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**PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR**

# Aspectos genéticos, ambientais e suas interações na suscetibilidade e farmacogenética da doença de Parkinson

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**Lista de Abreviaturas**

ABCB1 – glicoproteína-P

ALDH – aldeído desidrogenase

*ALDH2* – gene da aldeído desidrogenase

CL – corpos de Lewy

COMT – catecol o-metiltransferase

CYP1A1 – citocromo P 450 1A1

CYP1A2 – citocromo P 450 1A2

CYP1B1 – citocromo P 450 1B1

CYP2D6 –citocromo P 450 2D6

DAT1 – transportador de dopamina 1

DDC – dopa-decarboxilase

DP – doença de Parkinson

*GBA* – gene da glicocerebrosidade cerebral

GRIN2A – receptor inotrópico de glutamato

GWAIS – genome-wide association and interaction study

GWAS – genome-wide association study ''estudos de associação genômicos ''

HAPs – hidrocarbonetos aromáticos policíclicos

LRRK2 – quinase rica em leucina 2

MAOA – monoamina oxidase A

MAOB – monoamina oxidase B

MAPT – proteína tau

MPTP – 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine

NOS1 – óxido nítrico sintetase

OCT1 – transportador de cátions orgânico

PON1 – paraoxonase 1

SLC6A3 – transportador de dopamina 1

SNpc – *substantia nigra pars compacta*

SNCA – alfa-sinucleína

SV2C – proteína da vesícula sináptica 2C

# RESUMO

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais frequente na espécie humana. A etiologia da DP é multifatorial, com muitos fatores ambientais e genéticos atuando em conjunto para sua determinação. Ao nível patológico ela caracteriza-se pela destruição seletiva de neurônios dopaminérgicos da *substantia nigra pars compacta* e pelo acúmulo de corpos de Lewy no cérebro. Em aproximadamente 90% dos casos da DP, a suscetibilidade parece ser determinada por variantes comuns no genoma que podem interagir com o ambiente. Dentre os fatores ambientais que modulam o risco para a DP estão: uso de pesticidas, exposição ocupacional a tóxicos, consumo de café e cigarro. A variabilidade na resposta à principal medicação da DP, a levodopa, parece também estar relacionada à genética do indivíduo. Dessa forma, o presente trabalho teve como objetivo a melhor compreensão dos fatores genéticos e suas interações com o ambiente envolvidos na suscetibilidade à DP e no tratamento com levodopa. Pacientes com DP e seus controles foram recrutados no ambulatório de distúrbios do movimento e no ambulatório de medicina interna do Hospital de Clínicas de Porto Alegre e da Universidade Federal de Ciências da Saúde de Porto Alegre, respectivamente. Os resultados obtidos foram organizados em cinco artigos. No primeiro estudo, foram considerados oito polimorfismos previamente associados à DP. Na nossa população, os indivíduos que possuíam sete ou mais alelos de risco desses polimorfismos apresentaram um *odds ratio* de 2,54 quando comparados a quem possuía seis alelos ou menos (95% IC 1,66-3,89; P = 1,80E−05). Esse ponto de corte foi escolhido porque o número médio de alelos de risco na amostra foi 7. No segundo artigo, portadores do genótipo TT do polimorfismo rs1021463 e dos genótipos TT ou GT do polimorfismo rs30196 do gene *SV2C a*presentaram um maior risco a DP quando expostos ocupacionalmente a tóxicos, quando comparado a não-expostos (respectivamente, OR 2,53; 95% IC 1,33-4,69; $P_{interação}$ = 0,008 e OR 2,30; 95% IC 1,21-4,36; $P_{interação}$ = 0,033). O terceiro artigo mostra uma associação em que fumantes com o haplótipo T- não G -T do transportador *ABCB1* apresentaram menor risco a DP quando comparados a portadores do

haplótipo C-G-C (OR 0,34, 95% IC 0,15-0,72; $P_{interação}$ = 0,012). No quarto trabalho, foi constatada uma interação entre o gene *NOS1* e a cafeína modulando o risco da DP (OR 0,24; 95% IC 0,10-0,54; $P_{interação}$ = 0,0002). O último artigo trata da farmacogenética da levodopa, em que foi proposto um modelo com variáveis genéticas, biológicas e farmacológicas que explicou 23% da variabilidade na dose ($F = 11,54$; $P < 0,000001$). Observou-se uma redução da média de dose em aproximadamente 76 mg/dia por cada alelo C nos genótipos do polimorfismo rs30196 do gene *SV2C*. Estes trabalhos enriqueceram o conhecimento da variabilidade da DP tanto em aspectos de suscetibilidade quanto farmacogenética. Os dados obtidos serão importantes na continuação das pesquisas para identificar biomarcadores para prevenção e tratamento da DP.

# ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease in humans. PD etiology is multifactorial, due to several environmental and genetic factors. Pathologically, it is characterized by a selective destruction of dopaminergic neurons in *substantia nigra pars compacta* and by the accumulation of Lewy bodies in brain. In approximately 90% of PD cases, susceptibility seems to be driven by common variants in the genome that might interact with the environment. Among environmental factors that modulate PD risk, pesticides, occupational exposure to toxics, smoking and coffee consumption were identified. The variability in patients' response to the main medication of PD, levodopa, seems also to be related to genetics. Therefore, the present work had the objective to understand the genetic factors and their interaction with the environment involved in PD susceptibility and in the treatment with levodopa. Patients and controls were recruited at the movement disorders ambulatory and at the internal medicine ambulatory at the Hospital de Clínicas de Porto Alegre and at Universidade Federal de Ciências da Saúde de Porto Alegre respectively. The main results obtained were organized in five manuscripts. In the first, eight polymorphisms previously associated with PD were considered. In our population, individuals with 7 or more risk alleles presented an *odds ratio* of 2.54 for PD when compared to those with 6 or less alleles (95% CI 1.66-3.89; P = 1.80E−05). This cut off was chosen because the average number of risk alleles in the sample was 7. In the second manuscript, *SV2C* rs10214163 TT genotype carriers and *SV2C* rs30196 TT/GT genotypes carriers showed a higher PD risk in subjects exposed to environmental toxics compared to those not exposed (respectively, OR 2.53; 95% CI 1.33-4.69; $P_{interaction}$ = 0.008 and OR 2.30; 95% CI 1.21-4.36; $P_{interaction}$ = 0.033). The third manuscript shows the association between *ABCB1* T-non G-T haplotype and smoking (OR 0.34, 95% CI 0.15-0.72; $P_{interaction}$ = 0.012). In the fourth manuscript, an interaction between *NOS1* gene and caffeine, modulating PD risk, was observed (OR 0.24; 95% CI 0.10-0.54; $P_{interaction}$ = 0.0002). The last manuscript reports a

model with genetic, biological and pharmacological variants in response to levodopa. This model explained 23% of dose variability ($F = 11.54$; $P < 0.000001$). The presence of each rs30196 C allele reduced the average dose in approximately 76 mg/day. All these work enriched the knowledge of the variability of PD in both susceptibility and pharmacogenetic areas. The data obtained will be important to identify biomarkers for disease prediction and treatment.

# CAPÍTULO I

## *Introdução*

# A doença de Parkinson

## Histórico

Os primeiros seis casos de uma enfermidade diferente foram inicialmente descritos em 1817, em uma monografia denominada "An Essay on Shaking Palsy", por James Parkinson. Devido aos seus primeiros relatos, a doença posteriormente levou o seu nome. James Parkinson denominou a síndrome de paralisia agitante, caracterizando-a pela presença de movimentos tremulantes involuntários, diminuição da força muscular, uma tendência à inclinação do tronco para frente e alteração na marcha (Parkinson, 1817 citado por Przedborski, 2017).

Aproximadamente meio século após a descrição da doença por James Parkinson, o neurologista Jean-Martin Charcot proporcionou importantes contribuições para o estudo desta patologia, definindo os sintomas cardinais da doença: tremor, bradicinesia e rigidez. Além disso, nomeou a doença e a delimitou como uma doença única (Teive, 1998).

## Epidemiologia

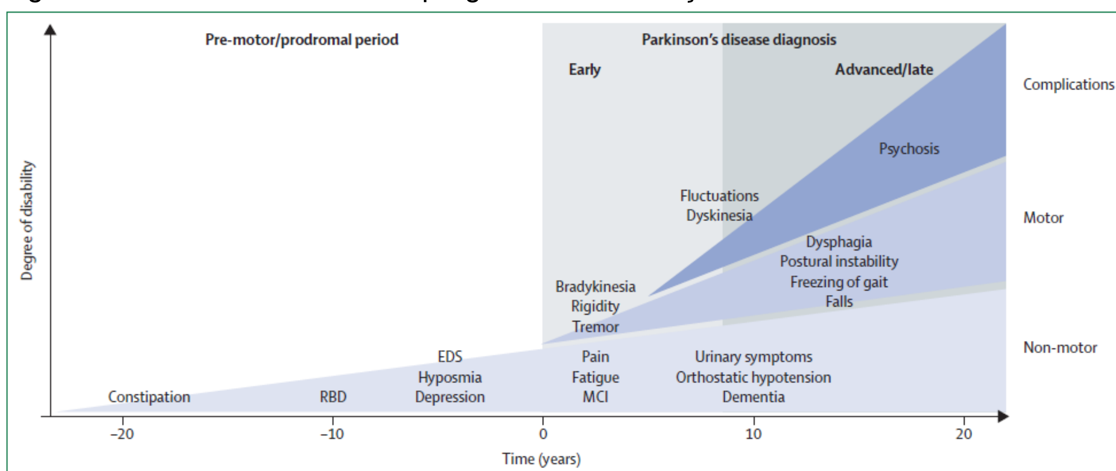
A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais frequente na espécie humana, ficando atrás apenas da doença de Alzheimer (Dorsey et al., 2007; Alzheimer's Association, 2014). Sua incidência e prevalência aumentam em sincronia com a idade. Uma meta-análise com dados epidemiológicos abrangendo vários países mostrou uma prevalência de DP de 425 por 100.000 indivíduos no grupo de idade de 60 a 69 anos, chegando a 1.087 por 100.000 indivíduos no grupo de 70 a 79 anos. Esse estudo mostra também que a prevalência é dependente da região geográfica. Na América do Sul, uma prevalência de 2.180 indivíduos na faixa de 70 a 79 anos por 100.000 indivíduos foi estimada, enquanto que na Europa foi de 1.602 indivíduos e na Ásia 646 indivíduos por

100.000 na mesma faixa de idade (Pringsheim et al., 2014). No Brasil, um estudo de base populacional em Bambuí, Minas Gerais, identificou uma prevalência de 3,3% para a doença de Parkinson entre maiores de 60 anos (Barbosa et al., 2006).

## Manifestações clínicas e patológicas

Até o final do século 20, a DP era considerada uma doença puramente motora, caracterizando-se pelos sintomas descritos por J. Parkinson e J.M. Charcot como: bradicinesia (lentidão anormal dos movimentos), tremor de repouso, instabilidade postural e rigidez muscular (Dauer & Przedborski, 2003). Todavia, hoje se sabe que a DP apresenta uma variedade de sintomas não motores, podendo eles se manifestar antes dos sintomas motores característicos (Figura 1). Os sintomas não motores compreendem disfunções autonômicas cardiovasculares, urogenitais e gastrointestinais, distúrbios do sono, hiposmia, transtornos de humor, psicose, ansiedade, déficit cognitivo e demência (Kalia & Lang, 2015; Jost, 2017).

Figura 1 - Sintomas clínicos e a progressão da doença




Kalia & Lang 2015

Acredita-se que os sintomas motores sejam resultantes de uma destruição seletiva de neurônios dopaminérgicos da *substantia nigra pars compacta* (SNpc), observada nos exames *post-mortem* dos pacientes com DP. Os núcleos dos neurônios dopaminérgicos se localizam na SNpc, e projetam suas aferências para o estriado: putamen e caudado. A diminuição desses neurônios dopaminérgicos determina uma despigmentação da SNpc, devido a falta da produção de neuromelanina, normalmente produzida por esses neurônios. Foi visto também que a perda neuronal ocorre em outras regiões cerebrais como: *locus ceruleus*, núcleo basal de Meynert, núcleo pedunculo pontine, núcleos da rafe, núcleo dorsal do vago, amígdala e hipotálamo e assim, envolvendo outros sistemas de neurotransmissores (noradrenérgico, colinérgico e serotorinérgico) (Dauer & Przedborski, 2003; Lees et al., 2009).

Várias evidências sugerem que a degeneração dos neurônios dopaminérgicos se inicia 6-8 anos antes dos sintomas clínicos. O início dos sintomas é tão gradual que torna difícil diagnosticar o início da doença. No momento do diagnóstico, a patologia da DP já se encontra estabelecida e os pacientes apresentam 50-60% de perda neuronal e 70-80% de depleção dopaminérgica. Essa latência entre o início da patologia e o da manifestação clínica possivelmente se deve a uma redundância das vias dopaminérgicas e mecanismos de compensação que mantêm a função dos núcleos da base estável por vários anos (Schapira & Obeso, 2006).

Outra característica clássica da DP é o acúmulo de inclusões citoplasmáticas conhecidas como corpos de Lewy (CL). Essas inclusões são formadas por material protéico. Trezentas proteínas diferentes foram identificadas nestas inclusões, predominando a alfa-sinucleína. Uma significante perda neuronal é encontrada em sítios em que se encontram os CL, incluindo *substantia nigra*, *locus ceruleus* e núcleo basal de Meynert. Porém, esse achado não implica que os corpúsculos sejam a causa da morte celular (Wakabayashi et al., 2013).

Estudos *post-mortem* demonstraram que os CL se formam progressivamente e gradualmente afetam o sistema nervoso central. Esses estudos contribuíram para um modelo de evolução de neurodegeneração proposto por Braak et al. (2003). Neste modelo, o estágio inicial (estágio 1) apresenta CL no sistema nervoso periférico, bulbo olfatório e núcleo motor dorsal dos nervos vago e glossofaríngeo. No estágio 2 há comprometimento da ponte até chegar ao mesencéfalo, onde se localiza a SNpc, caracterizando assim o estágio 3. Depois, a neurodegeneração chega até o prosencéfalo e mesocortex (estágio 4), culminando em múltiplas regiões corticais (estágios 5 e 6) (Braak et al., 2003; Kalia & Lang, 2015). O modelo de Braak et al. ganhou muita visibilidade porque a hipótese de progressão temporal e espacial conseguiu explicar o curso clínico da DP, em que não há acometimento exclusivo do sistema dopaminérgico, mas sim, de vários outros sistemas monoaminérgicos. Estágios 1 e 2 correspondem ao início dos sintomas motores, estágio 3 se caracteriza pela apresentação dos sintomas motores, devido à depleção dopaminérgica. Nos estágios 4 a 6, com o avanço da doença, e atingindo regiões corticais, ocorrem sintomas não motores como déficit cognitivo e demência. Porém, os CL não são específicos para DP, sendo encontrados em outras doenças como demência de corpos de Lewy, Doença de Gaucher e doença de Alzheimer com CL (Wakabayashi et al., 2013).

O diagnóstico definitivo da doença só ocorre após o exame do cérebro *post-mortem*, no qual se pode observar a degeneração da SNpc e os CL. Os critérios diagnósticos clínicos atuais utilizados para a DP são baseados no Banco de cérebro de Londres, que fez uma extensa correlação clínico-patológica de cérebros de pacientes com DP e sintomas característicos (Hughes et al., 1992).

## Etiologia da doença de Parkinson

### Fatores Ambientais

No final dos anos 70 uma neurotoxina que reproduz os sintomas da DP nos indivíduos intoxicados estimulou o interesse dos pesquisadores. Essa neurotoxina, conhecida como MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine), é um produto da síntese de opióides (Przedborski, 2017). Indivíduos intoxicados com MPTP apresentaram uma degeneração específica de neurônios dopaminérgicos, porém sem a presença dos CL. Ao nível molecular, o MPTP administrado em modelos animais consegue ultrapassar a barreira hematoencefálica, é convertido ao metabólito MPP+, que é transportado ativamente e seletivamente para neurônios dopaminérgicos pelos transportadores de dopamina (DAT), se acumulando nos neurônios dopaminérgicos (Blum et al., 2001). A toxicidade do MPTP é resultante de altos níveis de MPP+ que inibem o complexo I da cadeia de transporte de elétrons na matriz mitocondrial (Przedborski, 2017). Atualmente, essa substância é utilizada para produzir modelos animais para DP devido à alta similaridade neuropatológica e neurofisiológica da DP esporádica determinada nesses animais por esse composto (Winklhofer & Haass, 2010).

Após a descoberta do MPTP, várias outras substâncias foram associadas à DP sugerindo uma hipótese de que o ambiente poderia determinar ao menos em parte a DP. A influência ambiental na DP engloba tanto fatores mais gerais como industrialização, ambiente rural, mineração, utilização de água de poço, toxinas de plantas, microbiota intestinal e infeções bacterianas ou virais quanto mais específicos como alguns solventes orgânicos, medicamentos, monóxido de carbono e manganês (Bellou et al., 2015).

Muitos estudos apontam o uso de pesticidas como um dos principais fatores ambientais de suscetibilidade a DP (Nandipati & Litvan, 2016). Pesticidas compreendem uma gama de compostos desenhados para matar insetos (inseticidas), plantas (herbicidas), fungos (fungicidas) e roedores (rodenticidas). Uma meta-analise com 46 estudos mostrou um risco de 1,6 para exposição a qualquer tipo de pesticida (van der Mark et al., 2011). Os pesticidas que mais frequentemente são associados à DP são a rotenona e o paraquat. Outros pesticidas, pertencentes à classe dos organoclorados, carbamatos, organofosfatos e

piretroides, são pouco estudados e possuem poucas evidências de contribuição para a etiologia da DP (Goldman, 2014).

A rotenona é um composto derivado de planta que inibe o complexo I da cadeia transportadora de elétrons na mitocôndria, no mesmo local atacado pelo MPP+. É utilizada como inseticida há décadas na agricultura e jardinagem (Goldman, 2014). Estudos epidemiológicos mostram que quem aplica rotenona profissionalmente tem um maior risco à DP em relação a quem nunca aplicou (Dhillon et al., 2008; Tanner et al., 2011). Em ratos, a administração de rotenona desencadeou sintomas de bradicinesia, instabilidade postural e rigidez, com resposta à dopamina. Eles também apresentaram morte neuronal na *substantia nigra* e estriado (Nandipati & Litvan, 2016).

O paraquat é outro composto frequentemente associado a risco da DP. Ele é um dos herbicidas mais utilizados no mundo (Tanner et al., 2011). Sua estrutura é muito similar ao MPTP. A maioria dos estudos caso-controles mostra que o paraquat confere risco à DP, embora com resultados conflitantes (Elbaz et al., 2009; Tanner et al., 2011; Kamel et al., 2014).

Em contrapartida, existem dois fatores que mostraram consistentemente conferir proteção à DP: o café e o cigarro. Ambos parecem ser protetores de maneira dose-dependente (Wirdefeldt et al., 2011).

