18]. The single nucleotide polymorphism (SNP) selection investigated in this study was performed using the HapMap (HapMap Genome Browser release #24) (Phases 1 and 2 — full dataset) using the following settings for the tool "annotate TagSNP Picker": European population (CEU), minimum frequency of the rarer allele of 20% and a coefficient of determination (R2) of 80%. The five polymorphisms were genotyped with the use of TaqMan Genotyping Master Mix and TaqMan SNP Genotyping assays (Applied Biosystems). The *DNMT1* (rs2162560,

rs759920) and *DNMT3B* (rs998382, rs2424932 and rs2424913) genotyping were extensively described in previous report [3].

Statistical Analysis

Descriptive statistics were presented as means, standard deviations, and medians. All parameters were tested for normality of distribution with the Kolmogorov–Smirnov test. A non-parametric Mann–Whitney test was used to calculate the differences in age and education between cases and controls. For sex comparisons, a chi-squared association test was performed.

Allelic frequencies were obtained by direct counting throughout the genotype frequency. Chi-square testing was carried out to verify whether the genotypic frequencies were in agreement with Hardy-Weinberger equilibrium. The linkage disequilibrium between the polymorphisms in each genomic region was estimated with MLocus 3.0 [19].

Univariate analyses to verify the associations between the polymorphisms in the genes encoding the enzymes *DNMT1* and *DNMT3B* and PD were carried out by chi-square association test with a dominant model. The Benjamini and Hochberg false discovery rate procedure was performed for multiple testing corrections [20].

Multivariate logistic regression analysis was performed for PD outcome, with polymorphism as independent variable. The confounders entered in the model were education and sex.

Statistical analyses were performed with SPSS (version 18.0) (SPSS, Chicago, IL, USA), considering an alpha of 5% for all analyses.

RESULTS

Demographic sample' characteristics are summarized in Table 1. The mean age of PD patients and healthy controls were similar. Educational attainment was significantly lower in the PD group than in the control group. PD was more prevalent in male population (51.5%).

--- Insert Table 1 ---

The genotypic frequencies of *DNMT1* and *DNMT3B* polymorphisms were consistent with Hardy-Weinberg equilibrium ($P > 0.05$). For both genes, the polymorphisms were in linkage disequilibrium (D' > 0.8 and $P < 0.001$ in all comparisons).

The genotypic and allelic frequencies related to each gene polymorphism and their association with PD are described in Table 2. Univariate analyses showed that there was a significant association between the *DNMT3B* polymorphism rs2424913 genotype and PD ($P = 0.006$) and that the T allele of *DNMT3B* polymorphism rs2424913 was associated with PD ($P = 0.002$).

--- Insert Table 2 ---

In order to verify whether the effect of the *DNMT3B* rs2424913 was independent of age, sex and education, a multivariate logistic regression analysis was performed. For this analysis we categorized the *DNMT3B* rs2424913 in T carriers (individuals with genotypes TT and CT) and individuals with CC genotype. The PD group was associated with the presence of the T (TT+CT) (OR = 1.80, 95% CI 1.16 to 2.81, p = 0.009). These results are shown in Table 3.

--- Insert Table 3 ---

DISCUSSION

The present study evaluated the relation of polymorphisms in DNA methyltransferase enzymes, which are responsible for important epigenetic mechanism, and Parkinson disease. The main result described herein was the association between T allele of the rs2424913 polymorphism in the DNMT3B gene and PD. No association was found for polymorphisms in DNMT1.

To the best of our knowledge, this is the first study showing an association between polymorphisms of DNA methyltransferase and PD. Previous studies have shown a relationship between polymorphisms of DNA methyltransferases with aging, cancer, psychiatric and neurodegenerative syndromes. One study showed an association between suicide attempt and DNMT3B polymorphisms, and also

correlates this polymorphism with DNA methylation patterns [1]. Our previous study shows a positive association between DNMT3B TGG haplotype and Alzheimer's disease [3]. PD shares similar mechanisms with AD; both disorders are age-related and resulted from an abnormal handling of proteins. Considering these results, one might hypothesize that a common dysfunctional methylation pattern, influenced by *DNMT3B* polymorphisms, could contribute to abnormal protein accumulation and neurodegenerative process in a broader way.

A variety of studies suggested epigenetic mechanisms in PD. In the context of gene-specific methylation process, DNA methylation of CpG islands of human α-synuclein (SNCA) intron 1 and demethylation of the SCNA CpG in brains of PD patients can regulate SNCA gene expression [9, 10]. Besides, studies on the methylation status of α-synuclein (SNCA) in leukocytes have demonstrated controversial results. While one investigation in one hundred patients with PD, paired with one hundred healthy control subjects, indicated that CpG-2 island was hypomethylated in PD patients [11], other study reported no evidence for differential methylation of alpha-synuclein in leukocyte DNA of Parkinson's disease patients [21]. On the other hand, an increased global methylation was observed in aging animal model with a consequent dopamine, norepinephrine, and serotonin depletion and acetylcholine increase, causing hypokinesia and tremor. These findings suggested increased methylation as an inducing factor in parkinsonism [8]. Another potential epigenetic deregulation in PD was related to the brain-derived neurotrophic factor (BDNF) gene. Reduction of brain-derived neurotrophic factor (BDNF) levels has been demonstrated in patients with PD and other neurodegenerative disorders [22] and BDNF expression is regulated by histone acetylation as well as DNA methylation [23].

Interestingly, the presence of T allele of rs2424913 was previously associated with decreased risk of cancer, the same allele that in our sample was associated with AD and PD [24]. Recent epidemiological studies are depicting an inverse correlation between cancer and neurodegenerative disorders [25]. Epigenetic mechanisms are a possible explanation for the fact that individuals with neurodegenerative disorders, like PD, are protected against some types of cancer. The same methylation patterns that could predispose to cell death in PD might protect the individual against abnormal cell proliferation in cancer.

Some limitations need to be pointed out. There is a sex difference between two groups. However, we tried to control this possible confounding factor in the

multivariate analysis. This is an exploratory study, which suggests association between the polymorphism of DNMT3B and PD. This results need to be replicated in other samples.

The findings described in this paper suggest a role for an important DNA methylation enzyme in Parkinson disease. Understanding the complexity of PD neurodegeneration, the influence of environmental factors on phenotypic constitution as well as the attractive hypothesis of epigenetic mechanisms contributing to PD pathogenesis, justifies further researches on this field.

REFERENCES

[1] Murphy TM, Mullins N, Ryan M, Foster T, Kelly C, McClelland R, O'Grady J, Corcoran E, Brady J, Reilly M, Jeffers A, Brown K, Maher A, Bannan N, Casement A, Lynch D, Bolger S, Buckley A, Quinlivan L, Daly L, Kelleher C, Malone KM (2013) Genetic variation in DNMT3B and increased global DNA methylation is associated with suicide attempts in psychiatric patients. *Genes Brain Behav* **12**, 125-132.