É importante ressaltar que fumar cigarro, consumir tabaco sem a fumaça ou expor-se à fumaça do tabaco no ambiente estão associados a um menor risco de DP (Ma et al., 2017). Duas meta-analises mostraram que indivíduos fumantes possuem um risco de DP 40 a 50% menor do que não fumantes (Hernán et al., 2002; Ritz et al., 2007). Além disso, um grande estudo de coorte prospectivo realizado por Hernán et al. (2001) mostrou a relação inversa dose-dependente entre fumar cigarro e risco de DP, com riscos relativos de 0,8, 0,6, 0,5, e 0,4, para 1-9, 10-24, 25-44, e 45 ou mais maços/ano, respectivamente.

A exposição à fumaça do cigarro foi associada a um risco de DP 64% menor comparado a não expostos, sendo que nos fumantes passivos, o risco era inversamente

proporcional aos anos expostos à fumaça (Nielsen et al., 2012). Entretanto, o efeito do cigarro não parece influenciar na progressão da DP (Golbe, 1986; Alves et al., 2004; Kandinov et al., 2007).

Uma das hipóteses que explica a razão de cigarros conferirem proteção à DP é de que há uma menor resposta à nicotina na fase prodrômica da DP. Com isso, pacientes parariam de fumar antes do início da DP. E o parar de fumar seria um aspecto da DP pré-clínico. Porém essa hipótese não explicaria o baixo risco dos que nunca fumaram quando comparados aos que já fumaram, encontrado em vários estudos (Ascherio & Schwarzschild, 2016). Outra hipótese é que o tabaco e seus derivados possuem efeitos neuroprotetores, mas ainda não há evidências na literatura de quais mecanismos confeririam essa neuroproteção (Ascherio & Schwarzschild, 2016; Delamarre & Meissner, 2017).

Assim como o cigarro, o café é um fator protetor para a DP. Bebidas cafeinadas, como chá, café e chimarrão, contêm uma mistura de várias substâncias, dentre elas, a cafeína é a mais estudada. Meta-análises mostraram uma relação linear inversa entre dose de cafeína e risco de DP (Costa et al., 2010; Qi & Li, 2014). Apesar de se pensar que o consumo de cafeína e tabaco são altamente correlacionados, o efeito protetor da cafeína é visto mesmo em indivíduos que nunca fumaram cigarro (Kieburtz & Wunderle, 2013; Ascherio & Schwarzschild, 2016).

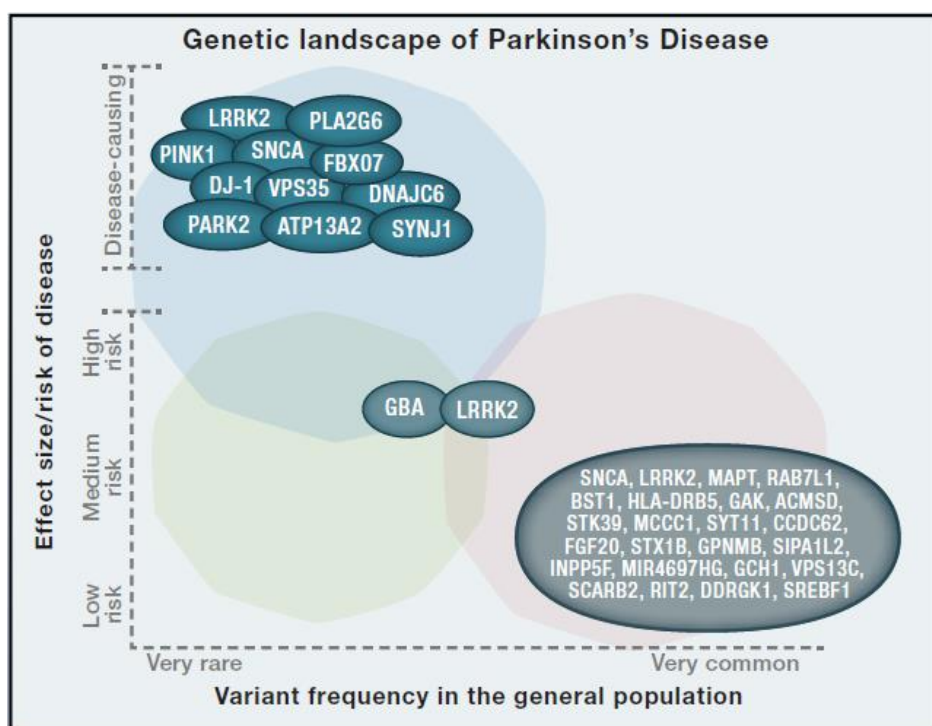
O efeito neuroprotetor na *substantia nigra* pode ser devido à inibição competitiva da cafeína e adenosina aos receptores de adenosina A2A neste local. Este processo de antagonismo que a cafeína produz parece diminuir a liberação de glutamato e atenua a citotoxicidade (Kolahdouzan & Hamadeh, 2017). Porém, os estudos ainda são incipientes, e não se sabe se a cafeína possa também atuar por outras vias.

### Fatores Genéticos

Apesar de as primeiras evidências de fatores ligados a DP sejam de origem ambiental, nos últimos vinte anos, variantes genéticas vem ganhando espaço no cenário de

suscetibilidade para a DP. A contribuição genética na DP parece abranger um amplo espectro (contínuo), onde a DP monogênica e a esporádica estariam em extremidades opostas, como pode ser visto na Figura 2. Mutações mendelianas estão ligadas à DP monogênica, que é caracterizada por um início mais precoce nos indivíduos. A DP monogênica é pouco frequente, abrangendo 5 a 10% dos casos de DP (Lill, 2016). Enquanto que à forma mais comum da doença (90% dos casos), é a DP esporádica. Essa forma está associada a variantes mais comuns da doença. Cada uma dessas variantes confere um efeito baixo no risco para desenvolver a DP. A presente tese detalha a seguir os principais genes desse espectro.

Figura 2 – Espectro de risco genético da DP




(Brás et al., 2015)

A primeira mutação ligada a DP, encontrada há mais de 20 anos, é de herança autossômica dominante, a Ala53Thr no gene da alfa-sinucleína (*SNCA*) (Polymeropoulos et al., 1996). Depois disso, mais quatro mutações já foram identificadas em estudos de ligação, entre elas, Ala30Pro, Glu46Lys, His50Gln e Gly51Asp (Trinh & Farrer, 2013). Mutações

nesse gene são raras e altamente penetrantes, apesar da alta variabilidade clínica observada nos pacientes portadores de mutação (Singleton et al., 2013). Duplicações e triplicações do *SNCA* também são frequentes na DP monogênica, chegando a 1 a 2 % dos casos, sendo descrita também em casos esporádicos da DP (Nuytemans et al., 2010; Singleton et al., 2013). Essas cópias a mais parecem adiantar o início da doença e agravar seus sintomas (Trinh & Farrer, 2013). Porém, alguns estudos mostraram que portadores dessas duplicações ou triplicações eram assintomáticos, portanto sua penetrância é reduzida. Biologicamente, a proteína SNCA mutante assim como a super-expressão do SNCA selvagem parecem ter uma maior propensão a não realizar um correto dobramento das mesmas. Esse processo gera o acúmulo de SNCA que se mostrou neurotóxico (Nuytemans et al., 2010).

Mutações no gene da quinase rica em leucina 2 (*LRRK2*) são as causas mais comuns da forma familial da DP, chegando até 10% dos casos de herança dominante e 2% nos casos esporádicos (Nuytemans et al., 2010; Spatola & Wider, 2014). *LRRK2* possui 51 exons e codifica uma proteína com duas subunidades enzimáticas, GTPase e quinase. Essa proteína está envolvida em vias celulares que regulam a formação e crescimento de dendritos neuronais, endocitose, autofagia e imunidade. Quase 80 mutações foram descritas, mas somente sete (Asn1437His, Arg1441Cys/Gly/His, Tyr1699Cys, Gly2019Ser e Ile2020Thr) parecem ser confirmadas como patogênicas a partir de suas análises de cosegregação e sua ausência em controles (Singleton et al., 2013). A mutação mais comum é a que determina uma substituição p.G2019S, descrita tanto em formas familiais (4 a 6,6% dos casos) quanto esporádicas da doença (1 a 4,2% dos casos) (Di Fonzo et al., 2005; Gilks et al., 2005; Healy et al., 2008; Cornejo-Olivas et al., 2017). No Brasil, esta mutação foi encontrada em 4,2% de casos com DP familial e 1,4% em casos esporádicos (Abreu et al., 2016; Cornejo-Olivas et al., 2017). Dessa forma, pode ser considerada uma mutação de médio efeito/risco para PD e de frequência não tão rara (Figura 2). Pacientes com esta

mutação foram indistinguíveis tanto na forma esporádica quanto na familiar em relação aos dados clínicos, apresentando boa resposta à levodopa (Healy et al., 2008).

Outros genes descritos na literatura foram identificados em menor número de casos. Seus efeitos na doença podem variar em relação aos sintomas, idade de início ou progressão da doença e as mutações causais podem ser recessivas ou dominantes (Tabela 1).

Tabela 1 – Genes de herança mendeliana ligados à doença de Parkinson.

| **Gene** | **Cromossomo** | **Função** | **Herança** |
|---|---|---|---|
| ***PARK2*/Parkin** | 6q25.2-q27 | • Mitocôndria<br>• Ubiquitinação<br>• Sinapses | AR |
| ***PINK1*** | 1p36 | • Mitocôndria | AR |
| ***DJ-1*** | 1p36.23 | • Sistema inflamatório<br>• Mitocôndria | AR |
| ***PLA2G6*** | 22q13 | • Mitocôndria | AR |
| ***FBXO7*** | 22q12.3 | • Ubiquitinação<br>• Mitocôndria | AR |
| ***VPS35*** | 16q12 | • Autofagia<br>• Lisossomo<br>• Endocitose | AD |
| ***ATP13A2*** | 1p36 | • Mitocôndria<br>• Autofagia<br>• Lisossomo | AR |
| ***DNAJC6*** | 1p31 | • Sinapses<br>• Endocitose | AR |
| ***SYNJ1*** | 21q22.2 | • Sinapses<br>• Endocitose | AR |

AD: autossômico dominante, AR: autossômico recessiva

A observação clínica de que pacientes com a doença de Gaucher e seus familiares possuíam sinais de parkinsonismo mais frequentemente do que o esperado levou a identificação de variantes genéticas no gene da glicocerebrosidade cerebral (*GBA*) em casos de DP (Lill, 2016). A GBA é uma enzima lisossomal responsável pela lise de glicocerebrosídeos em glicose e ceramida. Mutações em homozigoze na GBA são responsáveis pela principal doença de depósito lisossomal, a doença de Gaucher. Quase

300 mutações foram descritas em pacientes com a doença de Gaucher, porém as mais frequentes na população brasileira são N370S e L444P (Rozenberg et al., 2006; Sidransky et al., 2009; Siebert et al., 2012). A heterozigoze para essas mutações no *GBA* parecem ser um importante fator de risco para a DP. O risco acumulado para indivíduos portadores de mutações nesse gene foi estimado em 2,2% e 10,9% aos 65 anos e 85 anos, respectivamente (Rana et al., 2013). Em uma meta-análise de nove populações, verificou-se que as mutações N370S e L444P possuíam um *odds ratio* de 3,96 e 6,73 respectivamente para desenvolver a DP. Em uma amostra brasileira de São Paulo, com 65 indivíduos, 3% dos pacientes com DP apresentaram uma dessas duas mutações (Spitz et al., 2015). Os dados de mutações em *GBA* fazem com que este gene seja o fator de risco genético mais comum para a DP até o momento. Além disso, pacientes com DP que apresentam mutações no *GBA* parecem ter tremor, bradicinesia, rigidez em menor frequência, e alterações cognitivas em maior frequência (Sidransky et al., 2009;Alcalay et al., 2012; Spitz et al., 2015).

O gene da MAPT codifica a proteína tau que estabiliza microtúbulos, sendo essencial para o transporte axonal em neurônios (Garcia & Cleveland, 2001). Mutações nesse gene foram primeiramente encontradas em famílias com demência fronto-temporal com parkinsonismo ligado ao cromossomo 17 (FTD17) (Dumanchin et al., 1998; Hutton et al., 1998). Investigações sobre o papel de variantes genéticas no MAPT na DP foram motivadas pelas características compartilhadas entre a DP e outras doenças como doença de Alzheimer (Huang & Mucke, 2012), Paralisia Supranuclear Progressiva (Litvan et al., 1996) e degeneração cortico basal (Dickson et al., 2002). Todas essas doenças, também chamadas de taupatias, apresentam agregação intraneuronal de proteína tau hiperfosforilada. Dois haplótipos foram descritos nas populações caucasianas: H1 e H2. O H1 foi associado ao maior risco de DP (Golbe et al., 2001; Maraganore et al., 2001; Martin et al., 2001; Farrer et al., 2002). Interessantemente, indivíduos da população asiática são monomórficos para o

haplótipo H1, porém nenhuma associação a DP foi encontrada nessas populações (Satake et al., 2009).

Os estudos de associação genômicos (GWAS) forneceram uma nova dimensão na busca de variantes relacionadas às doenças comuns. Ao invés de se concentrar em genes candidatos, o genoma é rastreado sem qualquer preferência para regiões específicas, genes ou suas variantes, sendo descrito como uma abordagem "sem hipótese a *priori*" (Kitsios & Zintzaras, 2009). Os GWAS ofereceram a oportunidade de superar dificuldades e obstáculos impostos pela compreensão incompleta da fisiopatologia e etiologia da DP e de outras doenças. Em 2014, Nalls et al. geraram a maior meta-analise de dados genômicos da DP. Vinte e oito loci independentes foram associados à DP. Dentre eles, puderam-se confirmar genes que mesmo antes da “era GWAS” já tinham sido associados à DP, como SNCA, LRRK2, GBA e MAPT. Já os genes MCCC1, TMEM175 e RIT2 foram identificados como novos genes associados à DP. Polimorfismos nesses genes foram fortemente associados com DP nessa análise. Embora os estudos de GWAS tenham ajudado a identificar e estabelecer variantes de risco para a DP, as funções dos genes identificados permanecem não esclarecidas.

**Interação gene-ambiente**

Visto que a maioria dos casos de DP é esporádica e os fatores genéticos explicam pouco do risco para a doença, estudos de interação gene-ambiente começaram a ser propostos na busca de explicações para sua patogênese. Genes que afetam a absorção, metabolismo e excreção dos xenobióticos foram os mais estudados (Tsuboi, 2012; Ritz et al., 2016). Apesar do número considerável de estudo de interação gene-ambiente na DP disponíveis na literatura, eles ainda são muito controversos, heterogêneos e possuem pequeno tamanho amostral.

A família das enzimas do citocromo P450 é conhecida por metabolizar uma gama de xenobióticos. O CYP2D6 é um exemplo de enzima responsável por metabolizar xenobióticos no fígado, incluindo pesticidas. A interação do gene do CYP2D6 com a exposição a pesticidas mostrou um risco maior em pacientes com DP portadores de alelos de baixa metabolização (Deng et al., 2004; Elbaz et al., 2004). O CYP1A1 é um dos maiores metabolizadores de hidrocarbonetos aromáticos policíclicos (HAPs) como o benzopireno presente na fumaça do cigarro e no café (Kim et al., 1998). O CYP1A2 participa da metabolização de vários pró-carcinogênicos derivados da fumaça do cigarro e de algumas comidas. É responsável por metabolizar aproximadamente 95% da cafeina ingerida (Butler et al., 1989; Matthaei et al., 2016). Três meta-análises de GWAS apontaram regiões nos genes *CYP1A1* e *CYP1A2* associadas a um maior consumo de café (Cornelis et al. 2011; Sulem et al. 2011; Amin et al. 2012). Na DP, o efeito da interação de variantes no gene *CYP1A2* e da cafeína ainda é controverso. Um estudo mostrou uma interação *CYP1A2* e cafeína (Popat et al., 2011), enquanto que outros três estudos não observaram essa interação (Tan et al. 2007; Facheris et al. 2008; Hill-Burns et al. 2011). Apesar de ser um forte gene-candidato, não foram descritos na literatura estudos de interação gene-ambiente envolvendo o *CYP1A1* na DP.

O CYP1B1 está envolvido na ativação dos componentes da fumaça do cigarro, como os HAPs. Sua expressão é induzida com ativação de HAPs por meio dos receptores de hidrocarbonetos aromáticos (Christou et al., 1995). Uma variante nesse gene, CYP1B1*3 (rs1056836), foi associado a maior atividade da enzima (Shimada et al., 1999). O único estudo que avaliou a interação CYP1B1 e tabaco não encontrou efeito de variante CYP1B1*3 na DP (De Palma et al., 2010).

A enzima da monoamina oxidase B (MAOB) é responsável pela degradação da dopamina na fenda sináptica (Riederer et al., 1989). A evidência de que fumantes tinham 40% menor atividade da MAOB do que não fumantes (Fowler et al., 1996), levou a uma série de estudos de interação relacionados à DP. A primeira investigação relatou uma

interação entre o polimorfismo rs1799836 e fumo na suscetibilidade a doença (Checkoway et al., 1998). Depois, se constatou uma diferença de gênero na interação do fumo e esse polimorfismo da *MAOB* (Kelada et al., 2002). Contudo, outros quatro estudos não apoiam esses achados (Hernán et al., 2001; Tan et al., 2003; De Palma et al., 2010; Gu et al., 2010).

A glicoproteína-P (ABCB1), também conhecida como proteína de multirresistência a drogas (MDR1), é um transportador de várias substâncias para dentro e fora da célula e através da barreira hematoencefálica, com mais de uma centena de substâncias descritas (Amin, 2013). Dentre elas estão os compostos derivados do cigarro e pesticidas. Um haplótipo derivado dos polimorfismos rs1045642 e rs2032582 do *ABCB1* está associado a menor expressão da proteína (Hitzl et al., 2004). Os dois polimorfismos parecem ser modificadores do risco de DP em caso de exposição a pesticidas organoclorados (Dutheil et al., 2010; Narayan et al., 2015). Também foi constatada uma interação do polimorfismo rs1045642 e consumo de bebida alcoólica na DP (Kiyohara et al., 2013).

A paraoxonase 1 (PON1) participa da detoxificação de pesticidas no soro. Essa enzima hidroliza o composto oxon dos organofosfatos, presente em vários pesticidas (Gan et al., 1991; Androutsopoulos et al., 2011). O gene da PON1 foi o mais investigado nos estudos de interação com pesticidas. De uma forma geral os resultados são controversos e não conclusivos (Ritz et al., 2016). Estudos funcionais revelaram que duas variantes L55M e Q192R determinam menor metabolização dos substratos nos homozigotos “MM” ou “QQ’’ (Garin et al., 1997; Li et al., 2000; O’Leary et al., 2005). Um estudo de exposição, definido pelo local de residência, aos organofosfatos diazinon, clorpirifos e paration foi conduzido na Califórnia, EUA, e descreveu que indivíduos expostos aos organofosfatos com genótipos de metabolizadores rápidos da PON1 mostraram um risco menor de DP quando comparados aos metabolizadores lentos (Lee et al., 2013). Porém, outros dois estudos não observaram o efeito dessas variantes no risco para DP em pacientes com exposição ocupacional a pesticidas (Fong et al., 2005; Dick et al., 2007).

Alguns pesticidas específicos, pertencentes às classes ditiocarbamato, imidazol, dicarboximida, e organoclorados que possuem a propriedade de inibir a aldeído desidrogenase (ALDH) também foram investigados. Um estudo com o gene da ALDH (ALDH2) mostrou um aumento no risco de DP de 2 a 5 vezes em pacientes portadores do alelo C do polimorfismo rs737280 e com exposição a essas classes de pesticidas (Fitzmaurice et al., 2014).