[2] Saradalekshmi KR, Neetha NV, Sathyan S, Nair IV, Nair CM, Banerjee M (2014) DNA methyl transferase (DNMT) gene polymorphisms could be a primary event in epigenetic susceptibility to schizophrenia. *PLoS One* **9**, e98182.

[3] Pezzi JC, Ens CM, Borba EM, Schumacher-Schuh AF, de Andrade FM, Chaves ML, Fiegenbaum M, Camozzato AL (2014) DNA methyltransferase haplotype is associated with Alzheimer's disease. *Neurosci Lett* **579**, 70-74.

[4] Wang SC, Oelze B, Schumacher A (2008) Age-specific epigenetic drift in late-onset Alzheimer's disease. *PLoS One* **3**, e2698.

[5] Tanner CM, Ottman R, Goldman SM, Ellenberg J, Chan P, Mayeux R, Langston JW (1999) Parkinson disease in twins: an etiologic study. *JAMA* **281**, 341-346.

[6] Liao XX, Zhan ZX, Luo YY, Li K, Wang JL, Guo JF, Yan XX, Xia K, Tang BS, Shen L (2013) Association study between SNP rs150689919 in the DNA demethylation gene, TET1, and Parkinson's disease in Chinese Han population. *BMC Neurol* **13**, 196.

[7] Masliah E, Dumaop W, Galasko D, Desplats P (2013) Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. *Epigenetics* **8**, 1030-1038.

[8] Charlton CG, Crowell B, Jr. (1995) Striatal dopamine depletion, tremors, and hypokinesia following the intracranial injection of S-adenosylmethionine: a possible role of hypermethylation in parkinsonism. *Mol Chem Neuropathol* **26**, 269-284.

[9] Jowaed A, Schmitt I, Kaut O, Wullner U (2010) Methylation regulates alpha-synuclein expression and is decreased in Parkinson's disease patients' brains. *J Neurosci* **30**, 6355-6359.

[10] Matsumoto L, Takuma H, Tamaoka A, Kurisaki H, Date H, Tsuji S, Iwata A (2010) CpG demethylation enhances alpha-synuclein expression and affects the pathogenesis of Parkinson's disease. *PLoS One* **5**, e15522.

[11] Tan YY, Wu L, Zhao ZB, Wang Y, Xiao Q, Liu J, Wang G, Ma JF, Chen SD (2014) Methylation of alpha-synuclein and leucine-rich repeat kinase 2 in leukocyte DNA of Parkinson's disease patients. *Parkinsonism Relat Disord* **20**, 308-313.

[12] Pieper HC, Evert BO, Kaut O, Riederer PF, Waha A, Wullner U (2008) Different methylation of the TNF-alpha promoter in cortex and substantia nigra: Implications for selective neuronal vulnerability. *Neurobiol Dis* **32**, 521-527.

[13] Chen T, Li E (2006) Establishment and maintenance of DNA methylation patterns in mammals. *Curr Top Microbiol Immunol* **301**, 179-201.

[14] Baets J, Duan X, Wu Y, Smith G, Seeley WW, Mademan I, McGrath NM, Beadell NC, Khoury J, Botuyan MV, Mer G, Worrell GA, Hojo K, DeLeon J, Laura M, Liu YT, Senderek J, Weis J, Van den Bergh P, Merrill SL, Reilly MM, Houlden H, Grossman M, Scherer SS, De Jonghe P, Dyck PJ, Klein CJ (2015) Defects of mutant DNMT1 are linked to a spectrum of neurological disorders. *Brain* **138**, 845-861.

[15] Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* **55**, 181-184.

[16] Katz S, Akpom CA (1976) A measure of primary sociobiological functions. *Int J Health Serv* **6**, 493-508.

[17] Lino VTSP, S.R.M.; CamachoI, L.A.B.; FilhoII, S.T.R.; BuksmanII, S. (2008) Adaptação transcultural da Escala de Independência em Atividades da Vida Diária (Escala de Katz). *Cad. Saúde Pública* **24**.

[18] Lahiri DK, Nurnberger JI, Jr. (1991) A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* **19**, 5444.

[19] Long JC, Williams RC, Urbanek M (1995) An E-M algorithm and testing strategy for multiple-locus haplotypes. *Am J Hum Genet* **56**, 799-810.

[20] Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* **57**, 289-300.

[21] Richter J, Appenzeller S, Ammerpohl O, Deuschl G, Paschen S, Bruggemann N, Klein C, Kuhlenbaumer G (2012) No evidence for differential methylation of alpha-synuclein in leukocyte DNA of Parkinson's disease patients. *Mov Disord* **27**, 590-591.

[22] Zuccato C, Cattaneo E (2009) Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat Rev Neurol* **5**, 311-322.

[23] Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, Fan G, Sun YE (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* **302**, 890-893.

[24] Duan F, Cui S, Song C, Dai L, Zhao X, Zhang X (2015) Systematic evaluation of cancer risk associated with DNMT3B polymorphisms. *J Cancer Res Clin Oncol* **141**, 1205-1220.

[25] Driver JA (2014) Inverse association between cancer and neurodegenerative disease: review of the epidemiologic and biological evidence. *Biogerontology* **15**, 547-557.

Table 1: Demographic variables: descriptive and comparative analyses

Variable	Controls	PD	*P*
N	308	214	
Age (years), mean (SD)	66.7 (9.6)	67.8 (9.9)	0.190*
Sex (male), %	20.1	51.9	<0.001**
Education (years), median (p25-p75)	11 (5-13)	5 (4-9)	<0.001***

Note: PD: Parkinson disease. * Student t-test; ** Chi-square test; ***Mann-Whitney test.

Table 2: Genotype and Allelic Frequencies of *DNMT3B* gene polymorphisms rs2424913, rs998382, rs2424932 and *DNMT1* rs2162560, rs759920 in PD and healthy control groups: descriptive and univariate analyses.

		Genotype Frequency			**Allelic Frequency**	
		%	%	%	%	%
DNMT3B	**rs2424913**	CC	CT	TT	C	T
	Control	37.0	44.5	18.5	59.3	40.7
	Parkinson	24.2	50.2	25.6	49.3	50.7
	P		0.006[a]		0.002[a] (OR=1.5; 95% CI=1.16-1.94)	
	rs998382	AA	AG	GG	A	G
	Control	40.9	41.2	17.9	61.5	38.5
	Parkinson	31.9	48.1	20.0	56.0	44.0
	P		0.113[a]		0.082[a] (OR=1.26; 95%CI=0.97-1.63)	
	rs2424932	AA	AG	GG	A	G
	Control	15.3	46.1	38.6	38.3	61.7
	Parkinson	11.2	44.2	44.7	33.3	66.7
	P		0.258[a]		0.112[a] (OR=1.25; 95%CI=0.95-1.63)	
DNMT1	**rs2162560**	AA	AG	GG	A	G
	Control	16.6	48.7	34.7	40.9	59.1
	Parkinson	12.4	46.2	41.4	35.5	64.5
	P		0.236		0.093(OR=1.26; 95%CI=0.96-1.64)	
	rs759920	AA	AG	GG	A	G
	Control	24.0	51.3	24.7	49.7	50.3
	Parkinson	24.4	51.9	23.6	50.5	49.5
	P		0.958		0.849(OR=0.97; 95%CI=0.76-1.24	

Note: Control: Control Group. Parkinson: Parkinson´s disease Group.
[a] Benjamini and Hochberg

Table 3. Multiple logistic regression analysis for outcome PD

Variables in the Model	B	OR	95%CI	*P*
DNMT3B rs2424913*	0.590	1.80	1.16-2.81	0.009
Education (years)	-0.184	0.83	0.79-0.87	<0.001
Sex (male)	1.367	3.92	2.58-5.98	<0.001

Note: **DNMT3B* rs2424913 categories: T carriers (TT+CT) X CC (reference); OR: Odds Ratio; B: estimated coefficient; 95%CI: Confidence Interval 95%.

6 Conclusão

A presente tese avaliou polimorfismos das DNA-metiltransferases e sua correlação com as patologias neurodegenerativas doença de Alzheimer e Parkinson.

Tendo em vista a complexidade na etiopatogenia dessas doenças, estudos que se aprofundem no tema são de extrema relevância, e considerando a genética complexa destas doenças envolvendo a interação entre múltiplos fatores de risco genéticos e o ambiente, a hipótese de mecanismos epigenéticos associados torna-se muito atrativa.

Esta tese de doutorado gerou dois artigos científicos, um já publicado e outro submetido para publicação. O artigo científico 1 visou avaliar a relação das *DNMT1* e *DNMT3B* com a DA. O haplótipo TGG no gene *DNMT3B* mostrou associação significativa com a doença de Alzheimer. Os indivíduos que carreiam o haplótipo TGG apresentam um risco aumentado de doença de Alzheimer (OR = 3,03 , IC de 95% 1,63-5,63 , $p < 0,001$). O mesmo não foi observado em SNPs analisados para o gene da *DNMT1*. Outro artigo do nosso grupo de pesquisa, submetido e em revisão, investigou se ambos o polimorfismo da *APOE* e o haplótipo TGG da DMNT3 apresentavam efeito aditivo. Observou-se que a presença dos dois fatores aumentou o risco para DA, parecendo haver um efeito aditivo. Esse material pode ser encontrado no Anexo 2.

No artigo científico 2, investigou-se a associação dos mesmos polimorfismos das mesmas enzimas na doença de Parkinson. Nesse estudo, observou-se que a presença do rs2424913 do gene *DNMT3B* aumenta em 1,8 vezes o risco de um paciente possuir o diagnóstico da DP, sendo esse risco carreado pelo alelo T (Indivíduos CT e TT: OR = 1.80, IC de 95% 1.16 - 2.81, $p = 0.009$). Da mesma forma como ocorreu na DA, não foi observado associação significante com os *SNPs* analisados para o gene da *DNMT1*.

Até onde tem-se conhecimento, esse são os primeiros estudos que descrevem uma associação significativa entre polimorfismos das *DNMTs* e doenças neurodegenerativas. Já há evidências de alteração dos padrões de metilação de DNA com o envelhecimento, mas a possível contribuição de *SNPs* nesses genes, facilmente analisável, para a ocorrência desses padrões alterados e sua possível associação com o surgimento de DA e DP ainda é incipiente.

Pode-se hipotetizar que polimorfismos nos genes que codificam as *DNMT3B* sejam um marcador de traço em relação ao envelhecimento bem sucedido ou ao risco de desenvolvimento de doenças neurodegenerativas, tendo em vista que alterações globais nos padrões de metilação e gene-especificas de DNA possam conferir um risco para essas doenças. Estes polimorfismos da *DNMT3B* podem interagir com o impulso epigenético determinado pela idade, conferindo então um risco para DA e para DP. Assim, estas duas patologias compartilham o achado de deposição anormal de proteínas, elas parecem também compartilhar mecanismos epigenéticos, que, por sua vez, podem estar associados a neurodegeneração em geral ao invés de estar relacionados a uma patologia especifica.

Como perspectivas futuras o campo deverá buscar elementos de funcionalidade dos polimorfismos das *DNMTs* estudados, bem como a repercussão destes polimorfismos na metilação de genes envolvidos na etiopatogenia de DP e DA, além da relação com a metilação global do DNA. Além disso, é necessário aprofundar o estudo da epigenética em tecido cerebral é de extrema importância. Estudos em líquor talvez também sejam perspectivas mais factíveis em curto prazo. Há também a necessidade da replicação dos resultados desses estudo em diferentes amostras e em um número maior de indivíduos, para que possamos ter evidências consistentes.

Em suma, essa tese demonstrou a íntima relação entre polimorfismos das *DNMTs* e duas doenças neurodegenerativas mais prevalentes, ampliando a compreensão de aspectos genéticos associados a mecanismos de regulação gênica.

7 Anexos

7.1 Anexo 1: Aprovação do Comitê de Ética em Pesquisa

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Relação entre Polimorfismos da APOE e das DNA-metiltrasnferases em Pacientes com Doença de Alzheimer e Indivíduos com Envelhecimento Normal

Pesquisador: Analuiza Camozzato de Padua

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

Versão: 2

CAAE: 23144813.7.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

DADOS DO PARECER

Número do Parecer: 566.029

Data da Relatoria: 20/03/2014

Apresentação do Projeto:

A Doença de Alzheimer (DA) é uma doença neurodegenerativa primária caracterizada por deterioração adquirida da memória e de outras funções cognitivas que leva a morte em 3 a 9 anos após o diagnóstico. Essa doença compromete difusamente o córtex cerebral o que resulta em atrofia cerebral progressiva ¿ que afeta primariamente o hipocampo.Do ponto de vista genético a DA mais comum é a forma de início tardio para a qual existe uma crescente evidência de que estes casos são também influenciados por fatores genéticos. Os genes de risco são geralmente vários, apresentando padrões intrincados de interação entre si e não exibem um único modo de herdabilidade. Assim, a genética destas doenças tem sido chamada de "complexa". Uma hipótese é que estas doenças de genética "complexa" são governadas por variantes comuns de DNA (tais como polimorfismos de nucleotídeo único) e que estas variantes aumentam significativamente o risco, mas são insuficientes para causar a doença. O principal fator de risco genético para o desenvolvimento das placas amiloides na DA de início tardio tem sido relacionado à apolipoproteína E (APOE) que apresenta três formas alélicas: ¿2, ¿3 e ¿4, sendo o alelo ¿4 o que confere maior risco para a doença. A maioria das abordagens analíticas utiliza a análise de associação de marcador único, no

Endereço: Rua Sarmento Leite ,245
Bairro: **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (513)303 -8804 **E-mail:** cep@ufcspa.edu.br

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

entanto, evidências empíricas de organismos modelo e de estudos em humanos sugerem que as interações entre os loci contribuem amplamente para as características ¿traços¿. Diante disso, ppretendemos genotipar genotipar uma amostra quanto ao perfil do gene da APOE e, assim, associar os achados das DNMTs aos alelos de APOE, com o propósito de observar se em conjunto ambas variáveis têm poder para aumentar o risco para o desenvolvimento da DA.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar se a associação do alelo e4 do gene da APOE com os SNPs de DNMT3b aumentam o risco para a DA.