A NOS1 codifica a enzima óxido nítrico sintetase, que produz o óxido nítrico a partir do aminoácido L-arginina. No cérebro, o óxido nítrico possui propriedades de neurotransmissor e está associado às doenças neurodegenerativas (Echeverri et al., 2010). O uso de café parece alterar os níveis de óxido nítrico na circulação. Um estudo *in vitro* mostrou que metabólitos do café aumentam a atividade da NOS1 de maneira dose-dependente (Huang et al., 2004). Além disso, foi sugerido que a NOS1 e pesticidas poderiam agir sinergisticamente para aumentar o risco de DP, uma vez que ambos contribuem para o dano neuronal (Dauer & Przedborski, 2003; Paul et al., 2015). O uso de pesticidas em associação com três polimorfismos da NOS1 foi observado em famílias com casos de DP. Paul et al. (2015) na tentativa de reproduzir esse achado, relataram a interação entre pesticidas e o polimorfismo rs2682826 da NOS1 associado a casos de DP esporádica, porém na direção oposta ao previamente publicado.

Estudos de associação ao nível genômico, com interação com o ambiente ainda são pouco realizados. Há dois estudos de GWAIS (genome-wide association and interaction study) que evidenciaram genes que não tinham sido relacionados à DP. Um de interação com a cafeína obteve como maior sinal um polimorfismo no gene *GRIN2A* (rs4998386) em associação a DP. Portadores do alelo T possuem menor risco de DP comparados ao genótipo CC (Hamza et al., 2011). E o outro de interação com o fumo identificou o gene do SV2C, que codifica a proteína da vesícula sináptica 2C (Hill-Burns et al., 2013). Esse achado foi corroborado em uma analise de expressão gênica em *Drosophila melanogaster* utilizadas como modelo para DP. Quando as moscas eram tratadas com nicotina, o efeito

mais benéfico foi proporcionado por um gene análogo ao *SV2C* nesses animais. Pouco se sabia sobre o SV2C até o GWAIS publicado por Hill-Burns et al. (2013), somente que era altamente expresso na *substantia nigra* e que mediava a entrada de neurotoxinas como a neurotoxina botulínica A nos neurônios (Mahrhold et al., 2006; Dardou et al., 2011).

**Tratamento farmacológico**

A degeneração dos neurônios dopaminérgicos da SNpc provoca um déficit de dopamina no estriado, e esse fenômeno é o responsável pelos sintomas motores da DP. O tratamento da DP mais efetivo é pela reposição farmacológica do neurotransmissor dopamina, a partir de seu precursor, a levodopa. A levodopa é a principal medicação para o controle dos sintomas motores. Ela é administrada oralmente, sendo absorvida no duodeno, passa a barreira hematoencefálica e é convertida a dopamina nos neurônios da *substantia nigra*. Os efeitos adversos agudos da levodopa são náusea, vômitos e hipotensão postural, e se devem a sua conversão periférica para dopamina, através da enzima dopa-decarboxilase. Por essa razão, a levodopa é administrada juntamente com inibidores dessa enzima, para impedir a degradação periférica da droga e reduzir a intolerância gastrointestinal, assim permitindo a entrada da levodopa em maior concentração no cérebro (Kostrzewa et al., 2005). A levodopa é disponível em várias formulações e o manejo da medicação é feito de forma a obter menos efeitos adversos e a melhor resposta motora do paciente.

No início do tratamento essa medicação se mostra extremamente eficaz, reduzindo os sintomas da doença. Entretanto, o uso continuado da levodopa pode provocar o surgimento de fenômenos indesejados, como a flutuação motora, a discinesia e a alucinação. Foi estimado que aproximadamente 35% e 40% dos pacientes irão desenvolver flutuações motoras e discinesias, respectivamente, em 4 a 6 anos de tratamento dopaminérgico (Connolly & Lang, 2014). A flutuação motora é uma alternância da resposta

clínica da levodopa. O paciente no estado “on” apresenta boa resposta à medicação, com controle do tremor, da rigidez e da bradicinesia. No estado “off” ele não apresenta efeito da medicação. Esses estados podem ser imprevisíveis e iniciar de maneira abrupta nas flutuações motoras (Fox & Lang, 2008). A discinesia é complicação do estado "on", mais precisamente quando há um pico de dose, caracterizada por movimentos involuntários anormais e excessivos, podendo afetar qualquer parte do corpo (Sorbo & Albanese, 2008). Essas complicações motoras alteram a qualidade de vida do paciente com DP, aumentam os custos do tratamento e são um desafio ao médico assistente, uma vez que deverá utilizar outras medicações ou esquemas posológicos mais complexos (Oertel & Schulz, 2016).

O tratamento a partir de outros medicamentos como agonistas dopaminérgicos, inibidores da MAOB e da catecol o-metiltransferase (COMT), amantadina e anticolinergicos, administrados sozinhos ou em conjunto com a levodopa, também é realizado.

Os agonistas dopaminérgicos atuam diretamente no neurônio pós-sináptico no estriado. Dez agonistas dopaminérgicos estão disponíveis para tratamento da DP, sendo cinco derivados do ergot (bromocriptina, cabergoline, lisurida e pergolida) e cinco não derivados do ergot (piribedil, pramipexole, ropinirol, apomorfina e rotigotina). Normalmente são recomendados no início do tratamento como uma monoterapia ou conjuntamente com a levodopa para minimizar flutuações motoras (Olanow et al. 2009; Oertel & Schulz, 2016).

Inibidores de enzimas que degradam a dopamina, a MAOB e a COMT, prolongam o efeito da dopamina na fenda sináptica. Inibidores da MAOB podem ser usados no início da doença com monoterapia ou em conjunto com a levodopa para manejo das complicações motoras. Já os inibidores da COMT são mais utilizados quando há flutuações motoras (Factor, 2008; Olanow et al., 2009).

A amantadina é geralmente indicada em fases iniciais de casos de DP com sintomas leves em monoterapia e para reduzir discinesias em fases mais tardias. Eventualmente pode causar efeitos adversos como delírio, alterações cognitivas, agitação psicomotora e

mioclonias. Seu mecanismo de ação ainda não é bem conhecido, mas parece ter efeitos anticolinérgicos, bloqueio da recaptação dopaminérgica, aumento da liberação de dopamina e estímulo dos receptores dopaminérgicos (Hubsher et al., 2012).

Os anticolinérgicos são os medicamentos mais antigos para tratar DP. O uso destes foi muito reduzido depois da descoberta da levodopa. Ocasionalmente esses medicamentos são utilizados em pacientes mais jovens, os quais apresentem o tremor como principal sintoma e que não tenham algum déficit cognitivo. Os anticolinérgicos são de uso limitado, principalmente nos pacientes mais idosos, devido a seus efeitos adversos: perda de memória, confusão e alucinação (Olanow et al., 2009).

## Farmacogenética da doença de Parkinson

Embora muitos medicamentos sejam utilizados no tratamento da DP, nenhum deles parece diminuir ou parar a neurodegeneração ou a progressão dos sintomas. À medida que a DP progride, a eficácia dos fármacos vai diminuindo, necessitando de doses mais altas ou mudanças no esquema posológico (Payami, 2017). A resposta individual a levodopa e outros medicamentos antiparkinsonianos é muito variável, podendo ser em parte explicada por fatores genéticos. Nesse contexto, estudos de farmacogenética começaram a ser realizados, visando ao tratamento personalizado de acordo com o perfil genético individual. A maioria dos estudos de farmacogenética da DP é direcionada aos efeitos adversos da levodopa: discinesias, alucinações e flutuações motoras. Apenas 13% dos estudos focam na dose ideal de levodopa, e uma minoria foca em outros medicamentos antiparkinsonianos (Schumacher-Schuh et al., 2014a). Nesse tópico, serão abordados apenas os principais estudos farmacogenéticos relacionados à dose de levodopa já que esse foi o objetivo da presente Tese.

Lee et al. (2001), Contin et al. (2005) e Moreau et al. (2015) realizaram desafio oral agudo de levodopa nos pacientes, e não encontraram diferença na resposta motora em relação aos genótipos do polimorfismo Val158Met da COMT. Bialecka et al. (2004) constataram uma associação do genótipo Met/Met do mesmo polimorfismo com doses menores de levodopa nos primeiros cinco anos da doença. O mesmo grupo também encontrou um haplótipo de quatro polimorfismos da COMT, cujos alelos possuem maior atividade enzimática, associado a maiores doses de levodopa (Bialecka et al., 2008). Já Cheshire et al. (2013) observaram em sua amostra que somente genótipos de alta atividade da COMT em conjunto com genótipo de alta atividade da monoamina oxidase A (MAOA) estavam associados a maiores doses equivalentes de levodopa.

A levodopa parece ser substrato dos transportadores de cátions orgânicos (OCT1), envolvidos em transportar compostos endógenos como a dopamina e outros medicamentos antiparkinsonianos no intestino, fígado e cérebro (pramipexol e amantadina). O alelo C do polimorfismo rs622342 no gene do OCT1 foi associado a menores doses de fármacos antiparkinsonianos em geral (Becker et al., 2011).

A enzima dopa-decarboxilase também foi alvo de estudo de farmacogenética, uma vez que possui um importante papel, degradando a dopamina nas vias periféricas. Devos et al. (2014) avaliou a resposta motora em relação ao desafio oral de uma dose única de levodopa com benzerazida (inibidor da dopa-decarboxilase) em 39 pacientes genotipados para dois polimorfismos do gene da dopa-decarboxilase (*DDC*). A resposta motora foi inferior em indivíduos que possuíam o alelo C do rs921451 ou eram portadores de uma deleção no polimorfismo rs3837091. Esse achado não foi corroborado pelo estudo do desafio oral de levodopa/benzerazida de Moreau et al. (2015), que avaliou os mesmos polimorfismos rs921451 e rs3837091, mas não encontrou diferença na resposta motora dos pacientes.

O transportador de dopamina possui ação central na ação deste neurotransmissor por ser responsável pela sua recaptação pré-sináptica, regulando temporal e espacialmente a quantidade de dopamina na fenda sináptica. O gene desse transportador (*DAT1* ou *SLC6A3*) possui dois polimorfismos associados à eficácia da medicação dopaminérgica (rs3836790 e rs28363170) (Schumacher-Schuh et al., 2013; Moreau et al., 2015). No estudo de Schumacher-Schuh et al., o alelo de 9 repetições do rs28363170 estava associado a menores doses equivalentes de levodopa. Enquanto que no estudo de Moreau et al., melhor resposta motora após desafio oral de levodopa foi vista com o haplótipo de 6 e 10 repetições dos polimorfismos rs3836790 e rs28363170 respectivamente. Portanto, esse estudo sugere que os pacientes com genótipos 6/6 do rs3836790 ou 10/10 do rs28363170 exigiriam menores doses de levodopa e/ou teriam maior intervalo entre as doses.

O entacapone é um dos potentes inibidores da COMT. O efeito do polimorfismo Val158Met da *COMT* sobre o entacapone é controverso na literatura. Corvol et al. (2011) realizaram um ensaio clínico para observar o efeito da administração de levodopa em conjunto com entacapone ou placebo. Pacientes com genótipo Val/Val possuíram uma resposta motora mais duradoura, com mais tempo “on”. Já dois outros estudos realizaram ensaios parecidos, com mesmo tempo de administração de entacapone, e detectaram um maior tempo “on” independente dos genótipos da *COMT* (Lee et al., 2002; Seon et al., 2011).

Estudos com o pramipexol, um agonista dopaminérgico, mostraram que a resposta motora é dependente do polimorfismo Ser9Gli no gene DRD3. Liu et al. (2009) observaram que pacientes Ser/Ser tratados prospectivamente por dois meses com pramipexol, possuíam melhores taxas de resposta ao medicamento que pacientes portadores do alelo Gli. Em concordância, Xu et al. (2017), descreveram que pacientes com genótipos Ser/Ser e Ser/Gli utilizavam menores doses de pramipexol do que pacientes com genótipo Gli/Gli utilizando uma amostra maior do que a do estudo anterior. O polimorfismo Ser9Gli do DRD3 parece afetar a função/estrutura da proteína. A serina na posição 9 da proteína é a

responsável por uma maior afinidade à dopamina (Jeanneteau et al., 2006). Esse polimorfismo não parece afetar a dose de piribedil, outro agonista dopaminérgico (Xu et al., 2017).

# CAPÍTULO II

## *Justificativa e objetivos*

A primeira descrição da DP foi feita há dois séculos, mas a sua conceptualização continua evoluindo através dos anos. A DP é uma doença de alta prevalência, sem cura, com significativos prejuízos motores e não-motores para o paciente. Sua etiologia é complexa, e provavelmente resulta de interações do perfil genético do indivíduo e do ambiente. O estabelecimento do efeito das relações ambientais e genéticas tem se mostrado muito complicado para a maioria das doenças, e a DP não é exceção. Embora muito se discuta a interação de fatores genéticos e ambientais na etiologia da DP, poucos estudos investigaram essas interações até o presente.

Estudos de varredura genômica indicaram uma nova gama de possibilidades promissoras para estudos de suscetibilidade à DP. Esses estudos evidenciaram que os "genes mendelianos" parecem também estar envolvidos na etiologia da DP esporádica revelando um contínuo na causa da DP e não um quadro dicotômico entre os casos determinados por um único gene e aqueles dependentes de múltiplos fatores para se manifestar. Além disso, novos genes ganharam destaque com essa abordagem.

É importante determinar a contribuição conjunta de variações nesses genes na suscetibilidade a DP. O estudo de variantes genéticas ligadas a risco ou proteção à DP pode ser muito útil na determinação de possíveis biomarcadores para a doença. Dessa forma, o diagnóstico anterior aos sintomas podem melhorar qualidade de vida e o prognóstico dos pacientes com a doença e possivelmente levar a regressão da neurodegeneração. Além disso, a descoberta de fatores genéticos ou ambientais em associação/interação com a DP possibilita melhor entendimento da patogênese doença.

Outra perspectiva é a de entender o papel de variantes genéticas na dose da principal medicação da DP. A terapia dopaminérgica, baseada principalmente no uso de levodopa, gera varias complicações, efeitos adversos, que prejudicam muito a qualidade de vida dos pacientes. Determinar a terapia ideal individual é um importante passo para gerar menos efeitos tóxicos e aumentar a eficiência dos medicamentos, gerando menos efeitos

adversos. Essa análise possibilitaria um melhor prognóstico aos pacientes e menores custos para os sistemas de saúde.

Os resultados publicados até o presente não foram suficientes para determinar a base genética da DP nem esclarecer a variabilidade na dose de levodopa tomada pelos pacientes. Portanto os objetivos dessa tese visaram melhor compreensão dos fatores genéticos envolvidos na suscetibilidade à DP e no tratamento com levodopa.

Objetivos específicos

- Investigar se há interação de genes candidatos e fatores ambientais determinando risco para a doença de Parkinson.
- Investigar o efeito conjunto de variantes genéticas identificadas em GWAS na suscetibilidade à DP.
- Avaliar efeitos de variantes genéticas na dose de levodopa tomada pelos pacientes com doença de Parkinson.

# CAPÍTULO III

## *The additive effect of eight gene polymorphisms on Parkinson's disease susceptibility in a Brazilian cohort*

Manuscrito em preparação

**The additive effect of eight gene polymorphisms on Parkinson's disease susceptibility in Brazilian patients**

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**Abstract**

Parkinson's disease (PD) genetics is complex with Mendelian and non-Mendelian contribution factors. Most PD cases are considered sporadic and do not have a determined cause. Several genetic risk variants with small effects have been associated with PD so far. The aim of this study was to investigate if there are additive effects of the main genetic variants identified on PD risk. A total of 230 PD patients and 199 controls were included in this study. Eight polymorphisms were used for risk score calculations. The number of risk alleles in the total sample ranged from 2 to 10. O*dds ratio* for PD, considering one risk allele was 1.38 (95% CI 1.19-1.61; P = 3.50E−05). After controlling for age, gender, ancestry and smoking, individuals with 7 or more risk alleles presented a 2.54 higher risk for PD than those with 6 or less alleles (95% CI 1.66-3.89; P = 1.80E−05). This PD risk score model provided an AUC of 74.4%. The current study showed that evaluating risk with eight associated PD genetic variants was informative and might be useful for PD screening processes.

## Introduction

Substantial progress has been made in understanding Parkinson's disease (PD) genetics. In the last twenty years, several disease-causing mutations have been identified in families with PD. The first mutation elucidated was in the α-synuclein gene (*SNCA*)[1]. Later, other Mendelian inherited mutations were discovered in genes such as *LRRK2*, *VPS35*, *CHCHD2*, *EIF4G1*, *PARK2*, *PINK1* and *DJ1*[2]. Although PD monogenic forms are rare and altogether represent about 10% of all PD cases, they helped to improve knowledge about the pathogenesis of this complex disease[3].

Approximately 90% of PD cases are sporadic and do not have a defined genetic cause. Genome-wide association studies (GWAS) have identified multiple genetic variants associated with PD. A large GWAS meta-analysis resulted in 28 loci associated with this disease ($P < 5 \times 10^{-8}$), 22 of them were replicated in the same study[4]. As expected, an overlap of genes mutated in PD families with those genes previously detected in GWAS studies was observed. Mutations in genes such as *GBA* and *MAPT*, widely explored before GWAS studies, were also confirmed as PD risk factors. Moreover, some PD associated genes such as *MCCC1*, *RIT2* and *TMEM175* were novel and disclosed by GWAS.

As in most multifactorial disorders, these variants derived from GWAS confer small effects, but altogether they might represent an important risk for PD. In light of that, the aim of this study was to investigate additive effects of the main genetic variants identified by GWAS studies on PD risk.

## Results

The characteristics of the samples investigated are summarized in Table 1. Males were more frequent in PD patients (51.7%) than in controls (36.2%) (OR1.89; 95% CI 1.28-2.78; $P = 0.001$). As in previous investigations smoking cigarettes was associated with lower PD risk (OR 0.50; 95% CI 0.33 – 0.74; $P = 0.001$). Individuals who ever smoked were more frequent in controls (48.7%) than in PD patients (32.2%). Genotype and allele frequencies did not differ between ethnic groups ($P > 0.05$).

An allele was considered a risk allele if it was identified previously in GWAS or other studies as associated with PD. LRRK2 G2019S (rs34637584) was found in PD patients only (3 patients,

frequency in PD sample = 1.3%), therefore were not used for risk scores estimations. Eight SNPs were used for further analyses: *SNCA* (rs356182), *MAPT* (rs9468), *LRRK2* (rs1175964 and rs34637584), *GBA* N370S (rs7676375), GBA L444P (rs421016), *MCCC1* (rs12637471), *TMEM175* (rs34311866) and *RIT2* (rs12456492). Considering that individuals could have one risk allele (heterozygous) or two risk alleles (homozygous), the highest potential number of risk alleles, with eight polymorphisms, is 16. The number of risk alleles in the total sample ranged from 2 to 10. The distribution of risk alleles from cases and controls is shown in Figure 1. In logistic regression, the *odds ratio* for PD, considering one risk allele was 1.38 (95% CI 1.19-1.61; P = 3.50E−05). Mean risk alleles in the total sample was 6.7, and the median was 7.0. Therefore, using ≥7 or <7 risk alleles as a criterion, subjects were grouped creating a binary variable of risk alleles. After controlling for age, gender, ancestry and smoking, individuals with 7 or more risk alleles presented a 2.54 higher risk for PD than those with 6 or less alleles (95% CI 1.66-3.89; P = 1.80E−05 (Table 2). To evaluate the predictive power of the models, ROC analysis was applied (Figure 2). The AUC estimated was 0.744 (Standard Error = 0.024; 95% CI 0.70 - 0.79; P<0.001). Cumulative risk assessed by age divided by ≥7 or <7 risk alleles is presented in Figure 3, controlled for gender, ancestry and smoking. As age advances, individuals with ≥7 risk alleles have a higher hazard to develop PD compared to individuals with <7 risk alleles ($\chi^2 = 13.06$; P = 0.005).