Objetivo Secundário:

a) Avaliar as interações entre os loci dos alelos de APOE com os polimorfismos de DNMTs e suas contribuições para o desenvolvimento da DA.

b) Armazenamento das amostras de sangue (sob a forma de sangue total e plasma/soro) para serem utilizadas em estudos futuros (Biorrepositório), de acordo com a Conselho Nacional de Saúde na Resolução Nos 196/96 e 441/11 e 340/04 do Comissão Nacional de Ética em Pesquisa.

Avaliação dos Riscos e Benefícios:

Adequados à pesquisa.

Comentários e Considerações sobre a Pesquisa:

Todas as solicitações foram atendidas satisfatoriamente.

Considerações sobre os Termos de apresentação obrigatória:

Adequados.

Recomendações:

Aprovar o projeto.

Conclusões ou Pendências e Lista de Inadequações:

Pesquisa, atual e relevante.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Início do projeto: abril/2014

Endereço: Rua Sarmento Leite ,245
Bairro: **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303 -8804 **E-mail:** cep@ufcspa.edu.br

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Término do projeto: março/2016

PORTO ALEGRE, 24 de Março de 2014

Assinador por:
José Geraldo Vernet Taborda
(Coordenador)

Endereço: Rua Sarmento Leite ,245
Bairro: **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)303 -8804 **E-mail:** cep@ufcspa.edu.br

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA E COMITÊ DE ÉTICA EM PESQUISA

A Comissão Científica e o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP/HCPA), que é reconhecido pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institutional Review Board (IRB00000921) analisaram o projeto:

Projeto: 110060
Data da Versão do Projeto: 28/01/2011
Data da Versão do TCLE: 01/05/2011

Pesquisadores:
ANALUIZA CAMOZZATO DE PADUA
JULIO CARLOS PEZZI
MARCIA LORENA FAGUNDES CHAVES

Título: Nature and nurture na Doença de Alzheimer: Marcadores genéticos de traço ou de estado ou ambos? Associação de polimorfismos de DNA metiltranferases (DNMT1 e DNMT3B) com mudanças no padrão de metilação do gene BDNF.

Este projeto foi APROVADO em seus aspectos éticos e metodológicos, bem como o respectivo Termo de Consentimento Livre e Esclarecido, de acordo com as diretrizes e normas nacionais e internacionais de pesquisa clínica, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

- Os membros da Comissão Científica e do Comitê de Ética em Pesquisa não participaram do processo de avaliação dos projetos nos quais constam como pesquisadores.
- Toda e qualquer alteração do projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente ao CEP/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEP/HCPA.
- Somente poderá ser utilizado o Termo de Consentimento Livre e Esclarecido no qual conste o carimbo de aprovação do CEP/HCPA.

Porto Alegre, 15 de junho de 2011.

Profª Nadine Clausell
Coordenadora GPPG e CEP/HCPA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEP – COMITÊ DE ÉTICA EM PESQUISA

Protocolo para apresentação de Projeto de Pesquisa aprovado pelo CEP HCPA

Data de recebimento CEP UFCSPA	Número
19/05/2011	016/11

Nome do projeto: Nature and Nurture na Doença de Alzheimer: Marcadores genéticos de traço ou estado ou ambos? Associação de polimorfismo de DNA metiltransferase (DNMIT1 e DNMT3B)com mudanças no padrão de metilação do gene BDNF.

Autores: Analuiza Camozzato de Pádua, Márcia Lorena Fagundes, Marilu Fiegenbaun e Julio Carlos Pezzi

Protocolo: 110060

Parecer:

Data de Aprovação no CEP HCPA: 15/06/2011

Situação: Homologado

Data:21/07/2011

José Geraldo Vernet Taborda
Coordenador do CEP/UFCSPA

7.2 Anexo 2: Artigo Científico, outras produções

Title

The additive risk effect of apolipoprotein ε4 and *DNMT3B* haplotype for Alzheimer's disease

Authors

Cíntia Monique Boschmann Ens[1], Julio Carlos Pezzi[1], Ericksen Mielle Borba[1], Marcia Lorena Fagundes Chaves[2], Fabiana Michelsen de Andrade[3], Marilu Fiegenbaum[1], Analuiza Camozzato[1]

[1] Post Graduate Program in Health Sciences, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre/RS, Brazil.

[2] Dementia Clinic, Neurology Service, Hospital de Clínicas de Porto Alegre, Porto Alegre/RS, Brazil.

[3] UniRitter Laureate International Universities, Porto Alegre/RS, Brazil.

Corresponding Author:

Analuiza Camozzato

Rua: Taquara 564/306 Bairro Petrópolis – 90460210 – Porto Alegre – Brazil

Email: anacamoz@gmail.com

Phone 55-51-33353560

Paper Submited

Abstract

Background: Alzheimer's disease (AD) is a complex and multifactorial disease with the contribution of several genes and polymorphisms to its development. Among these genes, the *APOEε4* is a well known risk factor for AD. Methylation is associated with *APOE* expression and AD development. Recently, we found an association of the TGG haplotype in the *DNMT3B* gene, one of the catalyst enzyme for methylation, with AD. Therefore, the aim of the study is to investigate whether *APOE ε4* and TGG haplotype have an additive effect on AD.

Methods and Results: The sample was composed of 212 Caucasian subjects (108 healthy controls and 104 with AD by NINCDS-ADRDA and DSM-IV-TR criteria) from southern Brazil. The genetic analyses were performed by real time PCR for TaqMan® assay. Multivariate logistic regression was performed categorizing groups according to presence of *APOEε4* and/or TGG haplotype as an independent variable for outcome AD. The presence of TGG haplotype plus the allele *APOEε4* were strongly associated with the risk of developing AD [OR 11.13; CI 95% (4.25 to 29.16); p<0.001]. This association had a higher risk than each risk factor alone.

Conclusion: We found a strong association of the interaction of *DNMT3B* gene with the *APOEε4* in this sample of AD patients. The presence of TGG haplotype and *APOEε4* significantly increased the risk of developing the disease, showing an additive effect.

Keywords: *APOEε4*, *DNMT3B*, epigenetics, Alzheimer's disease.

Introduction

Alzheimer disease (AD), the most common form of dementia, is considered a complex disease influenced by both environmental and genetic mediators. Many genetic variations interactions increased the risk of sporadic AD [1]. The ε4 allele of the apolipoprotein E (*APOE*) is the major genetic risk factor for sporadic, late onset AD [1-3]. The genotype *APOE* ε3/ε4, ε4/ε4 yielded risks three and eight times greater than the risk of ε3/ε3 for the development of AD [1].