Even that no differences were observed between ethnic groups, the same analyses were performed with individuals with European ancestry only. The *odds ratio* for PD, considering one risk allele was: 1.38 (95% CI 1.17-1.63; P = 1.73E-04). As in the total sample, subjects could also be grouped considering ≥7 or <7 risk alleles, because the mean risk alleles in the sample was 6.7, and the median was 7.0. Binary logistic regression showed that individuals with 7 risk alleles or more presented an OR 2.66 (95% CI 1.66-4.27; P = 4.63E-05) (Supplementary Fig. S1). ROC curve presented an AUC = 0.746 (Standard Error = 0.026; 95% CI 0.70-0.80; P < 0.001) (Supplementary Fig. S2). The Cumulative risk estimation presented a similar graph as Figure 3 ($\chi^2 = 10.34$; P = 0.006) (Supplementary Fig. S3). Age, gender and smoking were considered covariates in all analyses.

## Discussion

In present case-control study, the combination of known PD risk alleles could predict risk for PD in our population. Alleles were associated in an additive or binary (more or less than 7 alleles) manner. A clinical perspective is the most expected result in human genetic studies. The discriminative power of a test or classifier is often expressed as the area under the curve (AUC). The AUC indicates whether a test is useful to identify individuals who are at increased risk of disease (screening; e.g., AUC 0.75–0.80) or to diagnose a disease before the onset of symptoms (presymptomatic diagnosis; AUC 0.99)[5]. In the present study, validity of prediction was evaluated by ROC curves along with AUC estimates. PD risk score considering genetics, age, gender, ancestry and smoking, provided an AUC of 74.4%. Although our results were close to screening ability, it could not be considered for diagnosis. Cumulative risk analysis enabled to see a differential genetic influence on individuals that had more than 7 risk alleles compared to individuals with 6 or less risk alleles. This difference remained significant when patients with European ancestry were selected.

PD GWAS meta-analyses studies compiled an enormous amount of data and then independently replicated the results in many thousands of patients and controls[4]. Reassuringly, meta-analyses are important because they confirmed previously known risk genes by gene-candidate and family-based approaches, but also revealed novel genes or loci associated with PD. Genes such as *SNCA*, *LRRK2*, *GBA* and *MAPT* were discovered to have a link with PD before GWAS studies. Their detection as risk factors in hypotheses-free approaches, gives the idea of biologically plausibility and credibility to these loci.

The results of GWAS are quite challenging, once variants with small effects are associated with the disease. From the 28 associated loci generated by Nalls et al.[4] GWAS, a genetic risk prediction analysis have yielded an AUC of 0.633. These investigators also calculated a genetic risk score with a less heterogenic cohort of 367 PD patients and 165 controls[6]. The genetic risk score was based on the 28 loci plus two rare variants from previous published research. In this model, considering age, family history, population substructure variables, and genetic risk score, the ability to predict PD had an AUC of 0.748 very similar to the figures obtained with 8 loci in the present study (AUC = 0.744)

Lill et al.[7] investigated PD genetic risk with 23 susceptibility loci on age at onset. Theses loci were derived from Nalls et al.[4] study as well. A genetic risk score was weighted by the sum of number of risk alleles by their β coefficient from GWAS meta-analysis. These investigators reported that their

model was significant only when *GBA* and *TMEM175* loci were included, otherwise the model was nonsignificant.

One of the main concerns of GWAS studies published is that the samples included are mainly from European populations. Nevertheless, the high risk variants are not presented in the same frequency in African, Amerindian or Asian populations. All individuals in the present study were sampled in South Brazil where a higher contribution of European ancestry and reduced Amerindian and African influences occur when compared to other Brazilian geographic regions[8,9]. Genotype and allelic frequencies did not differ between ethnic groups in this population, however to avoid hidden population stratification bias, a second analysis was conducted with individuals of European ancestry only. The results were very similar to those observed in the whole sample analysis.

The current study showed that evaluating risk with the key strongest associated PD genetic variants was informative and might be useful for PD screening processes. Cost-effective screening might not need all risk variants detected by GWAS studies, once the majority of them confer little risk for the disease. Further studies with larger samples are needed to provide a diagnostic and/or screening test to be applied in clinical practice.

**Materials and methods**

The study sample comprises 230 PD patients and 199 controls. PD patients were recruited at the Movement Disorder Clinics at Hospital de Clínicas de Porto Alegre, Brazil. Patients were examined as previously described[10], and diagnosed by use of UK Brain Bank criteria[11]. Patients with atypical manifestations, secondary parkinsonism or with familial history of the disease were excluded. Controls were sampled at the Geriatric Clinics from the same hospital, and at Universidade Federal de Ciências da Saúde de Porto Alegre. These individuals were recruited in the same city, to ensure they were from the same geographical area as cases. All individuals included in the control sample were not related to PD patients and did not have family history of PD or other neurological disorder. The hospital ethics committee approved the study, and all participants gave written informed consent.

All genes were selected based on the strongest association signals in PD GWAS meta-analyses[4]. Nine SNPs on those genes were selected based in a high minor allele frequency and/or strongest locus association with PD. *SNCA* (rs356182), *MAPT* (rs9468), *LRRK2* (rs1175964 and rs34637584), *GBA* (rs7676375), *MCCC1* (rs12637471), *TMEM175* (rs34311866) and *RIT2* (rs12456492) were genotyped using Taqman® SNP genotyping assays (Applied Biosystems, CA, USA), according to the manufacturer's recommended protocol. GBA L444P (rs421016) was determined by sequencing of a 670 pb region that belong exclusively to the GBA gene, excluding GBAP (pseudogene). Primers were designed with Primer3 software[12]. A polymerase chain reaction (PCR) reaction was run for 40 cycles: 94 ºC for 30 seconds, annealing for 30 seconds at 50 ºC and extension 72 ºC for 1 minute. After PCR, PCR products were purified and sequenced by Sanger methods (Macrogen, Korea).

Distribution normality was tested using the Kolmogorov-Smirnov test for quantitative variables.Differences in demographic characteristics between cases and controls were assessed using $\chi^2$-tests for qualitative variables, and Mann-Whitney test for quantitative variables. Differences of genotype and allele frequencies between ethnic groups were calculated by $\chi^2$-tests.

In order to observe the influence of each risk allele in PD, an additive model was created. In addition, a model was designed with binary variable divided by individuals who had more or less than 50% of risk alleles. Both models were generated by binary logistic regression. Covariates to be entered in models were defined based on conceptual analyses of the literature or if they were associated with study factor and outcome at $P \leq 0.10$.

ROC curve was calculated from the expected values obtained in binary logistic model. An equation derived from the binary logistic analysis was used for expected values estimation for each patient. Area under curve (AUC) estimates along with Wald confidence intervals for ROC curves were calculated as indicators of validity for each risk model.

Cox regression was selected for cumulative hazard analysis of risk variants controlled by covariates. Time to event (to have PD) was scaled in years. For all analysis, the significance level accepted was 0.05, all tests were two-tailed.

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**Author contributions**

V.A., A.F.S., C.R.M.R. and M.H.H. wrote manuscript and designed research. V.A., A.F.S., C.R.M.R., M.H.H., M.F. and E.H.M.acquired the data. Analyzes and interpretation of data were performed by V.A., S.M.C and M.H.H. All authors discussed the results and contributed in this manuscript.

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Table 1 – Characteristics of Parkinson's disease patients and controls.

| | PD patients N=230 | Controls N=199 | P | OR (95% CI) |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 119 (51.7%) | 72 (36.2%) | 0.001 | 1.89 (1.28 – 2.78) |
| Female | 111 (48.3%) | 127 (63.8%) | | |
| **Age** | 69.0 (±11.7) | 71.1 (±7.8) | 0.218 | - |
| **European descent** | 198 (86.1%) | 157 (78.9%) | 0.055 | 1.65 (0.99 – 2.74) |
| **Smoking cigarettes** | | | | |
| Ever smoker | 74 (32.2%) | 97 (48.7%) | 0.001 | 0.50 (0.33 – 0.74) |
| Never smoker | 156 (67.8%) | 102 (51.3%) | | |

PD, Parkinson's disease. Gender, ancestry and smoking cigarettes frequencies were compared by $\chi^2$-test. Mean age was compared by Mann-Whitney-test.

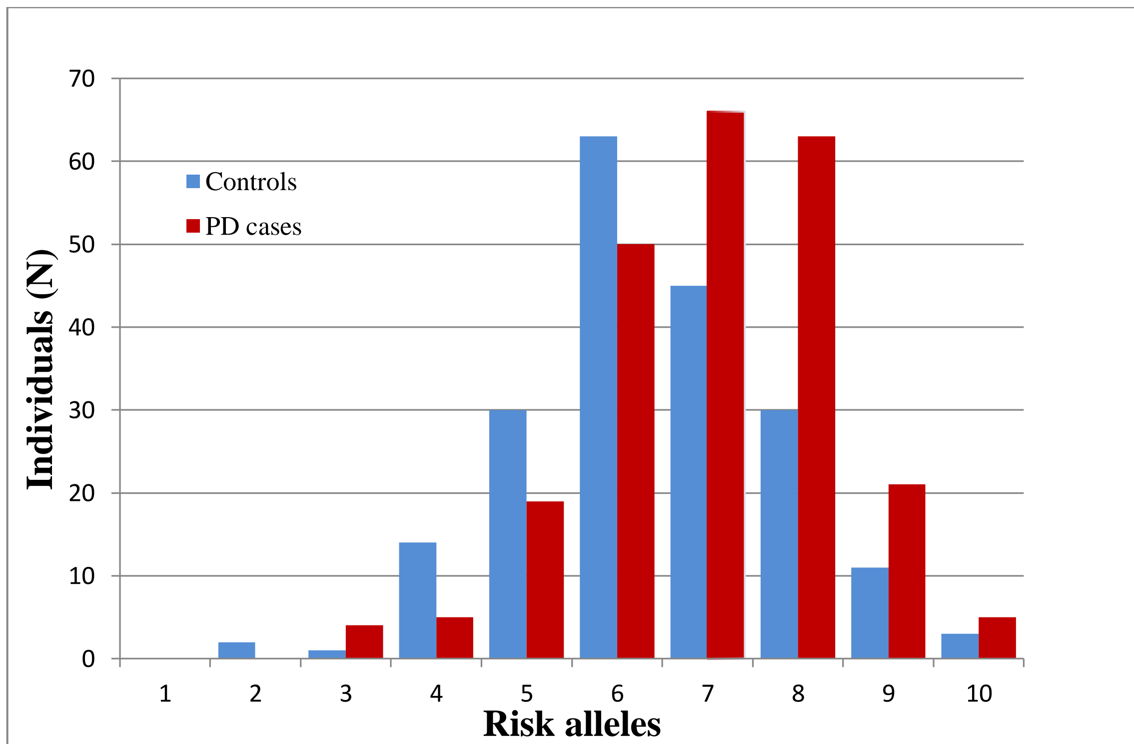


Figure 1 – Number of risk alleles in PD patients and controls

Table 2 – Binary logistic regression predicting PD risk

| | B | Standard Error | P | OR | 95% CI |
|---|---|---|---|---|---|
| Gender (male) | 1.12 | 0.240 | 2.78E−06 | 3.07 | 1.92-4.92 |
| European ancestry | 0.24 | 0.291 | 4.14E−01 | 1.27 | 0.72-2.24 |
| Age | -0.05 | 0.012 | 2.41E−06 | 0.95 | 0.93-0.97 |
| ≥7 risk variants | 0.93 | 0.217 | 1.80E−05 | 2.54 | 1.66-3.89 |
| Smoking | -1.06 | 0.243 | 1.20E−05 | 0.34 | 0.21-0.56 |
| Constant | 3.17 | 0.867 | 2.62E−04 | 23.70 | |

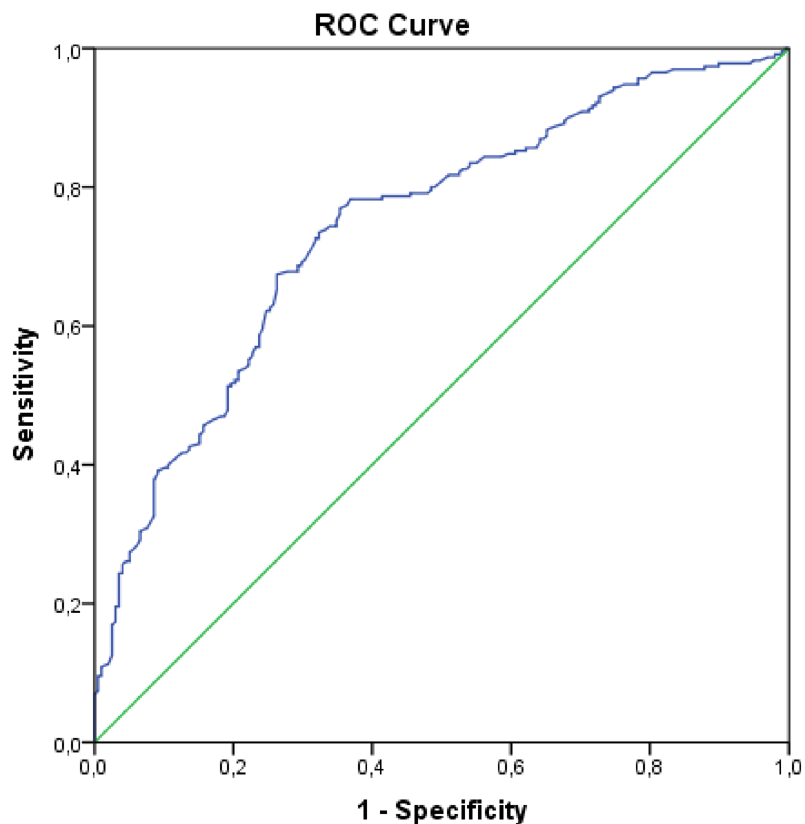


Figure 2 – ROC curve for prediction of PD was calculated with expected values obtained in binary logistic model shown in Table 2. AUC = 0.744 (Standard Error = 0.024; 95% CI 0.70-0.79; P < 0.001)

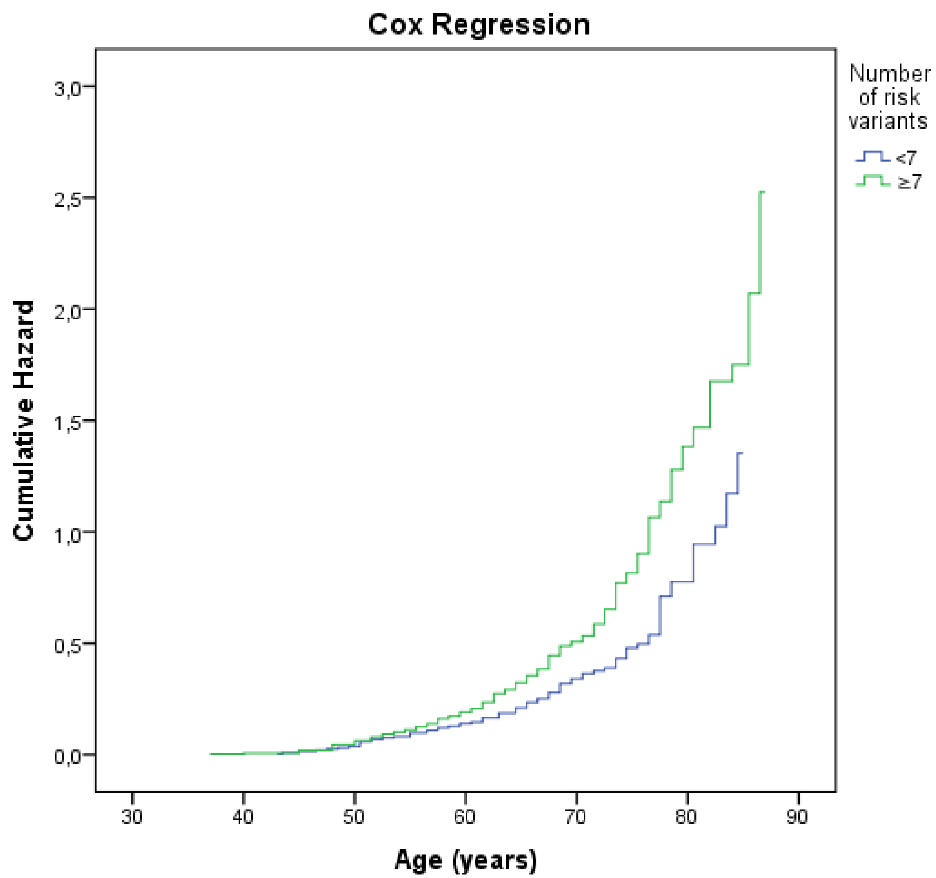


Figure 3 – Cumulative hazard for PD divided by more or less than 7 risk alleles. Cumulative hazard was calculated by Cox regression analysis controlled by age, gender, ancestry and smoking cigarettes. $\chi^2 = 13.06$; P = 0.005.

Supplementary material

Table S1 - Binary logistic regression predicting PD risk in individuals with European ancestry

| | B | Standard Error | P | OR | 95% CI |
|---|---|---|---|---|---|
| Gender (male) | 1.05 | 0.26 | 7.34E−05 | 2.87 | 1.70-4.83 |
| Age | -0.06 | 0.01 | 1.72E−06 | 0.94 | 0.92-0.96 |
| ≥7 risk variants | 0.98 | 0.24 | 4.63E−05 | 2.66 | 1.66-4.27 |
| Smoking | -1.09 | 0.27 | 5.60E−05 | 0.34 | 0.20-0.57 |
| Constant | 3.98 | 0.94 | 2.25E−05 | 53.56 | |

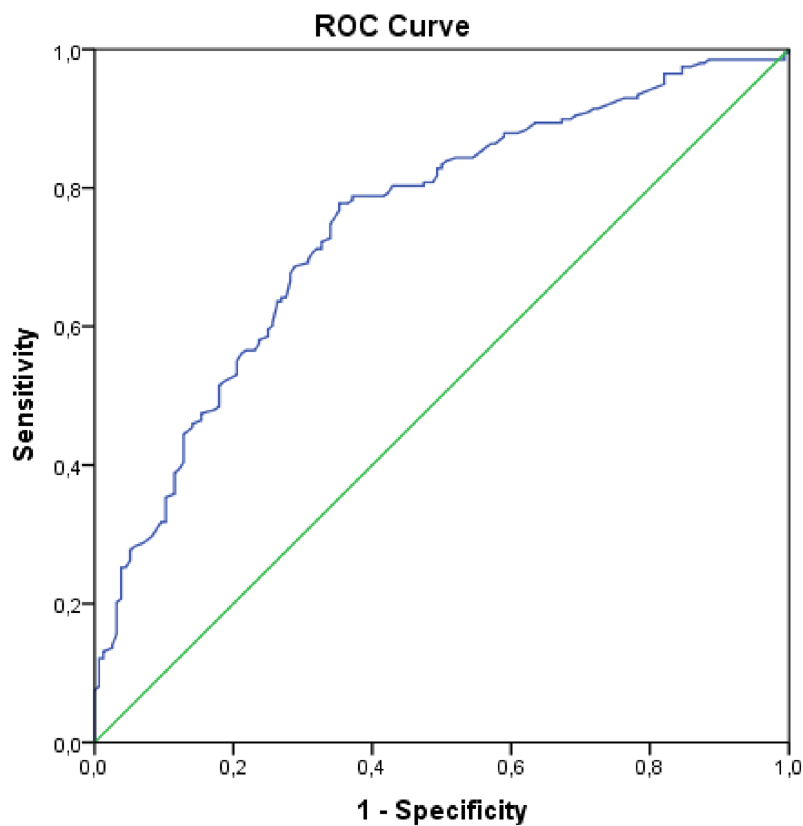


Figure S2 - Figure 2 – ROC curve for prediction of PD in individuals with European ancestry was calculated with expected values obtained in binary logistic model shown in Table S1. AUC = 0.746 (Standard Error = 0.026; 95% CI 0.70-0.80; P < 0.001).

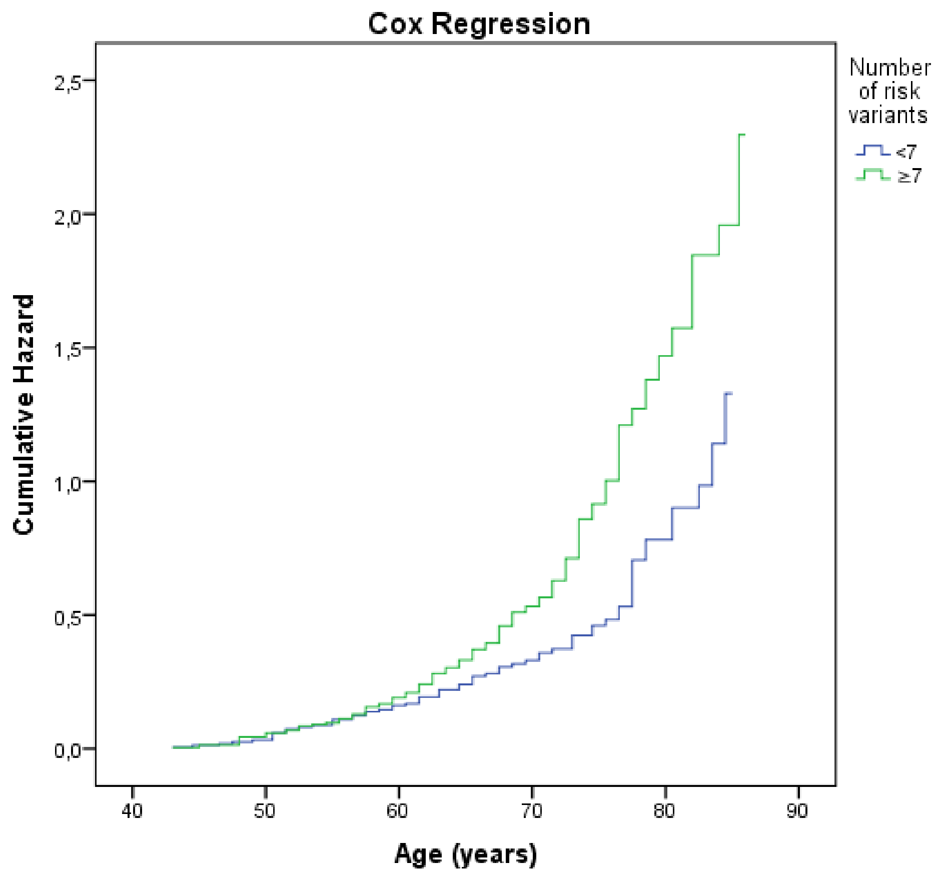


Figure S3 – Cumulative hazard for PD in individuals with European ancestry divided by more or less than 7 risk alleles. Cumulative hazard was calculated by Cox regression analysis controlled by age, gender and smoking cigarettes. $\chi^2 = 10.34$; P = 0.006

# CAPÍTULO IV

## *Occupational exposure and SV2C and PON1 interaction on idiopathic Parkinson's disease*

Artigo submetido à revista Neuromolecular medicine

**Occupational exposure and SV2C and PON1 interaction on idiopathic Parkinson's disease**

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**Abstract**

Genetic and environmental factors are likely involved in Parkinson's disease (PD) etiology. Exposure to pesticide chemicals, welding, industry and mining have been pointed as PD risk factors. The objective of this study was to investigate the role of gene-environment interactions regarding *SV2C* and *PON1* genes and toxic exposure on PD susceptibility. Parkinson's disease patients and controls were genotyped for *SV2C* and *PON1* polymorphisms by allelic discrimination assays. Occupational exposure to environmental agents was recorded from each patient. Interaction results were assessed by binary logistic regression, controlling for age, gender and ancestry. *SV2C* rs10214163 TT genotype carriers showed a higher PD risk in subjects exposed to environmental toxics compared to those not exposed (OR 2.53; 95% CI 1.33-4.69; $P_{interaction}$ = 0.008). *SV2C* rs30196 TT/GT genotypes carrier in interaction with toxics presented also a higher PD risk (OR 2.30; 95% CI 1.21-4.36) compared to individuals not exposed ($P_{interaction}$ = 0.033). *PON1* L55M was associated with higher risk of PD disease *per se* (OR 2.34; 95% CI 1.21-4.53; $P_{adjusted}$ = 0.012), but no interaction with toxics was observed. The results obtained in this study suggest that *SV2C* variability might have an important role in the identification of individuals at risk for PD development due to occupational exposure.

Keywords: PON1, SV2C, Parkinson's disease, environment, interaction, pesticide

## Introduction

Parkinson's disease (PD) is a common, progressive neurodegenerative disease, with increasing age being its major risk factor. The worldwide PD incidence rate for people with 40 years or older is 37.55 in females and 61.21 in males per 100,000 person-years (Hirsch and Steeves 2016). PD is characterized by symptoms of bradykinesia, tremor and rigidity. A selective death of dopaminergic neurons in *substantia nigra pars compacta* (SNpc) has long been considered as the main feature of the disease. Although, in PD neurodegeneration, a widespread involvement of other central nervous system (CNS) structures and peripheral tissues (Dauer and Przedborski 2003) have been observed, the causes associated with PD neurodegeneration are still not known. Over decades, several studies have been pointing to a mixed etiology, involving environmental and genetic factors.

The first environmental factor related to PD discovered was 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This compound is a neurotoxicant that selectively damages dopaminergic neurons. It has been shown that it increases reactive oxygen species through inhibition of mitochondrial complex I (Hare et al. 2013). Since this discovery, many epidemiological studies associated environmental toxicants to PD. Among them, pesticides are the most investigated. Occupational exposure to pesticides seems to increase the risk of PD in 1.7 to 5.6 times (Mostafalou and Abdollahi 2017). In addition to pesticides, environment toxicant exposure in occupations such as welding, mining and industry are beginning to be explored as PD-causing factors (Caudle et al. 2012; Tanner et al. 2014).

Human serum paraoxanase (PON1) participates in pesticides detoxification in serum. This enzyme hydrolyzes organophosphate metabolites, which is the basis of many pesticides (Gan et al. 1991). PON1 activity was reported to be significantly reduced in male and female PD patients (Ikeda et al. 2011). Two common variants in *PON1* that alters enzyme function were reported. The leucine (L) to methionine (M) substitution at codon 55 and a glutamine (Q) to arginine (R) substitution at codon 192 reduce PON1 metabolizing capacity (Garin et al. 1997; Li et al. 2000).

SV2C is a synaptic vesicular protein that is expressed mainly in SNpc and in the striatum (Dardou et al. 2011; Janz and Südhof 1999). PD animals models treated with MPTP presented an increase in SV2C mRNA expression (Dardou et al. 2013). The function of this protein is not well elucidated, but it seems to mediate the entry of toxic substances into neurons, as the botulinum neurotoxin A, leading to

its toxic effect (Mahrhold et al. 2006). A recent investigation showed that SV2C is a mediator of dopamine homeostasis and could be involved in PD pathogenesis (Dunn et al. 2017). Hill-Burns et al. (2013) reported an interaction among rs30196 and rs10214163 and smoking conferring risk to PD. Whereas rs30196 was also associated with variation in levodopa dose in PD patients (Altmann et al. 2016).

Epidemiological studies point to a variety of environment factors linked to PD. Gene-environment interactions in biological pathways as risk modifiers might be important for Parkinson's disease susceptibility. Therefore, the objective of this study was to investigate the role of gene-environment interactions regarding *SV2C* and *PON1* genes and environmental factors on PD susceptibility.

## Materials and Methods

### Subjects

The investigated sample included 183 sporadic cases of PD recruited at the Movement Disorder Clinics at Hospital de Clínicas de Porto Alegre, Brazil. Patients were examined as previously described (Rieck et al. 2012), and diagnosed based on UK Brain Bank criteria (Hughes et al. 1992). Patients with atypical manifestations, secondary parkinsonism or with familial history of the disease were excluded.

A total of 162 controls were recruited at the Geriatric Clinics from the same hospital to ensure they were from the same geographical area as cases. All individuals included in this sample were not related to PD patients and did not have family history of PD or other neurological disorder.

A questionnaire with environmental questions was developed in Portuguese and applied to all patients and controls. This questionnaire involved questions about dietary habits as alcohol and caffeine consumption, cigarette smoking, and use of drugs (cannabis, cocaine, crack, LSD, heroin). It also included occupational exposure to environmental toxics (agriculture, mining, industry and welding), pesticides (including all types of pesticides: herbicides, fungicide, insecticide, rodenticide and fumigants) and heavy metals. Exposure to environmental toxics and pesticides was considered for this

study as ever or never exposed. The hospital ethics committee approved the study. Informed consent was obtained from all individual participants included in the study.

Genotyping

Peripheral blood samples were collected from all patients and controls. DNA was extracted by standard procedures (Lahiri and Nurnberger 1991). *PON1* (rs854560 and rs662) and *SV2C* (rs10214163 and rs30196) were genotyped using Taqman® SNP genotyping assays (Applied Biosystems, CA, USA), according to the manufacturer's recommended protocol.

Statistical Analysis

Allele and genotype frequencies were estimated by gene counting. Deviation from Hardy–Weinberg equilibrium was assessed by $\chi^2$-tests. *SV2C* genotypes were grouped pooling the minor genotype with the heterozygous genotype. *PON1* genotypes were grouped based on functional studies. MM (rs854560 or L55M) and QQ (rs662 or Q192R) genotypes are considered PON1 poor metabolizers.
Distribution normality was tested using the Kolmogorov-Smirnov test for quantitative variables. Differences in demographic characteristics and allele frequencies between cases and controls were assessed using $\chi^2$-tests for qualitative variables, and Mann-Whitney test for quantitative variables. Univariate analyses of risk were performed by Fisher's exact test. Multivariate analyses of risk were performed by binary logistic regression, controlling for statistical confounders and conceptual confounders (age and gender). Gene-environment interactions were estimated by binary logistic regression model, controlling for confounders. Variables were considered statistical confounders when $P \leq 0.1$ in the univariate analysis.
All statistical analyses were performed with the SPSS 18.0.0 statistical package and a significance level of 5% was set in all analyses with two-tailed tests.

## Results

Demographic and occupational exposure to toxics/pesticides data are shown in Table 1. No differences in age, gender and education were observed between PD patients and controls. European ancestry was more frequent in PD patients than in controls (90.2% vs. 77.8%, respectively). Therefore, ancestry was used as a confounder in all multivariate tests. Table 1 shows that exposure to toxics was more frequent in PD patients (34.6%) than in controls (21.6%; P = 0.01). However, time of exposure to toxics was similar between patients and controls. To have ever been exposed to pesticide exposure and time of exposure to pesticides did not differ between cases and controls.

Forty six individuals were occupationally exposed to pesticides (28 cases and 18 controls). Among them, 95.6% knew what type of pesticide they were exposed, and 28.3% remembered the name/brand of the pesticide. Percentage of pesticides types reported were: herbicide (46.4% cases vs. 22.2% controls), fungicide (28.6% cases vs. 11.1% controls), insecticide (75.0% cases vs. 77.8% controls), rodenticide (17.8% cases vs. 0% controls) and fumigation (17.8% cases vs. 0% controls). A total of 47.7% of individuals reported to be exposed to more than one type of pesticide. Occupational exposure to other environmental toxics was mainly due to agriculture (18.3%) followed by industry (7.2%), mining (2%) or welding (1.7%). Given the small groups by exposure types further analyses were made without partitioning.

*SV2C* (rs10214163 and rs30196) and *PON1* (L55M and Q192R) genotype frequencies are presented in Table 2. The genotype frequencies observed for all studied polymorphisms did not deviate to those expected under Hardy–Weinberg equilibrium. Allele and genotype frequencies for all polymorphisms did not differ between individuals of European descent and those with other ancestries (P>0.05).

*PON1* MM genotype carriers were associated with a higher risk of PD (Table 2; OR 2.34; 95% CI 1.21-4.53; $P_{adjusted}$ = 0.012). Individuals with MM genotype were more frequent in PD patients when compared to controls (19.7% vs. 9.3%, respectively). The same result was observed in unadjusted tests. The other polymorphisms investigated were not associated with PD (Table 2).

Logistic regression with or without confounders showed a statistically significant interaction between SV2C polymorphisms and environmental toxics (Table 3). *SV2C* rs10214163 TT genotype carriers, after controlling for age, gender and ancestry, showed a higher PD risk in subjects exposed to environmental toxics compared to those not exposed (OR 2.53;95% CI 1.33-4.69; $P_{interaction}$ = 0.008).

*SV2C* rs30196 TT/GT genotypes carriers also showed a higher PD risk in interaction with toxics compared to individuals not exposed (OR 2.30; 95% CI 1.21-4.36; $P_{interaction}$ = 0.033). No interaction between PON1 polymorphisms and pesticides were observed.

When analyzing only individuals with European ancestry, the results were similar to those observed in the whole sample. *PON1* MM genotype carriers were associated with a higher risk of PD (Supplemental table 1). Similar findings were observed in interaction analyses between *SV2C* polymorphisms and environmental toxics (Supplemental table 2).

**Discussion**

In this study, *SV2C* and *PON1* polymorphisms and their interaction with environmental factors as possible risk factors for PD susceptibility were investigated. The major finding reported herein was that SV2C polymorphisms rs30196 and rs10214163 in interaction with environmental toxics were PD risk factors.

Several evidences showed that SNpc is susceptible to many toxics, which accumulate its effects over time, generating mitochondrial dysfunction and oxidative stress, abnormal protein degradation, misfolding and aggregation and other forms of subcellular dysfunction. These alterations might lead to dopaminergic neurons death (Dauer and Przedborski 2003; Obeso et al. 2010). SV2C is localized and densely expressed in SNpc and in the ventral tegmental area (Dardou et al. 2011). Dunn et al. (2017) demonstrated that α-synuclein coimmunoprecipitates with SV2C, suggesting a functional interaction.

SV2C knock-out animals have increased expression of the multimeric form of α-synuclein over the decreased monomeric form. This altered ratio might be important in the induction of α-synuclein aggregation and toxicity. SV2C protein was shown to interact with neurotoxin proteins such as botulinum toxin A by GST-pull-down experiments and crystal structure analysis (Benoit et al. 2013; Mahrhold et al. 2006). Moreover, Dardou et al. (2013) treated mice with neurotoxic agents such as MPTP and 6-OHDA, and observed differences in SV2C mRNA expression. These studies suggested *SV2C* as a candidate gene for toxic interaction in PD affected regions.

Although little is known about SV2C, structural studies suggested that it could act as a transporter, scaffold protein or as an exocytosis modulator (Dardou et al. 2011). The rs30196 polymorphism in *SV2C* has been associated with levodopa dose in PD patients (Altmann et al. 2016). A recent study

reported that SV2C is involved in dopamine homeostasis in SNpc, regulating dopamine release (Dunn et al. 2017). When the pattern of SV2C expression of human striatum and midbrain samples from tissue banks were compared among PD, Alzheimer's disease, Multiple System Atrophy and progressive supranuclear palsy cases and controls, only PD showed a different pattern. Abnormal punctate SV2C-positive staining in the SNpc and the dorsal striatum, including the putamen were described in PD brain-tissues (Dunn et al. 2017). These studies suggested that SV2C might play a role in PD pathogenesis. However, an association of SV2C and PD has not been reported so far. Our results suggest that *SV2C* polymorphisms *per se* are not PD susceptibility factors, nevertheless it became a susceptibility factor in those individuals exposed to environmental toxics.

Although, the last meta-analysis (Liu et al. 2012) reported that *PON1* L55M and Q192R were not PD risk factors, several studies observed an association between these markers and PD (Akhmedova et al. 2001; Carmine et al. 2002; Clarimon et al. 2004; Kelada et al. 2003; Momose et al. 2002). Since the last meta-analysis, Belin et al. (2012) found an association between a *PON1* promoter polymorphism, rs854571, with a PD protective effect. The present results show that *PON1* L55M was associated with PD risk. These discrepancies might be attributable to heterogeneity among populations, environment and genetics factors.

Some studies pointed that the *PON1* association with PD risk was due to an interaction with pesticides. Benmoyal-Segal et al. (2005) reported that polymorphisms in *ACHE/PON1* locus were overrepresented in PD patients overexposed to a large variety of pesticides in rural communities from Israel. These investigators observed that *PON1*C-108T polymorphism in combination with *ACHE* haplotype interacted with pesticides to confer PD risk. Lee et al. (2013) showed after calculating exposure to pesticides by geographic information models in California, USA, that L55M and Q192R, but not PON1 C-108T, interacted with chlorpyrifos pesticide on PD susceptibility. Both studies considered exposure due to place of living independent of occupational use. The frequency distribution of *PON1* L55M genotypes did not differ significantly between patients and controls among those with occupational exposure to pesticides in Taiwan (Fong et al. 2005). Dick et al. (2007) also did not find a higher risk for PD considering *PON1* L55M and Q192R genotypes vs. exposure to pesticides based on occupational use in individuals from Scotland, Italy, Sweden, Romania and Malta.

The present investigation is in line with these two last studies because we considered as pesticide use only those subjects that reported to have occupational exposure in the questionnaire.