Furthermore, emerging data have also demonstrated that the gene-environment process is probably associated with sporadic AD supporting the hypotheses of epigenetic processes enrolled in this neurodegenerative disease [4,5]. Additionally, age, the main risk factor for AD, was associated with an epigenetic drift in late-onset Alzheimer's disease (LOAD) [6] and with APOE methylation [7]. Epigenetic refers to changes in gene activity independent of primary DNA sequences. The DNA-methyltransferases (DNMTs) are the enzymes responsible for catalyzing the methylation of DNA, which is the most common epigenetic mechanism. Methylation occurs mainly on CpG islands by the addition of a methyl group (-CH3) at the 5-carbon of the pyrimidine ring of cytosine, resulting in 5-methylcytosine (5-mC) [8]. Recently, our research group observed that that the carriers of the haplotype TGG in polymorphisms of *DNMT3B* gene (derived from rs2424913, rs998382 and rs2424932) showed an association with AD (OR = 3.03, 95% CI 1.63 to 5.63, p <0.001) [9].

Additionally, the *APOE* ε2/ε3/ε4 isoforms showed distinct methylation patterns modifying the *APOE* gene expression. The *APOE* SNPs that define APOE ε2/ε3/ε4 isoforms (rs429358 and rs7412) were located in a CpG island located in the exon 4, with ε4 carriers bearing the greatest number of CpG sites while ε2 carriers had the smallest number [6,7]. Therefore, the aim of the present study is to evaluate whether the risk of *APOE*ε4 and *DNMT3B* polymorphisms for AD is additive or solely mediated by the effect of methylation on *APOE* expression.

Method and Materials

Data from the current case-control study were derived from a previous study of our research group. That database provided demographic, clinical information and *DNMT3B* haplotype distribution (derived from rs2424913, rs998382 and rs2424932) of 212 European-descendants subjects (104 subjects with AD diagnosis according to NINCDS-ADRDA and DSM-IV-TR [10], and 108 healthy subjects) recruited by convenience in a southern Brazilian city. A more detailed sample description can be obtained in Pezzi and colleagues [9]. The DNA was extracted using the Lahiri & Nuremberg protocol [11] and an amount of DNA were stored in -20ºC. For the present investigation we further carried out the *APOEε4* genotyping.

The *APOE* SNPs rs7412 and rs429358 were genotyped by TaqMan® Genotyping Master Mix and TaqMan SNP Genotyping assays (Applied Biosystems®). Allelic frequencies were obtained by direct counting throughout the genotype frequency.

Frequencies were described as proportions for categorical variables and as mean and standard deviation for quantitative variables. The non-parametric Mann–Whitney test was used to calculate the differences in age and education between cases and controls. The chi-square association test was used to analyze sex distribution between groups.

To investigate the genetic risk for AD, we sub-classified participants into four groups according to the genetic profile of *DNMT3B* and *APOE*:

1. Participants who do not present either polymorphism (TGG haplotype of *DNMT3B* and *APOE*ε4 allele) – this group was set as the reference for statistical analysis;

2. Participants who present TGG haplotype of *DNMT3B* but do not have the *APOE*ε4 allele;

3. Participants who do not have TGG haplotype of *DNMT3B* but have *APOE*ε4 allele;

4. Participants who present both polymorphism risks (TGG haplotype of *DNMT3B* and *APOEε*4 allele. These groups were entered in a multivariate logistic regression analysis as independent variable for the outcome AD. Confounders entered in the model were age and education, because of their known importance as risk for the development of AD.

A two-tailed P < 0.05 was considered significant for all analyses. The statistical package SPSS® version18.0 was used to perform the analyses.

This study was approved by the Ethic Committee (protocol number 566.029) and it was designed to comply with the terms of the Declaration of Helsinki. All participants and, their proxies, for AD subjects, signed the consent form.

Results and Discussion

The comparison of demographic data between AD subjects and healthy controls is showed in Table 1. Individuals with AD showed significantly lower scores on Mini Mental State Examination (MMSE) (P<0.0001) and lower educational level (P<0.0001) than healthy control subjects. *APOEε4* allele was more frequent among AD patients (33.1%) than in control group (12.0%, P<0.0001), resulting in a risk 3.63 higher for AD (OR=3.63, 95%CI = 2.19-6.03, P<0.0001).

----- Insert Table 1 here -----

Table 2 shows the frequency of each genetic category (according to *DNMT3B* TGG haplotype and *APOEε4* allele) in AD and healthy controls, as well as age, and education adjusted risk of each genetic category for AD. Both genetic risks kept their associations with AD independently. The chance for AD was 3.55 higher given the presence of *DNMT3B* TGG haplotype and absence of *APOEε4* allele compared with absence of both *DNMT3B* TGG haplotype and *APOEε4* allele. The odds ratio for AD was even higher (4.75) given the presence of *APOEε4* allele and the

absence of *DNMT3B* TGG haplotype compared with absence of both *DNMT3B* TGG haplotype and *APOEε4* allele. Subjects with both genetic risk factors (TGG *DNMT3B* haplotype and *APOEε4*) showed an odds ratio 11.13 higher for AD in comparison with those individuals who do not present any of these genetic risk factors (reference group).

----- Insert Table 2 here -----

In a previous study of our research group [9] we observed that the presence of the TGG haplotype in the *DNMT3B* gene increased the risk of AD to 3.55 times. Since the ε4 allele of the *APOE* gene is the most important genetic risk of developing sporadic AD [2,4,12] and recent findings suggests that methylation had a potential role in modifying *APOE* isoforms[7], we evaluate the gene-gene interaction of these two risk factors. Results from this study show that the combination of both polymorphisms greatly increases the risk of developing AD (OR = 11.13, 95% CI 4.25 to 29.16, P <0.001), i.e., there was an additive risk for AD in those subjects who presented TGG haplotype of *DNMT3B* plus *APOEε4* allele. So, we can hypothesize that, besides the risk of the *APOEε4* allele, which can also be mediated by age-associated changes acting upon its methylation, the TGG haplotype of *DNMT3B* confers a risk for global epigenetic disruption affecting any gene AD-associated.

Alzheimer's disease is considered a multifactorial disease with factors such as environmental, individual and genetic having an effect on its development [1]. Furthermore, among the myriad of genetic factors, gene variants, for example, SNPs, may also be associated with diseases. However, the interaction of different genes and their polymorphisms between themselves has been less studied. It is suggested that APOE is involved in the clearance of soluble Aβ and Aβ aggregation, modulating processes that lead to the accumulation of β-amyloid plaques in the brain. Furthermore, it is responsible for interruption of the normal transport and catabolism of cholesterol necessary for the growth and neuronal repair and regeneration of nerves. And, it is also associated with immune response, activation of lipolytic enzymes and vascular diseases affecting the brain [13,14]. In the present investigation we find an expected risk of *APOEε4* allele, similar to those observed in other studies [3,12]. However, the magnitude of this risk may vary. For example, no signs of dementia were found in

90-years-old individuals who have the *APOEε4* allele. Furthermore, only about 50% of subjects with AD were *APOEε4* allele carriers, so probably genetic and environmental factors, yet unidentified, were acting [14,15]. Our results show an increased risk for AD (OR = 11.13, 95% CI 4.25 to 29.16, P <0.001) in those subjects who present both polymorphism risks (TGG haplotype of DNMT3B plus *APOEε4* allele), stressing the relevance of analyzing gene interactions in complex diseases such as AD.