The present findings should be understood in the light of certain limitations. First, the sample is of moderate size. However, occupational toxic and pesticides data are very difficult to obtain and are valuable information for PD research. The current study presented a sample size similar to investigations already published by other groups (Ritz et al. 2016). The second limitation of this study is that pesticides were not discriminated by chemical group or name. There are already some studies linking only groups of pesticides such as paraquat (Costello et al. 2009; Tanner et al. 2011), rotenone (Kamel et al. 2014; Tanner et al. 2011), diazinon and chlorpyrifos (Manthripragada et al. 2010) with PD risk. Whereas, many studies reported pesticides in general as risk factors in their populations (Dick et al. 2007; Elbaz et al. 2009; Hancock et al. 2008). The region where the current study was performed is surrounded by agricultural areas. Individuals and agricultural workers are frequently exposed to many different pesticides or pesticide mixtures. The widespread use of pesticides in agriculture, homes and gardens makes it virtually impossible to completely estimate exposure. Moreover, the target population in studies with PD is old and might not remember all pesticides they used in the past. Lee et al. (2013) developed exposure estimation computer models based on geographic information system of individuals' homes or workplaces. This method might help to estimate exposure by food, soil or air but it is not a perfect model, since it does not consider occupational exposure and individual habits. Therefore how to estimate the effect of pesticides on PD risk is still an open question.

In conclusion, *SV2C* and *PON1* seem to influence PD susceptibility on different ways. Whereas *SV2C* polymorphisms are PD risk factors only in interaction with environmental toxics, *PON1* seems to be a genetic risk factor *per se* independent of pesticide exposures. Larger studies are needed, specially designed to identify the complicated environmental and genetic factors that can, in a combinatorial manner, determine a more complete picture of PD etiology.

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Table 1 –General and environmental exposure characteristics of PD patients and controls.

| | **PD patients** n=184 | **Controls** n=162 |
|---|---|---|
| Age (years) | 69.0(7.9) | 71.5(11.8) |
| Gender (male) | 92(50.3) | 65(40.1) |
| Descent (European) | 165(90.2) | 126(77.8)[A] |
| Education (years) | 6.6(4.2) | 6.6(4.2) |
| Exposure to toxics | 63(34.6) | 35(21.6) [A] |
| Time of exposure (years) | 20.7 (17.0) | 18.0 (18.7) |
| Exposure to pesticides | 28(15.4) | 18(11.1) |
| Time of exposure (years) | 20.07 (20.9) | 15.1 (13.0) |

PD, Parkinson's disease.

Data are given as number (percentage) or mean (±SD).

[A]$P<0.05$

Table 2 - Distribution of genotype frequencies between PD patients and controls.

| | Count (Frequencies) | | $P^A$ | $OR^A$ (95% CI) | $P^B$ | $OR^B$ (95% CI) |
|---|---|---|---|---|---|---|
| | Case | Controls | | | | |
| SV2C | | | | | | |
| rs10214163 | | | | | | |
| CC/CT | 125 (68.3) | 113 (69.8) | | 1 | | 1 |
| TT | 58 (31.7) | 49 (30.2) | 0.816 | 0.94 (0.59-1.48) | 0.847 | 0.95 (0.60-1.53) |
| rs30196 | | | | | | |
| TT/GT | 128 (69.9) | 119 (73.5) | | 1 | | 1 |
| GG | 55 (30.1) | 43 (26.5) | 0.476 | 0.84 (0.53-1.35) | 0.913 | 0.97 (0.60-1.59) |
| PON1 | | | | | | |
| L55M | | | | | | |
| LL/LM | 147(80.3) | 147(90.7) | | 1 | | 1 |
| MM | 36 (19.7) | 15 (9.3) | 0.009 | 2.40 (1.26-4.57) | 0.012 | 2.34 (1.21-4.53) |
| Q192R | | | | | | |
| QR/RR | 107 (58.5) | 98 (60.5) | | 1 | | 1 |
| QQ | 76 (41.5) | 64 (39.5) | 0.742 | 1.09 (0.71-1.67) | 0.842 | 1.05 (0.67-1.63) |

P, P-value; OR, *odds ratio*; PD, Parkinson’s disease.

[A]Difference between groups was tested by $\chi^2$-tests

[B]Difference between groups was tested through binary logistic regression model. P-value was adjusted for gender, age and descent.

Table 3 – Interaction Analyses of environmental factors and *SV2C* and *PON1* polymorphisms on PD risk.

| | Environmental factor | $P^A$ | $OR^A$(95% CI) | $P^B$ | $OR^B$(95% CI) |
|---|---|---|---|---|---|
| SV2C | Toxics | | | | |
| rs10214163 | | 0.025 | | 0.008 | |
| TT | Absent | | 1 | | 1 |
| TT | Present | | 2.76 (1.53-4.96) | | 2.53 (1.36-4.69) |
| CC/CT | Absent | | 1 | | 1 |
| CC/CT | Present | | 0.82 (0.34-1.98) | | 0.51 (0.18-1.43) |
| rs30196 | | 0.015 | | 0.033 | |
| GG | Absent | | 1 | | 1 |
| GG | Present | | 0.79 (0.33-1.85) | | 0.73 (0.29-1.83) |
| TT/GT | Absent | | 1 | | 1 |
| TT/GT | Present | | 2.87 (1.58-5.23) | | 2.30 (1.21-4.36) |
| PON1 | Pesticides | | | | |
| L55M | | 0.090 | | 0.106 | |
| MM | Absent | | 1 | | 1 |
| MM | Present | | 0.36 (0.06-2.05) | | 0.25 (0.36-1.77) |
| LL/LM | Absent | | 1 | | 1 |
| LL/LM | Present | | 1.81 (0.92-3.61) | | 1.37 (0.66-2.81) |
| Q192R | | 0.531 | | 0.435 | |
| QQ | Absent | | 1 | | 1 |
| QQ | Present | | 1.14 (0.45-2.74) | | 1.38 (0.54-3.54) |
| QR/RR | Absent | | 1 | | 1 |
| QR/RR | Present | | 1.85 (0.75-4.59) | | 0.82 (0.31-2.16) |

P, P-value; OR, *odds ratio*; PD, Parkinson's disease.

$^A$Difference between groups was tested through $\chi^2$-tests

$^B$Difference between groups was tested through binary logistic regression model. P-value was adjusted for gender, age and descent

P-value corresponds to overall interaction of genotypes and environmental factor.

# CAPÍTULO V

## *The interplay between cigarette smoking and genetic factors on Parkinson's disease susceptibility*

Manuscrito em preparação

**The interplay between cigarette smoking and genetic factors on Parkinson's disease susceptibility.**

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## ABSTRACT

**Introduction**: The interplay of genetic and environment has been hypothesized to have a major role in Parkinson's disease (PD) susceptibility. While there is compelling evidence for protective effects of smoking in PD, the genetic involvement that modulates this feature is poorly understood. Therefore the aim of this investigation was to disclose the influence of genes related with smoking in PD patients.

**Methods**: Two hundred and thirty PD patients and 199 controls were ascertained. A questionnaire that includes smoking habits questions were applied in all investigated subjects. Individuals were genotyped for polymorphisms in genes associated with smoking (*ABCB1*, *CYP1A1*, *CYP1B1* and *MAOB*).

**Results**: CYP1B1 rs1056836 in interaction with smoking was associated nominally with PD ($P_{interaction}$ = 0.044) whereas no association was observed with *CYP1A1* and *MAOB.* Evidence of a statistically significant gene-smoking interaction with *ABCB1* was observed. Smokers with T-non G-T *ABCB1* haplotype showed lower PD risk (OR 0.34, 95% CI 0.15-0.72; $P_{interaction}$ = 0.012) compared with carriers of a C-G-C haplotype.

**Conclusions**: *ABCB1* T-non G-T haplotype was associated with PD in interaction with smoking. Replication of these findings is very important before any causal inference can be postulated.

## IMPLICATIONS

The results from this work show that Parkinson's disease susceptibility is associated with smoking consumption in the Brazilian population. Moreover, Parkinson's disease risk is modulated by ABCB1 gene and cigarette smoking altogether. *CYP1A1*, *CYP1B1* and *MAOB* were not associated with this disease in our population.

## INTRODUCTION

Despite the known harmful effects of cigarette smoking to human health, many evidences support cigarette smoking as a protective factor for Parkinson's disease. Meta-analyses consistently showed cigarette smoking associated with a lower risk of PD, with OR for ever smokers ranging from 0.55 to 0.59[1,2] and for current smokers from 0.31 to 0.41[2,3]. This inverse association of smoking and PD seems to be dose-dependent[4,5] but independent of gender or education[4]. Evidences linking smokeless tobacco use to a lower risk for PD has also been reported[6–8]. Moreover, environmental tobacco smoke exposure was considered a protective factor for PD[9], passive only smokers have similar reduced risk for PD as ever active smokers.

A recent finding in this field is that gene-environment interactions contribute more to the pathogenesis of PD than do genetic factors or environmental factors alone. Interactions of genes and environmental factors, however, have rarely been examined in epidemiological studies[10]. Few investigations, with contradictory findings, reported interaction of smoking related genes on PD susceptibility. Therefore the aims of this study is to evaluate the interaction of genes related to cigarette smoking metabolism in PD patients in order to disclose which genes could be involved in lower PD risk.

## MATERIALS AND METHODS

### Subjects

This study included 230 patients recruited and evaluated at the Movement Disorder Clinics at Hospital de Clínicas de Porto Alegre, Brazil, from 2006–2016. The inclusion criteria were PD diagnosis according to UK Brain Bank criteria[11] as previously described[12]. Patients with atypical manifestations, secondary parkinsonism or with familial history of the disease were excluded.

A total of 199 individuals were recruited as controls. These subjects were recruited at the Geriatric Clinics from the same hospital, and at Universidade Federal de Ciências da Saúde de Porto Alegre. These individuals were recruited in the same city, to ensure they were from the same geographical area as cases. All individuals included in the control sample were not related to PD patients and did not have family history of PD or other neurological disorder.

A questionnaire with environmental questions was developed in Portuguese and applied to all patients and controls. This questionnaire involved questions about cigarette smoking in lifetime. Cigarette smoking was defined as ever smoker (at least 100 cigarettes in life) vs. never smoker. The hospital ethics committee approved the study, and all participants gave written informed consent.

### Genes and SNP selection

*ABCB1*, *CYP1A1*, *CYP1B1* and *MAOB* were related to cigarette smoking metabolism in previous studies[13–18]. Single nucleotide polymorphisms (SNPs) selected from those genes are considered functional polymorphisms in the literature, all of them were associated with different enzyme activity levels between alleles[19–23]. The following SNPs were selected: *ABCB1* (rs2032582, rs1128503 and rs1045642), *CYP1A1* (rs4646903), *CYP1B1* (rs1056836), *MAOB* (rs1799836).

### Genotyping

Peripheral blood samples were collected from patients and controls. DNA was extracted by standard procedures[24]. All SNPs were genotyped by Taqman® SNP genotyping assays (Applied Biosystems, CA, USA) according to manufacturer's recommended protocol.

### Statistical analyses

Categorical demographic and environmental factors were evaluated by Fisher's exact test (gender, European ancestry and smoking cigarettes), or by non-parametric Wilcoxon-Mann-Whitney *U*-test if quantitative (age).

Allele frequencies were estimated by gene counting. Agreement of genotype frequencies to Hardy–Weinberg expectations was tested using a goodness of fit $\chi^2$-test. Frequencies were compared with data extracted from the HapMap. Linkage disequilibrium was estimated using the Multiple Locus Haplotype Analysis (Mlocus 3.0)[25,26]. Differences of genotype frequencies between ancestries were calculated by $\chi^2$-tests.

In order to investigate differences in genotype/haplotype frequencies between cases and controls, associations were performed by multivariate analyses using Binary Logistic Regression, adjusting for possible confounders. A dominant model was used for all SNPs and haplotypes. Reference alleles were taken from dbSNP website (https://www.ncbi.nlm.nih.gov/projects/SNP). *ABCB1* was evaluated by testing the risk haplotype against all other haplotypes. Once *MAOB* is an X-linked gene, *MAOB* rs1799836 heterozygous (AG) women were excluded from analyses. Smoking interaction with genotypes or haplotypes was also tested with Binary Logistic Regression adjusted for possible confounders.

Potential confounders to be entered in models were defined based on conceptual analyses of the literature or if they were associated with study factor and outcome at $P \leq 0.10$. The significance level accepted was 0.05, tests were two-tailed, and the adjustment for multiple testing was performed with Bonferroni correction. All tests were performed with SPSS version 18 software (SPSS Inc., Chicago, IL, USA).

## RESULTS

Demographic and consumption data from patients and controls are presented in Table 1. Cigarette smokers were more frequent in controls (48.7%) than PD cases (32.2%; P = 0.001). Cigarette smoking conferred protection for PD (OR = 0.50; 95% CI 0.33-0.74; P = 0.001). Males were more frequent in PD patients (51.7%) than in controls (36.2%; P = 0.001).

All *ABCB1* SNPs were in linkage disequilibrium. *ABCB1* rs1045642 was in linkage disequilibrium with rs2032582 and rs1128593 (D' = 0.848 and D' = 0.754; P<0.001). *ABCB1* rs2032582 was also in linkage with rs1128503 (D' = 0.883; P<0.001). Eight haplotypes were derived from *ABCB1* SNPs, haplotypes with alleles C-G-C and T-A/C-T (called as T-non G-T) from SNPs rs1045642, rs2032582 and rs1128503, respectively were the most frequent accounting for 83% of all chromosomes investigated. The observed genotype distributions were in agreement with Hardy-Weinberg equilibrium for all SNPs after Bonferroni correction. Observed genotype and allele frequencies were similar to those described in HapMap for individuals of European ancestry. ABCB1, CYP1B1 and MAOB genotypes or haplotypes frequencies did not differ between ethnic groups (P > 0.05). CYP1A1

genotype frequencies were different between Europeans and African derived subjects ($\chi^2 = 16.71$; P = 0.001).

The genotype frequencies for all SNPs tested in this study are presented in Table 2. After controlling for gender, age and ancestry no association with the disease was observed for all SNPs.

Table 2 also summarizes the gene-environment interaction analyses of each genotype or haplotype vs. smoking, controlled for age, gender and ancestry. *ABCB1* haplotype T-nonG-T in interaction with smoking was associated with PD ($P_{interaction} = 0.012$). Smokers with T-non G-T haplotype showed higher protection (OR 0.34; 95% CI 0.15-0.72) compared to those with C-G-C haplotype. The significance level accepted after Bonferroni correction was: $P_{Bonferroni\ corrected} < 0.0125$. CYP1B1 CG and GG in interaction with smoking was only associated nominally with PD ($P_{interaction} = 0.044$). We did not identify statistically significant interactions between the other SNPs examined and cigarette smoking. Considering only individuals with European ancestry, no polymorphism was associated with PD (Table S1).

## DISCUSSION

This study simultaneously considered genetic and environmental factors in PD risk. We evaluated potential interactions between key genes in smoking metabolism and their relation with smoking in PD. Consistent with many other studies, smoking was protective factor for PD in this Brazilian cohort. It is not known how cigarette smoking/smoke might contribute to PD development. Recent findings suggest that, PD starts in the gut, with environmental factors acting primarily via the gut. Gut microbiota seems to be altered in PD[27,28]. Beta diversity analyses and analysis on the bacterial family level showed a significant difference between certain bacterial families abundance of PD cases and controls[29]. Even before patients started treatment with oral PD medication, the composition of PD gut microbiota was significantly different from control at all taxonomic levels[30]. Smoking is reported to affect the microbiome composition[28], and could explain the participation of these factors in PD. However, these studies are still at initial levels.

ABCB1 protein, called P-glycoprotein (P-gp), was demonstrated to be a transporter of compounds present in smoke such as naphthalene, 7,12-dimethylbenzanthracene and 2-amino-1-methyl-6-

phenylimidazo[4,5-b]pyridine[14,31,32]. However, Kyiohara et al.[33] reported that ABCB1 rs1045642 polymorphism did not interact with smoking but with alcohol in Japanese population. P-gp is known to be an efflux pump to a wide range of structurally diverse compounds, with over hundreds described[34]. Thus, it is expected that *ABCB1* interaction studies present different findings in different populations.

In the present study only *ABCB1* T-nonG-T haplotype was associated with PD in interaction with smoking. This haplotype which presents the derived alleles, showed a higher protection in interaction with smoking when compared to haplotype with the ancestral alleles (C-G-C) only. However, when only individuals with European ancestry were considered, this interaction was not observed. This lack of association could be explained by smaller sample size when part of the sample was excluded but it could also suggest that other genes or environmental factors would participate in this interaction. However population stratification could also be an issue in this finding, therefore more studies with larger and more homogenous samples are warranted.

Fowler et al.[16] reported an overall 40% decrease in brain MAOB activity of current smokers compared with non-smokers or former smokers. Nevertheless, monoamine oxidase B is an enzyme with particular importance in PD, given that it catabolizes dopamine at the synaptic cleft[35]. The interaction of smoking and *MAOB* polymorphism in PD showed contradictory findings[36–42]. Checkoway et al.[36] reported for the first time an interaction of *MAOB* rs1799836 and smoking. In that study G allele carriers were associated with PD protection, and A allele carriers were associated with PD risk. Whereas, Mellick et al.[37] described the G allele as a protection factor, but no association with the A allele. Kelada et al.[42] found a gender difference in the interaction of smoking and *MAOB*. In that study, G allele confers protection in men only. In contrast to these studies, several other investigations did not support findings for *MAOB* genotypes/alleles interacting with smoking in PD[38–41]. In line with these investigations, the present study did not observe a relation among smoking, MAOB genotypes and PD susceptibility.

*CYP1A1* and *CYP1B1* are genes from the same family which encode cytochrome P450 enzymes. *CYP1A1* was evaluated in this study for cigarette smoking interaction, because it is known to be the highest metabolizer of polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene, present in

cigarette smoke[18]. CYP1B1 is the second highest metabolizer of benzo(a)pyrene, right after CYP1A1, and it also activates many structurally diverse compounds, including heterocyclic and aryl amines, and nitroaromatic hydrocarbons present in smoking[17,18]. CYP1A1 rs4646903 and CYP1B1 rs1056836 were associated with a variety of smoking-related diseases such as some types of cancers[43–46], myocardial infarction[47] and coronary artery disease[48,49], but yet, an association with PD was not found. The present investigation also did not support a role for CYP1A1 and CYP1B1 as susceptibility factors for PD modulated by cigarette smoking.

Based on a statistical definition, an interaction means that the effect of genes depends on the environment and/or the effect of the environment depends on genotype. In present study it is important to notice that none of the SNPs were associated with PD *per se*. Therefore, PD risk was modulated by genotypes only when the environmental factor was present. Although the sample size investigated in the present study may not be adequate to detect moderate gene-environment interactions with small effects, an association of *ABCB1* haplotype in interaction with smoking was reported.

Findings from gene-environment interaction analyses must be interpreted with caution due to differences in populations for habits and/or genetics. Replication of findings is very important before any causal inference can be postulated. Replication in different and larger samples is warranted. For all associations, P-values were adjusted for multiple comparisons, thereby minimizing false-positive results.

This work, given the novel results obtained, could be considered as exploratory study. Therefore, replication is warranted before a better PD susceptibility picture can be drawn.

## FUNDING

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## DECLARATION OF INTERESTS

No conflicts of interest, financial or otherwise, are declared by the authors.

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Table 1 – Demographic and environmental factors of Parkinson's disease patients and controls.

| | PD patients N=230 | Controls N=199 | P | OR (95% CI) |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 119 (51.7%) | 72 (36.2%) | 0.001 | 1.89 (1.28 – 2.78) |
| Female | 111 (48.3%) | 127 (63.8%) | | |
| **Age** | 69.0 (±11.7) | 71.1 (±7.8) | 0.218 | - |
| **European descent** | 198 (86.1%) | 157 (78.9%) | 0.055 | 1.65 (0.99 – 2.74) |
| **Smoking cigarettes** | | | | |
| Ever smoker | 74 (32.2%) | 97 (48.7%) | 0.001 | 0.50 (0.33 – 0.74) |
| Never smoker | 156 (67.8%) | 102 (51.3%) | | |

Number of patients, gender, European descent and smoking cigarettes are shown in absolute number (percentage). Age is shown in mean (± standard deviation). Association calculated by Fisher's exact test (categorical variables) and non-parametric Wilcoxon-Mann-Whitney *U*-test (quantitative variable).

Table 2 - Multivariate and Interaction analysis of Parkinson's disease risk

| | Genotype/Haplotype | | Frequency of studied genotype/haplotype | | $P_{adjusted}$ | $OR_{adjusted}$ (95% CI) | $P_{interaction}$ |
|---|---|---|---|---|---|---|---|
| | Reference | Studied | PD patients | Controls | | | |
| **ABCB1** | C-G-C | T-nonG-T | 54.6 | 62.8 | 0.152 | 0.72 (0.47-1.11) | 0.012 |
| **MAOB** | AA | GG | 42.9 | 50.4 | 0.242 | 0.74 (0.47-1.17) | 0.351 |
| **CYP1B1** | CC | CG and GG | 83.6 | 76.4 | 0.097 | 1.72 (0.89-2.46) | 0.044 |
| **CYP1A1** | TT | CC and CT | 35.0 | 33.2 | 0.746 | 1.08 (0.71-1.65) | 0.777 |

PD, Parkinson's disease. Differences in genotype/haplotype frequencies between cases and controls were performed by Binary Logistic Regression, adjusting for age, gender and ancestry. Interactions were calculated by Binary Logistic Regression adjusted for age, gender and ancestry. For all the tests was used studied Genotype/Haplotype vs Reference.

**Supplementary material**

Table S1 - Multivariate and Interaction analysis of Parkinson's disease risk in individuals with European ancestry

| | Genotype/Haplotype | | Frequency of studied genotype/haplotype | | $P_{adjusted}$ | $OR_{adjusted}$ (95% CI) | $P_{interaction}$ |
|---|---|---|---|---|---|---|---|
| | Reference | Studied | PD patients | Controls | | | |
| **ABCB1** | C-G-C | T-nonG-T | 57.4 | 64.3 | 0.275 | 0.75 (0.46-1.21) | 0.121 |
| **MAOB** | AA | GG | 41.2 | 47.0 | 0.434 | 0.79 (0.47-1.31) | 0.259 |
| **CYP1B1** | CC | CG and GG | 84.8 | 78.8 | 0.162 | 1.50 (0.86-2.56) | 0.150 |
| **CYP1A1** | TT | CC and CT | 30.3 | 30.6 | 1.000 | 0.98 (0.62-1.55) | 0.862 |

PD, Parkinson's disease. Differences in genotype/haplotype frequencies between cases and controls were performed by Binary Logistic Regression, adjusting for age and gender. Interactions were calculated by Binary Logistic Regression adjusted for age and gender. For all the tests was used studied Genotype/Haplotype vs Reference.

# CAPÍTULO VI

## *The variable effect of caffeine consumption on Parkinson's disease is influenced by NOS1 genotypes*

Manuscrito em preparação

**The variable effect of caffeine consumption on Parkinson's disease is influenced by *NOS1* genotypes**

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**Abstract**

Parkinson's disease (PD) is an age related neurodegenerative disease, with high prevalence among elderly. Consistently, caffeine has been associated as a neuroprotective factor for PD. Caffeine's mechanism of action in this disease is not fully understood, but accumulated research indicates that nitric oxide synthase might be involved. Here we investigated the interaction between neuronal nitric oxide synthase gene (*NOS1*) polymorphism and caffeine consumption in Parkinson's disease. For this purpose, this study included 183 PD patients and 162 controls, with caffeine daily consumption data (coffee and yerba mate). *NOS1* polymorphism rs478597 was genotyped for patients and controls. Caffeine interaction with *NOS1* genotypes was tested with Binary Logistic Regression adjusted for age, gender, ancestry and smoking. High caffeine consumers presented a lower PD risk when compared to low consumers (OR 0.56, 95% CI 0.36 – 0.88). *NOS1* genotypes in interaction with caffeine consumption were associated with PD ($P_{interaction}$= 0.0002). High consumers of caffeine that are *NOS1* rs478597 CC genotype carriers presented lower PD risk when compared to those with low consumption (OR 0.24, 95% CI 0.10 – 0.54). This exploratory study shows that NOS1 genotypes might be a useful marker for PD pharmacogenomic studies involving caffeine.

Keywords: Parkinson's disease, caffeine, NOS1, susceptibility.

## Introduction

Parkinson's disease (PD) is a common and worldwide distributed disease. PD prevalence can reach 1,087 individuals per 100.000 at age 70-79 years [1]. Loss of dopaminergic neurons in *substantia nigra pars compacta* and depletion of dopamine in the striatum are responsible for the cardinal motor manifestations of this disease [2].

Multiple environment and genetic factors seems to influence PD pathogenesis. Association studies evidenced several risk and protective factors for PD. Risk factors identified include herbicides and pesticides (e.g., paraquat, rotenone, and maneb), metals (e.g., manganese and lead), head trauma, and well water. In contrast, coffee/caffeine consumption has been consistently reported as a PD protective factor [3]. A meta-analysis of 26 epidemiological studies estimated a risk ratio between coffee drinkers and PD of 0.75 [4]. Although caffeine has been associated as an important protective factor for this disease, its mechanisms of action is still poorly understood.

Three NOS isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), endothelial NOS (eNOS) [5] has been identified. nNOS is localized in neurons, neutrophils and astrocytes [6,7]. Higher nNOS expression was observed in dopaminergic pathways, nigrostriatal region and basal ganglia in PD post mortem brains [8,9]. Caffeine might exert its protective effect on PD susceptibility through the involvement of nitric oxide synthase (NOS). Some studies suggested that caffeine affects NOS expression [10,11], including its neuronal form (nNOS) [12]. Moreover, nitric oxide (NO) is down-regulated by caffeine in PD animal models [13].

The causes of sporadic PD are still unknown but many evidences suggest that PD risk probably results from a complex interplay between genetic and environmental factors. Caffeine seems to have an important role in PD protection. Moreover, caffeine seems to modulate nNOS expression in mice [12]. Although localized in an intron with unknown functionality, rs478597 polymorphism in nNOS gene (NOS1) has already been associated with ADHD [14,15]. Therefore, we hypothesized that an interaction between this NOS1 genetic variant and caffeine consumption could be associated with PD susceptibility.

## Materials and Methods

### Subjects

The present study included 183 PD patients from a sample previously described by Rieck et al. [16]. Briefly, patients were recruited and evaluated in the Movement Disorder Clinics at Hospital de Clínicas de Porto Alegre, Brazil, from 2006–2016. The inclusion criteria were PD diagnosis according to UK Brain Bank criteria [17]. Patients with atypical manifestations, secondary parkinsonism or with familial history of the disease were excluded.

A total of 162 individuals were recruited as controls. These subjects were ascertained at the Geriatric Clinics from the same hospital, from 2014–2015. All individuals included in the control sample were not related to PD patients and did not have family history of PD or other neurological disorder.

A questionnaire with environmental questions was developed in Portuguese and applied to all patients and controls. This questionnaire involved questions about dietary habits such as alcohol and caffeine consumption, cigarette smoking, and use of drugs (cannabis, cocaine, crack, LSD, heroin). Caffeine consumption questions involved the main sources of caffeine because in south Brazil: coffee and yerba mate (a traditional hot beverage consumed in southern Brazil and neighboring countries, which is prepared from the leaves of *Ilex paraguariensis*) are very popular. Caffeine consumption was defined as high consumers (2 or more cups of coffee and 2 or more cups of yerba mate per week) vs. low consumers (Never consumers or 1 cup per week of Coffee or yerba mate) or ever vs. never consumers. The hospital ethics committee approved the study, and all participants gave written informed consent.

### Genotyping

Peripheral blood samples were collected from patients and controls. DNA was extracted by standard procedures [18]. *NOS1* (rs478597) was genotyped by TaqMan® (Applied Biosystems, CA, USA) according to manufacturer's recommended protocol.

Statistical analyses

Categorical demographic and environmental datawere evaluated by Fisher's exact test if qualitative (gender, European ancestry, caffeine consumption and smoking), or by non-parametric Wilcoxon-Mann-Whitney *U*-test if quantitative (age).

Allele frequencies were estimated by gene counting. Agreement of genotype frequencies to Hardy–Weinberg expectations was tested using a goodness of fit $\chi^2$-test. Frequencies were compared with data extracted from the HapMap. The comparison among genotype frequencies between different ethnic groups were performed by goodness of fit $\chi^2$-test.In order to investigate differences in genotype frequencies between cases and controls, associations were performed by Fisher's exact test.

Caffeine interaction with genotypes was tested with Binary Logistic Regression adjusted for possible confounders. Confounders to be entered in models were defined based on conceptual analyses of the literature or if they were associated with study factor and outcome at $P \le 0.10$. Tests with $P < 0.05$ significance level were accepted. All tests were performed with SPSS version 18 software (SPSS Inc., Chicago, IL, USA).

**Results**

Demographic and consumption data from patients and controls are presented in Table 1. Higher caffeine consumers were more frequent in controls (44.4%) when compared to PD patients (31.1%, P = 0.014). Individuals who were caffeine high consumers presented a lower PD risk when compared to low consumers (OR 0.56, 95% CI 0.36– 0.88). Never consumers were more frequent in patients (15.8%) then in controls (4.3%) (OR 0.24, P = 0.001, 95% CI 0.10 – 0.56). Smoking was also associated with PD, being more frequent in PD patients (70%) than in controls (49.4%).

The observed genotype distributions were in agreement with Hardy-Weinberg equilibrium. Observed genotype and allele frequencies were similar to those described in HapMap for individuals of European ancestry. The genotype frequencies of *NOS1* rs478597 are presented in Table 1. Genotype frequencies for rs478597 did not differ between ethnic groups ($\chi^2$= 1.39, P = 0.520). NOS1 genotypes were not associated with the disease (P = 0.664, OR 0.91, 95% CI 0.59 – 1.39).

Given the small number of control individuals who had never consumed caffeine, interaction analyses were made only with high or low consumers. Table 2 summarizes the gene-environment interaction

analyses of genotype vs. caffeine, controlled by age, gender, ancestry and smoking. *NOS1* genotypes in interaction with caffeine consumption were associated with PD ($P_{interaction}$= 0.0002). Carriers of the *NOS1* rs478597 CC genotype that are high caffeine consumers presented lower PD susceptibility when compared to those with low caffeine consumption (OR 0.21, 95% CI 0.09 – 0.43). However, individuals with CT and TT genotypes that are high consumers did not present PD protection. When only the group of high caffeine consumers was analyzed, the *NOS1* rs478597 CC genotype conferred protection to PD compared to those with CT and TT genotypes (OR 0.34, 95% CI 0.15 – 0.78).

When only individuals with European ancestry were considered, similar results were observed. *NOS1* genotypes were not associated with the disease (P = 0.285, OR 0.77, 95% CI 0.48 – 1.23). Interaction analyses showed that *NOS1* genotypes in interaction with caffeine consumption, controlled by age, gender and smoking, were associated with PD ($P_{interaction}$= 0.001), as observed in the whole sample (supplementary material - Table S1). High consumers of caffeine with *NOS1* rs478597 CC genotype presented lower PD risk when compared to those with low consumption (OR 0.24, 95% CI 0.10 – 0.54). This effect was not observed in individuals with CT and TT genotypes that were high caffeine consumers

**Discussion**

This study shows that the effect of caffeine on PD risk reduction is modulated by *NOS1* genotypes. *NOS1* rs478597 CC genotype carriers showed lower PD risk than their counterparts with other genotypes when they are high consumers. This finding was also observed when we considered only individuals with European ancestry.

Convergent caffeine intake epidemiological studies showed an inverse association with PD [4]. The current study corroborated these findings. A recent investigation has demonstrated that caffeine metabolites in serum could be a PD biomarker [19]. That investigation showed that caffeine concentrations and 9 of its downstream metabolites were significantly lower in PD patients than in controls. The caffeine neuroprotection against dopaminergic degeneration seems to be restricted to PD susceptibility and do not have a role in improving PD symptoms or progression, as recently reported [20,21].

The relation between neurodegeneration and NO is not restricted to PD, it has already been reported in other neurodegenerative disorders, like Alzheimer's disease [22,23]. NO can inhibit and induce apoptosis, depending on its concentration [24]. NO can also damage reversibly and irreversibly respiratory complexes of neuronal and astrocyte mitochondria and can act in inflammatory and anti-inflammatory pathways in neurons [22]. In PD, the high production of nNOS compared to controls [8,9] and the neuroprotection exhibited in nigral neurons of PD-animal models when in use of nNOS inhibitors [25,26] suggested a relation of NO and subsequently NOS in PD development. Leveque et al. found an association of rs1047735 and rs2682826 with PD. In an attempt to replicate those findings, four studies [27–30] did not found an association of those two polymorphisms and PD in their populations. The present study investigated another polymorphism and also did not find an association of NOS1 genotypes and PD *per se*. The association of caffeine consumption with PD was genotype–specific and varied from highly protective to neutral depending on NOS1 genotypes.

The primary candidate component that is believed to be responsible for the neuroprotective effect of coffee is caffeine. Nevertheless this study included an important source of caffeine in our population, yerba mate. However, it cannot be underestimated that coffee and yerba mate are complex chemical mixtures reported to contain more than a thousand different chemicals, including carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids, and phenolic compounds [4,31,32]. One of these compounds is eicosanoyl-5-hydroxytryptamide (EHT). EHT has been demonstrated to repress LPS-induced iNOS induction and nitric oxide production in astrocytes. These findings indicated that EHT could contribute indirectly to its neuroprotective activity *in vivo* [33].

Another explanation for neuroprotective effects of caffeine in PD is the caffeine's competitive inhibition of adenosine 2A receptor (A2aR), reducing glutamate release and diminishing excitotoxicity [34]. Although adenosine 2A receptor gene (*ADORA2A*) polymorphisms seem to influence individual caffeine sensitivity [35,36], no relation was observed between caffeine and PD susceptibility [19,37,38]. *ADORA2A* seems to be related to PD motor complication occurrence instead [39,40].

This study had a number of limitations that should be addressed in future studies. The interaction of NOS1 genotype with the protective effect of caffeine consumption must be independently replicated

with larger sample sizes. The molecular mechanism of the observed interactions should also be better understood.

The present study is the first to test the effect of NOS1 modulated by caffeine in PD and suggest a role in the pathogenesis of the disease. NOS1 genotypes might be a useful marker for pharmacogenomics studies of PD involving caffeine; therefore, the findings presented herein if replicated has the potential to make a significant positive impact on personalized prevention and treatment strategies for PD.

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Table 1 – Demographic data, caffeine intake and *NOS1* genotypes in PD patients and controls.

| | PD patients N=183 | Controls N=162 | P | OR (95% CI) |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 92 (50.3%) | 65 (40.1%) | 0.066 | 1.51 (0.98 – 2.31) |
| Female | 91 (49.7%) | 97 (59.9%) | | |
| **Age** | 69.0 (±11.7) | 71.4 (±7.9) | 0.142 | - |
| **European descent** | 165 (90.2%) | 126 (77.8%) | 0.002 | 2.62 (1.42 – 4.83) |
| **Smoking** | 128 (70.0%) | 80 (49.4%) | 0.001 | 0.42 (0.27 – 0.65) |
| **Caffeine** | | | | |
| High consumption | 57 (31.1%) | 72 (44.4%) | 0.014 | 0.56 (0.36 – 0.88) |
| Low consumption | 126 (68.9%) | 90 (55.6%) | | |
| Ever consumer | 154 (84.2%) | 155 (95.7%) | 0.001 | 0.24 (0.10 – 0.56) |
| Never consumer | 29 (15.8%) | 7 (4.3%) | | |
| ***NOS1* genotypes** | | | | |
| CC | 80 (43.7%) | 67 (41.4%) | 0.664 | 0.91 (0.59 –1.39) |
| CT and TT | 103 (56.3%) | 95 (58.6%) | | |

PD, Parkinson's disease; NOS1, nitric oxide synthase 1. Gender, European ancestry, caffeine consumption and smoking association with PD were calculated byFisher's exact test. Age differences between patients and controls were calculated by non-parametric Wilcoxon-Mann-Whitney *U*-test.

Table 2 – PD risk with *NOS1* genotypes in interaction with caffeine intake.

| | ***NOS1* genotype CC** | | ***NOS1* genotypes CT and TT** | | |
|---|---|---|---|---|---|
| **Caffeine consumption** | Frequency PD/controls | OR (95% CI) | Frequency PD/controls | OR (95% CI) | $\mathbf{P_{interaction}}$ |
| Low | 64/29 | reference | 62/61 | reference | 0.0002 |
| High | 16/38 | 0.21 (0.09-0.46) | 41/34 | 1.39 (0.75-2.61) | |

PD, Parkinson's disease. Interaction was calculated by Binary Logistic Regression adjusted for age, gender, smoking and ancestry.

**Supplementary material**

Table S1 -PD risk with *NOS1* genotypes in interaction with caffeine intake, only in individuals with European ancestry.

| | ***NOS1* genotype CC** | | ***NOS1* genotypes CT and TT** | | |
|---|---|---|---|---|---|
| | Frequency PD/controls | OR (95% CI) | Frequency PD/controls | OR (95% CI) | $\mathbf{P_{interaction}}$ |
| **Caffeine consumption** | | | | | |
| Low | 61/24 | reference | 54/51 | reference | 0.001 |
| High | 15/26 | 0.24 (0.10-0.54) | 35/25 | 1.60 (0.81-3.19) | |

PD, Parkinson's disease. Interaction was calculated by Binary Logistic Regression adjusted for age, gender and smoking.

# CAPÍTULO VII

## *Influence of genetic, biological and pharmacological factors on levodopa dose in Parkinson's disease*

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Research Article

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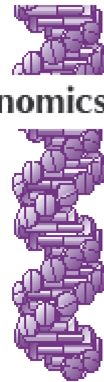

Pharmacogenomics

# Influence of genetic, biological and pharmacological factors on levodopa dose in Parkinson's disease

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**Aim:** Levodopa is first-line treatment of Parkinson's disease motor symptoms but, dose response is highly variable. Therefore, the aim of this study was to determine how much levodopa dose could be explained by biological, pharmacological and genetic factors. **Patients & methods:** A total of 224 Parkinson's disease patients were genotyped for *SV2C* and *SLC6A3* polymorphisms by allelic discrimination assays. Comedication, demographic and clinical data were also assessed. **Results:** All variables with $p < 0.20$ were included in a multiple regression analysis for dose prediction. The final model explained 23% of dose variation ($F = 11.54$; $p < 0.000001$). **Conclusion:** Although a good prediction model was obtained, it still needs to be tested in an independent sample to be validated.