In the context of gene-gene and gene-environment interactions, epigenetic mechanisms may have a pivotal role in AD pathogenesis [16]. DNA methylation in neurons appeared to play an important role in the encoding process of the memory [17] and in long-term memory [18,19] through the regulation of signaling pathways of various genes in neural network [20]. Aberrant methylation is mediated by folate levels that act as a cofactor in AD, and a marked reduction in methylation of cortical neurons is observed in AD post-mortem studies [16]. Recent studies have pointed an age-related change in the methylation pattern of *APOE* that can be also associated with its risk factor for AD [7].

Considering that our results showed an addictive risk for AD in those subjects who presented TGG haplotype of *DNMT3B* plus *APOEε4* allele, we can hypothesize that the TGG haplotype also has an effect on the methylation process of other important genes for AD development. Indeed, recently Yu et al. [21] evaluated the association of brain DNA methylation in 28 reported AD loci discovered by GWAS studies with AD pathologies. They found that DNA methylation of CpG islands in genes *SORL1*, *ABCA7*, *HLA- DRB5* and *BIN1* associated with pathological AD. Additionally, they observed that RNA expression transcripts of *SORL1* and *ABCA7* were associated with paired helical filament tau tangle density, while expression of *BIN1* was associated with amyloid load. According to those authors, these data supported the hypothesis that methylation had an independent effect on both amyloid process and on tau pathology [21]. These findings may explain the *DNMT3B* TGG haplotype and *APOEε4* allele independent and additive risk found in our study, since the first genetic risk factor can also impact other pathways than amyloid route. Moreover, Di Francesco et al. [22] observed an increase in overall DNA methylation,

in gene expression of *DNMT1* and *DNMT3B* and protein levels in blood cells of patients with AD.

Despite the statistical significance of our results, one of the limitations of the present study was the small sample size. Therefore, studies with larger samples should be performed in order to reach a stronger power. Replication of this study in different populations is also suggested, since gene-gene interaction can occur in different ways according to the genetic background found in every ethnic group. In conclusion, in our study we found that the interaction between TGG haplotype of *DNMT3B* gene and allele ε4 of *APOE* is a genetic risk factor that increased the chance of developing AD in 11-fold, suggesting that both factors are at least partially independent and addictive. To our knowledge, this is the first study aiming to investigate the association of *APOEε4* allele with the TGG haplotype in the *DNMT3B* gene in the Alzheimer's disease. How the interaction between these polymorphisms works in the development of AD should be investigated in future studies.

Acknowledgements

We are grateful for the support given to this research by National Council for Scientific and Technological Development (CNPq).

Conflict of Interest

Regarding research, article writing and/or publishing the authors state no potential conflict of interests.

References

1. Loy CT, Schofield PR, Turner AM, Kwok JB (2014) Genetics of dementia. Lancet 383:828-840. doi.org/10.1016/S0140-6736(13)60630-3

2. Montag C, Kunz L, Axmacher N, Sariyska R, Lachmann B, Reuter M (2014) Common genetic variation of the APOE gene and personality. BMC Neurosci 15:64. doi: 10.1186/1471-2202-15-64

3. Michaelson DM (2014) APOE ε4: the most prevalent yet understudied risk f actor Alzhemier's disease. Alzheimers Dement. 10:861-868. doi: 10.1016/j.jalz.2014.06.015

4. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, Rogers J (2011) Epigenetic mechanisms in Alzheimer's disease. Neurobiol Aging 32:1161-1180. doi: 10.1016/j.neurobiolaging.2010.08.017

5. Wang J, Yu JT, Tan MS, Jiang T, Tan L (2013) Epigenetic mechanisms in Alzheimer's disease: implications for pathogenesis and therapy. Ageing Res Rev 12:1024-1041. doi: 10.1016/j.arr.2013.05.00

6. Wang S-C, Oelze B, Schumacher A (2008) Age-specific epigenetic drift in late-onset Alzheimer’s disease. Plos ONE 3:e2698. doi: 10.1371/journal.pone.0002698

7. Ma Y, Smith CE, Lai CQ, Irvin MR, Parnell LD, Lee YC, Pham L, Aslibekyan S, Claas SA, Tsai MY, Borecki IB, Kabagambe EK, Berciano S, Ordovás JM, Absher DM, Arnett DK (2015) Genetic variants modify the effect of age on APOE methylation in the Genetics of Lipid Lowering Drugs and Diet Network study. Aging Cell 14:49-59. doi: 10.1111/acel.12293

8. Fitzsimons CP, van Bodegraven E, Schouten M, Lardenoije R, Kompotis K, Kenis G, van den Hurk M, Boks MP, Biojone C, Joca S, Steinbusch HW, Lunnon K, Mastroeni DF, Mill J, Lucassen PJ, Coleman PD, van den Hove DL, Rutten BP

(2014) Epigenetic regulation of adult neural stem cells: implications for Alzheimer's disease. Mol Neurodegener 9:25. doi: 10.1186/1750-1326-9-25

9. Pezzi JC, Ens CM, Borba EM, Schumacher-Schuh AF, de Andrade FM, Chaves ML, Fiegenbaum M, Camozzato AL (2014) DNA methyltransferase haplotype is associated with Alzheimer's disease. Neurosci Lett 579:70-74. doi: 10.1016/j.neulet.2014.07.013

10. American Psychiatric Association (2013) Diagnostics and statistical manual of mental disorders, 5ed. Washington, DC.

11. Lahiri DK, Nurnberger JI Jr (1991) A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res 19:5444.

12. Verghese PB, Castellano JM, Holtzman DM (2011) Apolipoprotein E in Alzheimer's disease and other neurological disorders. Lancet Neurol 10:241-252. doi: 10.1016/S1474-4422(10)70325-2

13. Karch CM, Cruchaga C, Goate AM (2014) Alzheimer's disease genetics: from the bench to the clinic.Neuron 83:11-26. doi: 10.1016/j.neuron.2014.05.041

14. Lyall DM, Royle NA, Harris SE, Bastin ME, Maniega SM, Murray C, Lutz MW, Saunders AM, Roses AD, del Valdés Hernández MC, Starr JM, Porteous DJ, Wardlaw JM, Deary IJ (2013) Alzheimer's disease susceptibility genes APOE and TOMM40, and hippocampal volumes in the Lothian birth cohort 1936. PLoS One 8:e80513. doi: 10.1371/journal.pone.0080513

15. Bekris LM, Yu CE, Bird TD, Tsuang DW (2010) Genetics of Alzheimer disease. J Geriatr Psychiatry Neurol 23:213-227. doi: 10.1177/0891988710383571

16. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, Rogers J (2010) Epigenetic changes in Alzheimer's disease: decrements in DNA methylation. Neurobiol Aging 31:2025-2037. doi: 10.1016/j.neurobiolaging.2008

17. Miller CA, Sweatt JD (2007) Covalent modification of DNA regulates memory formation. Neuron 53:857-8569.

18. Levenson JM, Choi S, Lee SY, Cao YA, Ahn HJ, Worley KC, Pizzi M, Liou HC, Sweatt JD (2004) A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel. J Neurosci 24:3933-3943.

19. Levenson JM, Sweatt JD (2005) Epigenetic mechanisms in memory formation. Nat Rev Neurosci 6:108-118.

20. Roberson ED, Sweatt JD (1999) A biochemical blueprint for long-term memory. Learn Mem 6:381-388.

21. Yu L, Chibnik LB, Srivastava GP, Pochet N, Yang J, Xu J, Kozubek J, Obholzer N, Leurgans SE, Schneider JA, Meissner A, De Jager PL, Bennett DA (2015) Association of Brain DNA methylation in SORL1, ABCA7, HLA-DRB5, SLC24A4, and BIN1 with pathological diagnosis of Alzheimer disease. JAMA Neurol 72:15-24. doi: 10.1001/jamaneurol.2014.3049

22. Di Francesco A, Arosio B, Falconi A, Micioni Di Bonaventura MV, Karimi M, Mari D, Casati M, Maccarrone M, D'Addario C (2015) Global changes in DNA methylation in Alzheimer's disease peripheral blood mononuclear cells. Brain Behav Immun 45:139-144. doi:10.1016/j.bbi.2014.11.0

Table 1: Demographic and clinical data: comparison between AD subjects and healthy controls

	Control Group (N=108)	AD Group (N=104)	*P*
Age (years) (mean/SD)	74.96 (7.73)	76.67 (7.34)	0.099*
Sex (female) N (%)	77 (71)	64 (61.5)	0.146**
Education(years)	7.95 (4.17)	5.03 (3.06)	<0.0001*
MMSE (mean/SD)	27.55 (2.02)	12.72 (5.55)	<0.0001*
APOE alleles			<0.0001**
ε 2	7.4%	5.8%	
ε 3	80.6%	61.1%	
ε 4	12.0%	33.1%	

Note:AD: Alzheimer's disease. MMSE: Mini Mental State Examination score * Mann-Whitney Test; **Chi-square Test

Table 2: Frequencies and education-adjusted risk of each TGG/APOE category for AD: multiple binary logistic regression

Group	Control Group	AD Group	Adjusted OR[a] (CI 95%)
		P	
Reference	49 (45.8%)	15 (15.2%)	1
TGG+/APOE-	33 (30.8%)	29 (29.3%)	3.55 (1.57-8.00)
		0.002	
TGG-/APOE+	12 (11.2%)	20 (20.2%)	4.75 (1.80-12.57)
		0.002	
TGG+/APOE+	13 (12.1%)	35 (35.4%)	11.13 (4.25-29.16)
		<0.001	

Reference group: subjects without TGG haplotype and *APOE ε4* allele, [a]OR adjusted by age and education

7.3 Anexo 3: Normas da revista a qual o Artigo Científico 2 foi submetido

ELSEVIER

NEUROSCIENCE LETTERS

The rapid communication journal for the neurosciences.

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0304-3940

DESCRIPTION

Neuroscience Letters is devoted to the rapid publication of short, high-quality papers of interest to the broad community of neuroscientists. Only papers which will make a significant addition to the literature in the field will be published. Papers in all areas of **neuroscience** - **molecular, cellular, developmental, systems, behavioral** and **cognitive**, as well as **computational** - will be considered for publication. Submission of laboratory investigations that shed light on disease mechanisms is encouraged. Clinical studies will also be published if they provide new information about organization or actions of the nervous system, or provide new insights into the neurobiology of disease. NSL does not publish case reports.

Papers that are primarily devoted to psychological or philosophical questions, that use unvalidated methodology, or that fall outside of the realm of neuroscience, will not be published.

Neuroscience Letters is published both on the web (in ScienceDirect - http://www.sciencedirect.com), and as a print journal. Publication in the web version of *Neuroscience Letters* will occur within five weeks of acceptance, and in the hard copy within seven weeks. Particularly important papers may be published more rapidly within a Plenary Article section of the journal.

Benefits to authors
We also provide many author benefits, such as free PDFs, a liberal copyright policy, special discounts on Elsevier publications and much more. Please click here for more information on our author services .

Please see our Guide for Authors for information on article submission. If you require any further information or help, please visit our support pages: http://support.elsevier.com

AUDIENCE

Neuroscientists, neurologists

IMPACT FACTOR

2014: 2.030 © Thomson Reuters Journal Citation Reports 2015

ABSTRACTING AND INDEXING

BIOSIS
Elsevier BIOBASE
Chemical Abstracts
Current Contents/Life Sciences
MEDLINE®
EMBASE
Pascal M
Reference Update
Science Citation Index
Scopus

EDITORIAL BOARD

J. Veliskova, New York Medical College, Valhalla, New York, USA
D.R. Weinberger, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
J. Wood, University College London (UCL), London, UK
C.-L. Zhang, University of Texas Southwestern Medical Center, Dallas, Texas, USA
J. Zhao, University College London (UCL), London, UK
H.Y. Zoghbi, Baylor College of Medicine, Houston, Texas, USA

GUIDE FOR AUTHORS

INTRODUCTION

Neuroscience Letters is devoted to the rapid publication of short, high-quality papers of interest to the broad community of neuroscientists. Only papers which will make a significant addition to the literature in the field will be published. Papers in all areas of neuroscience - molecular, cellular, developmental, systems, behavioral and cognitive, as well as computational - will be considered for publication. Submission of laboratory investigations that shed light on disease mechanisms is encouraged. Clinical studies will also be published if they provide new information about organization or actions of the nervous system, or provide new insights into the neurobiology of disease. NSL does not publish case reports.

Papers that are primarily devoted to psychological or philosophical questions, that use unvalidated methodology, or that fall outside of the realm of neuroscience, will not be published.

The Neuroscience Peer Review Consortium

Neuroscience Letters is a member of the Neuroscience Peer Review Consortium (NPRC). The NPRC has been formed to reduce the time expended and, in particular, the duplication of effort by, and associated burden on reviewers involved in the peer review of original neuroscience research papers. It is an alliance of neuroscience journals that have agreed to accept manuscript reviews from other Consortium journals. By reducing the number of times that a manuscript is reviewed, the Consortium will reduce the load on reviewers and Editors, and speed the publication of research results.

If a manuscript has been rejected by another journal in the Consortium, authors can submit the manuscript to *Neuroscience Letters* and indicate that the referees' reports from the first journal will be made available to the Editors of Neuroscience Letters.

It is the authors' decision as to whether or not to indicate that a set of referee's reports should be forwarded from the first journal to *Neuroscience Letters*. If an author does not wish for this to happen, the manuscript can be submitted to *Neuroscience Letters* without reference to the previous submission. No information will be exchanged between journals except at the request of authors. However, if the original referees' reports suggested that the paper is of high quality, but not suitable for the first journal, then it will often be to an author's advantage to indicate that referees' reports should be made available.

Authors should revise the original submission in accordance with the first journal's set of referee reports, reformat the paper to *Neuroscience Letters*' specification and submit the paper to *Neuroscience Letters* with a covering letter describing the changes that have been made, and informing the Editors that the authors will ask for the referees' reports to be forwarded from the first Consortium journal. The authors then must contact the first journal, and ask that reviews be forwarded, indicating they have submitted to *Neuroscience Letters*, and providing the new manuscript ID number.

The Editors of *Neuroscience Letters* will use forwarded referees' reports at their discretion. The Editors may use the reports directly to make a decision, or they may request further reviews if they feel such are necessary.

Visit http://nprc.incf.org for a list of Consortium journals, as well as further information on the scheme.

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see https://www.elsevier.com/publishingethics and https://www.elsevier.com/journal-authors/ethics.

Policy and ethics

The work described in your article must have been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans* http://www.wma.net/en/30publications/10policies/b3/index.html; *EC Directive 86/609/EEC*

for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm; *Uniform Requirements for manuscripts submitted to Biomedical journals* http://www.icmje.org. This must be stated at an appropriate point in the article.

For other policy issues, authors are referred to the policy guidelines of the Society for Neuroscience (see their website http://www.jneurosci.org/misc/itoa.shtml).

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also https://www.elsevier.com/conflictsofinterest. Further information and an example of a Conflict of Interest form can be found at: http://service.elsevier.com/app/answers/detail/a_id/286/supporthub/publishing.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see https://www.elsevier.com/sharingpolicy), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck https://www.elsevier.com/editors/plagdetect.

Contributors

Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see https://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult https://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult https://www.elsevier.com/permissions.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see https://www.elsevier.com/OAauthoragreement). Permitted third party reuse of open access articles is determined by the author's choice of user license (see https://www.elsevier.com/openaccesslicenses).

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. For more information see https://www.elsevier.com/copyright.

Role of the funding source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some authors may also be reimbursed for associated publication fees. To learn more about existing agreements please visit https://www.elsevier.com/fundingbodies.

Open access
This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse
• An open access publication fee is payable by authors or on their behalf e.g. by their research funder or institution
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs (https://www.elsevier.com/access).
• No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 2150**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access
Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information (http://elsevier.com/greenopenaccess). Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver

value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form.

This journal has an embargo period of 12 months.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Submit your article
Please submit your article via http://ees.elsevier.com/nsl/
For submission queries, please contact the Editorial Office (NSL@elsevier.com).

Referees
Authors must send the names, addresses and email addresses for 8-10 potential referees that meet the following criteria: potential referees must be experts or active workers in the field, must not be current or prior mentors or collaborators, and must have institutional email addresses (e.g., xx.yy@zz.edu) and not generic email addresses (e.g., xx.yy@163.com or xx.yy@gmail.com). Although the journal does not guarantee these reviewers will be used, the Editors take these suggestions under consideration. These recommendations help the journal speed the editorial process.

Additional information
Length of manuscripts will in no case be more than 6 printed pages (5000 words) of the journal. As an approximate guide to authors for judging the length of their paper, the following estimation may be used: heading + abstract = 0.5-0.6 pages; 3 type-written (double-spaced) pages = 1 printed page; (when using a word-processor) 850 words or 5300 characters = 1 printed page; 3 single-column wide or 2 double-column wide figures plus legends = 1 printed page; 3 single-column wide or 2 double-column wide tables = 1 printed page; 17 references = 0.5 printed page.

PREPARATION

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: https://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision
Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply " the text" .

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is in very occasional cases appropriate, although in general, Results and Discussion should be presented as distinct sections of the manuscript. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See https://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See https://www.elsevier.com/highlights for examples.

Keywords
Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Artwork
Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website:
https://www.elsevier.com/artworkinstructions.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. For further information on the preparation of electronic artwork, please see https://www.elsevier.com/artworkinstructions.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles (http://citationstyles.org), such as Mendeley (http://www.mendeley.com/features/reference-manager) and Zotero (https://www.zotero.org/), as well as EndNote (http://endnote.com/downloads/styles). Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/neuroscience-letters
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video data
Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at https://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides
The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at https://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary material
Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at https://www.elsevier.com/artworkinstructions.

Data in Brief
Authors have the option of converting any or all parts of their supplementary or additional raw data into one or multiple Data in Brief articles, a new kind of article that houses and describes their data. Data in Brief articles ensure that your data, which is normally buried in supplementary material, is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. Authors are encouraged to submit their Data in Brief article as an additional item directly alongside the revised version of their manuscript. If your research article is accepted, your Data in Brief article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the new, open access journal, *Data in Brief*. Please note an open access fee is payable for publication in *Data in Brief*. Full details can be found at http://www.journals.elsevier.com/data-in-brief. Please use the following template to write your Data in Brief: https://www.elsevier.com/dib-template.

Database linking
Elsevier encourages authors to connect articles with external databases, giving readers access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See https://www.elsevier.com/databaselinking for more information and a full list of supported databases.

Submission checklist
The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.
Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address
All necessary files have been uploaded, and contain:
• Keywords
• All figure captions
• All tables (including title, description, footnotes)
Further considerations
• Manuscript has been 'spell-checked' and 'grammar-checked'
• References are in the correct format for this journal
• All references mentioned in the Reference list are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
Printed version of figures (if applicable) in color or black-and-white
• Indicate clearly whether or not color or black-and-white in print is required.
For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Use of the Digital Object Identifier
The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. Example of a correctly given DOI (in URL format; here an article in the journal *Physics Letters B*):
http://dx.doi.org/10.1016/j.physletb.2010.09.059
When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

Online proof correction
Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints
The corresponding author, at no cost, will be provided with a personalized link providing 50 days free access to the final published version of the article on ScienceDirect. This link can also be used for sharing via email and social networks. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for

publication. Both corresponding and co-authors may order offprints at any time via Elsevier's WebShop (http://webshop.elsevier.com/myarticleservices/offprints). Authors requiring printed copies of multiple articles may use Elsevier WebShop's 'Create Your Own Book' service to collate multiple articles within a single cover (http://webshop.elsevier.com/myarticleservices/booklets).

AUTHOR INQUIRIES

You can track your submitted article at https://www.elsevier.com/track-submission. You can track your accepted article at https://www.elsevier.com/trackarticle. You are also welcome to contact Customer Support via http://support.elsevier.com.