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**Keywords:** algorithm • dopamine transporter • levodopa • Parkinson's disease • pharmacogenetics • synaptic vesicle isoform C

Parkinson's disease (PD) is an age-related neurodegenerative disorder caused by the interaction of multiple environmental and genetic factors [1]. PD pathology is characterized by a loss of dopamine-producing neurons in *substantia nigra pars compacta* [2]. Levodopa, a dopamine precursor that, since the late 1960s, is considered the 'gold standard' to treat PD. Levodopa ameliorates the primary motor dysfunction, but its continued use and disease progression are associated with motor fluctuations and levodopa-induced dyskinesia [3]. It is estimated that between 35 and 40% of treated patients will develop these adverse effects after 4–6 years [4]. Combination therapy of levodopa and other drugs such as entacapone and dopamine agonists have demonstrated to improve quality of life [5] and to reduce 'off' time [6] in PD patients, respectively. However, Cilia *et al.* [7] concluded that motor fluctuations and dyskinesias are not associated with the duration of levodopa therapy, but rather with longer disease duration and higher levodopa daily dose.

Few studies have been conducted to assess the genetic influence on levodopa dose response, moreover most of them focused on cathecol-*O*-methyltransferase (*COMT*) polymorphisms [8]. Studies of acute levodopa challenge showed an association between greater efficacy of motor symptoms treatment and dopa-decarboxylase (*DDC*) and the dopamine transporter (*SLC6A3*) gene polymorphisms [9,10]. The 9R allele in the 3′ UTR VNTR at the *SLC6A3* gene was associated with lower levodopa equivalent doses in one investigation [11]. The dopamine transporter is a key regulator of the dopamine system, controlling extracellular dopamine levels in the striatum. This protein is a transmembrane transporter localized in presynaptic neurons and is responsible for dopamine uptake from the synaptic cleft.

The synaptic vesicle isoform C (SV2C) is a member of SV2 transmembrane pro-

teins which are found on small clear vesicles containing neurotransmitters [12] and is highly expressed in *substantia nigra* and *striatum*, regions affected by PD [13,14]. Although Vesicular storage of dopamine is a crucial step in the dopaminergic pathway and could potentially be involved on levodopa dose, its relation with levodopa response was never assessed. *SV2C* gene selection was based on Hill-Burns *et al.* study [15]. The authors used a genome-wide approach, identifying this gene as one of highest top hit for Parkinson's disease risk in interaction with smoking.

Variability in levodopa dose response impacts clinical practice however its determinants are not fully established. Therefore, the aim of this study was to investigate a possible role for *SV2C* and *SLC6A3* gene polymorphisms in levodopa dose variability in order to implement an algorithm that uses clinical and genetic factors to guide levodopa dose prescriptions.

## Patients & methods

### Sample characteristics

This study included 224 patients recruited and evaluated at the Movement Disorder Clinics at Hospital de Clínicas de Porto Alegre, Brazil, from 2006–2013. The inclusion criteria were PD diagnosis according to UK Brain Bank criteria [16]; to be in use of levodopa for at least 1 year; and to have had a favorable initial response to the drug. Patients with secondary parkinsonism or with atypical manifestations were excluded. Levodopa daily dose of each patient was defined based on the best motor response based on clinical judgment by one of us (CRM Rieder), a movement disorders specialist. Each participant underwent a clinical and demographical assessment described in detail elsewhere [11,17]. Briefly, clinical data collected for this study were age at onset of the symptoms, disease duration, first motor symptom noticed and dosage per day of antiparkinsonian drugs in use. One patient was excluded from multivariate analysis because his/her first symptom data record was not available. The hospital Ethics Committee approved the study, and all participants gave written informed consent.

### SNP selection & genotyping

DNA was isolated from peripheral blood samples by standard procedures. The *SV2C* rs30196 and rs10214163 polymorphisms were chosen because they were considered GWAS top hits [15]. These polymorphisms were genotyped with TaqMan SNP genotyping assays (Applied Biosystems, CA, USA), according to the manufacturer recommended protocol. The *SLC6A3* 3′ UTR VNTR (rs2836371) polymorphism was genotyped as previously described [11].

### Statistical analyses

Allele frequencies were estimated by gene counting. Agreement of genotype frequencies to Hardy-Weinberg expectations was tested using a goodness of fit $\chi^2$ test. Linkage disequilibrium was estimated using the Multiple Locus Haplotype Analysis (Mlocus 3.0) [18]. Because levodopa dose did not follow a normal distribution, univariate analyses between genotypes and levodopa daily dose were performed by Kruskal–Wallis. Comparisons of levodopa dose between gender and first motor symptom were assessed by Mann–Whitney U test. Correlations between continuous variables were evaluated with Spearman coefficient. For the multiple regression analyses levodopa dose was log-transformed because of the assumption of the test, which requires that the dependent variable has normal distribution, but untransformed means are shown in text and tables.

Confounders to be included in the multiple linear regression model were determined conceptually or with an association ($p \leq 0.20$) with levodopa dose and genotypes. Confounders for these analyses were gender, first motor symptom, disease duration, entacapone dose and pramipexole dose. An algorithm was elaborated with variables that presented at least $p < 0.05$ in the multiple linear regression model. Spearman correlation was used to compare the observed levodopa daily dose of patients and the predicted dose by the algorithm. Mean absolute difference of levodopa dose estimates was calculated by predicted dose minus observed dose. The observed levodopa daily dose was defined as the amount of levodopa that the patients was taking to control their symptoms, whereas the predicted dose was based on the expected dose that the patients would take based on the model developed in the present study.

All statistical analyses were performed with the SPSS 16.0 statistical package and a significance level of 5% was set in all analyses with two-tailed tests.

## Results

The demographic and clinical characteristics of the investigated subjects are shown in Table 1. About 52% of the patients were males, and their mean age was 66.8 ± 10.7. Tremor was the most common first symptom noticed (71.7%). All patients were in levodopa usage for at least 1 year; some patients were using other antiparkinsonian drugs: pramipexole (n = 46), bromocriptine (n = 2), entacapone (n = 22), tolcapone (n = 5), selegiline (n = 3) and amantadine (n = 41). The average levodopa dose in the studied sample was 715.4 ± 297.5 and ranged from 200 to 2000 mg/day.

Genotype frequencies for the two *SV2C* polymorphisms (rs10214163 and rs30196) are presented in Table 2. *SV2C* polymorphisms were not in linkage dis-

Table 1. Clinical and demographical characteristics of the study sample.

| Variables | Mean ± standard deviation | Range |
|---|---|---|
| **Characteristics** | | |
| Age | 66.8 ± 10.7 | 37–94 |
| Age at onset | 58.0 ± 11.5 | 28–84 |
| Disease duration | 8.7 ± 5.0 | 2–30 |
| **Medications (n)** | | |
| Levodopa (224) | 715.4 ± 297.5 | 200.0–2000.0 |
| Pramipexole (46) | 2.0 ± 1.1 | 0.25–4.0 |
| Bromocriptine (2) | 20.0 ± 7.0 | 15.0–25.0 |
| Tolcapone (5) | 262.5 ± 47.8 | 200.0–300.0 |
| Entacapone (22) | 495.4 ± 205.8 | 200.0–1000.0 |
| Selegiline (3) | 8.3 ± 2.9 | 5.0–10.0 |
| Amantadine (41) | 260.1 ± 87.5 | 25.0–400.0 |

Age, age at onset and disease duration are measured in years. Medications are measured by mg/day. Characteristics in table were calculated from total sample (n = 224), except for medications.

equilibrium (D′ = 0.177; p = 0.071) as expected since they were mapped in two distinct haploblocks [18]. *SLC6A3* 3′ VNTR (rs2836371) repeat alleles varied from 3 to 11. Genotypes 9R/9R 9R/10R and 10R/10R were the most frequent (Table 2). Other *SLC6A3* less frequent genotypes were grouped. Frequencies of *SV2C* and *SLC6A3* genotypes were in Hardy–Weinberg equilibrium.

Univariate analyses showed that levodopa dose was associated with *SV2C* rs30196 genotypes (p = 0.024) (Table 2). The presence of each C allele reduced the average dose in approximately 76 mg/day. *SV2C* rs10214163 and *SLC6A3* 3′VNTR were not associated with levodopa dose. At the univariate analysis *SLC6A3* VNTR was not associated with levodopa dose, but since in our previous study *SLC6A3* polymorphism was associated with levodopa dose in a multivariate analysis, controlling for other factors as gender, disease duration and tremor as first symptom [11], a pooled genetic variable was created including *SV2C* rs30196 genotypes and the presence of the *SLC6A3* 9R allele. Considering both genes the pooled variable was coded as 0 (no risk allele in both genes), 1 (one risk allele in either gene), 2 (two risk alleles) and 3 (three risk alleles). Levodopa dose varied from 820.1 ± 352.9 mg/day in those without risk alleles to 630.2 ± 297.5 mg/day (p = 0.075) in those with three risk alleles (Table 2).

Biological and pharmacological factors influencing levodopa dose were assessed by univariate analysis. Males used a higher levodopa daily dose (779.4 ± 308.9 mg/day) than females (646.6 ± 269.6 mg/day; p = 0.001). Patients with tremor as first symptom were on lower levodopa dose compared with those who presented rigid/bradykinesia as first symptom (p = 0.004). Positive correlations between levodopa dose and disease duration ($r_s$ = 0.354; p < 1 × $10^{-7}$), entacapone dose ($r_s$ = 0.455; p = 0.033) and pramipexole dose ($r_s$ = 0.397; p = 0.006) were found. Negative correlation between age at onset and levodopa dose was observed ($r_s$ = −0.247; p = 0.001). Tolcapone, selegiline, bromocriptine and amantadine dose and age were not correlated with levodopa dose.

The variables selected by univariate analyses (p ≤ 0.20) were: gender (p = 0.001), PD first motor symptom (p = 0.004), disease duration (p < 1 × $10^{-7}$), entacapone dose (p = 0.033), pramipexole dose (p = 0.006) and the pooled genetic variable (p = 0.075). Age at onset was not included in the model because it is highly correlated with disease duration. The factors that were statistically significant (p < 0.05) in the multiple linear regression are presented in Table 3. The model explained 23% of levodopa dose variation (F = 11.54; p < 0.000001; adjusted $R^2$ = 0.23). The number of risk alleles varied from 0 (no risk allele) to 3 (three risk alleles) (p = 0.005; B = -0.04; 95% CI: -0.07 to -0.01). Mean levodopa doses varied from 670.0, 632.1 and 572.6 mg/day in patients with 1, 2 or 3 risk alleles, respectively. Therefore *SLC6A3* 9R allele or *SV2C* rs30196 C allele influence were considered additive.

The correlation between the predicted dose and the observed dose in patients was statistically significant (p < 0.0001; 95% CI: 0.4–0.6) with a Spearmans correlation coefficient $r_s$ = 0.51 (Figure 1). The levodopa dose estimates mean absolute difference was 201.1 mg/day.

In the present study, we decided to present levodopa dose results since our objective was to study factors asso-

Table 2. Frequencies of *SV2C* and *SLC6A3* genotypes and their relation with levodopa dose in patients.

| Polymorphisms | | Frequencies (n) | Levodopa daily dose (mg) | p-value |
|---|---|---|---|---|
| *SV2C* rs30196 | AA | 25.5 (57) | 791.2 ± 333.4 | 0.024 |
| | AC | 46.4 (104) | 719.9 ± 292.0 | |
| | CC | 28.1 (63) | 639.3 ± 255.1 | |
| *SV2C* rs10214163 | TT | 68.3 (153) | 696.4 ± 274.5 | 0.359 |
| | CT | 27.7 (62) | 736.9 ± 324.1 | |
| | CC | 4.0 (9) | 890.2 ± 434.4 | |
| *SLC6A3* rs2836371 | 10R/10R | 50.0 (112) | 728.2 ± 298.9 | 0.547† |
| | 9R/10R | 38.8 (87) | 695.5 ± 296.1 | |
| | 9R/9R | 5.4 (12) | 654.1 ± 322.6 | |
| | Other | 5.8 (13) | 794.2 ± 281.7 | |
| Pooled genetic variable (*SLC6A3/SV2C*) | 0 | 14.7 (33) | 820.1 ± 352.8 | 0.075 |
| | 1 | 35.3 (79) | 733.1 ± 302.6 | |
| | 2 | 37.1 (83) | 686.7 ± 249.5 | |
| | 3 | 12.9(29) | 630.2 ± 318.5 | |

Difference in levodopa daily dose between genotypes calculated by Kruskal–Wallis. Levodopa daily doses are expressed in mean ± standard deviation.
†The sample had 45.1% of 9R allele presence and 54.9% of 9R allele absence. Difference of mean levodopa daily dose between subjects with or without of 9R allele was calculated by Mann–Whitney U test (694.2 ± 298.2 mg/day versus 732.8 ± 296.2 mg/day; p = 0.201).

ciated with levodopa dose prescription, this approach seems to be more relevant to clinical practice; however, similar results were also obtained when equivalent doses were tested (data not shown but available upon request).

## Discussion

In this study, the influence of common SNPs in *SV2C* and *SLC6A3* genes and their influence on levodopa dose variation were investigated. Several environmental factors that could potentially influence levodopa dose were also investigated. Based on univariate findings an algorithm was created by multiple regression analysis to predict dose variation. Overall the model explained 23% of dose variability.

In this study, a *SV2C* variant was suggested to influence levodopa dose. *SV2C* rs30196 C allele was associated with lower doses of this drug in PD patients. *SV2C* gene variation appears to modulate the protective effect of nicotine on PD risk [15]. The strongest protective effect was observed in homozygous individuals for the rs30196 allele C [15]. SV2C expression is inversely linked to tyrosine-hydroxilase (TH) expression, SV2C knockout mice showed a larger production of TH mRNA [19]. As TH is the rate-limiting enzyme in the production of dopamine, SV2C may play a critical role in dopamine activities [19]. However the exact function of SV2 proteins is still not clear. Feany *et al.* [20], considered that it might be a transporter of neurotransmitters into vesicles based on its structure. But, several evidences suggested that this protein might be involved in calcium-dependent exocytosis regulation [21–23]. Studies in SV2 knockout mice showed calcium accumulation and abnormal increase of neurotransmitter release [21]. Taken together these findings suggest that endogenous levodopa levels might be modulated by SV2C variants, therefore it is possible to hypothesize that rs30196 C allele carriers may need less levodopa than their counterparts with the AA genotype. Notice however that functional studies are clearly needed to explain the role of *SV2C* gene variants in levodopa treatment response.

The *SV2C* genotypes contribution for levodopa dose variation was evaluated pooled with *SLC6A3* 3′ VNTR polymorphism because a previous study in the same population reported that the presence of the *SLC6A3* 9R allele was associated with lower levodopa equivalent doses in a multivariate model [11]. Few genes were previously associated with levodopa dose [8]. DRD2 variants were not included because it was not associated with levodopa dose in our sample [17]. *COMT, MAOB, TH, ADDC* has already been linked to levodopa dose in some studies but not in all [8], and have not been explored in this sample.

Several *in vitro* studies reported that cells containing the 10R allele had higher expression of SLC6A3 pro-

| Table 3. Multiple linear regression analyses of predicting factors in levodopa dose variability. | | | |
|---|---|---|---|
| **Covariates** | **Regression coefficient (B)** | **95% CI for B** | **p-value** |
| Constant | 2.788 | 2.71–2.86 | <0.0001 |
| Gender (male) | 0.071 | 0.03–0.11 | 0.002 |
| Tremor as first motor symptom | -0.076 | -0.11 to -0.03 | 0.004 |
| Sum of alleles (SLC6A3/SV2C) | -0.038 | -0.07 to -0.01 | 0.003 |
| Entacapone dose (mg/day) | 0.001 | 0.0005–0.0030 | 0.008 |
| Pramipexol dose (mg/day) | 0.026 | 0.03–0.05 | 0.030 |
| Disease duration (years) | 0.010 | 0.05–0.01 | <0.0001 |
| $R^2$ | 0.252 | | |
| Adjusted $R^2$ | 0.230 | | |

$p < 0.000001$.
F = 11.54.
SLC6A3: Dopamine transporter; SV2C: Synaptic vesicle isoform C.

tein compared with 9R or other alleles [24,25]. In contrast, the functionality of the VNTR alleles was inconclusive based on SPECT studies meta-analysis [26], but two PET scan studies with young healthy individuals reported that *SLC6A3* 9R carriers presented higher protein availability [27,28]. Shingai *et al.* [29] described that SLC6A3 levels decline 3.4% per-decade in *substantia nigra*, reaching 7.6 and 7.7% per-decade in caudate and putamen, respectively. This age related decrease seems to be higher in 9R/9R homozygotes [28]. Therefore, we can hypothesize that carriers of 9R may present lower levels of SLC6A3, and consequently PD patients with this genotype would reuptake less levodopa from the synaptic cleft, and thus, would need less levodopa.

Among the demographic and clinical variables included in the model, gender was associated with levodopa dose as in other studies [30,31], but it remains unclear if this association was due to patients weight or if it is linked to gender differences. Tremor as first motor symptom was associated to lower levodopa doses. In a longitudinal study, tremor-dominant subtype was associated with slower rate of disease progression and a better improvement with levodopa drug compared with akinetic/rigid subtype [32]. We have also shown that co-medication is an important issue on levodopa dosing. Entacapone and pramipexole are important to determine levodopa dose. These associations could represent those patients in need for a variety of agents that probably presents higher dopaminergic demand. The use of MAOB and COMT inhibitors that were not associated with levodopa dose probably did not presented significant associations due to the low number of patients in use of them (three in use of MAOB inhibitors and five in use of tolcapone, Table 1). Disease duration was also associated with levodopa dose variation in this model. It seems that the longer the disease progresses, higher doses are needed by patients.

The correlation between observed levodopa dose and that predicted by the algorithm was good, considering that the model explained only part of dose variation. The mean absolute difference was 201.1 mg/day and could be interpreted as small, once the standard deviation from levodopa mean dose in the population was higher (297.5 mg/day).

This work should be interpreted in the context of some limitations. First, information about weight, height, and diet from patients were not available. Weight is an important factor inversely correlated with levodopa plasma concentrations [33,34]. Rich protein diet is reported to have a relation with lower levodopa intake because levodopa competes with large neutral amino acids for uptake [35]. But, all patients were from the same outpatient clinic and as routine they were oriented to taken levodopa at least 1 h away from meals. Second, dyskinesia that might be related to levodopa dose was not included in the model, because our pri-

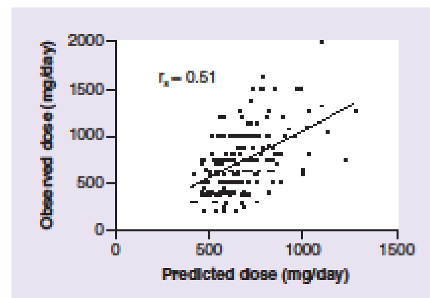


**Figure 1. Relationship between the predicted dose and observed dose in the investigated patients.**

mary goal was levodopa dose, moreover *SVC2* and *SLC6A3* polymorphisms were not associated with the occurrence of dyskinesia. Our study proposed a regression model that explained 23% of the levodopa dose variation only. Taking into account that levodopa dose variability is a multifactorial feature, there are many other factors and genes that might have an effect. The present results could be considered as a first attempt to understand levodopa dose variability. Nevertheless, more studies are needed with more genes, clinical, demographic and pharmacological variables to obtain an algorithm that could explain at least 80% of the variability to be useful in clinical practice.

## Conclusion

Our findings reveal the influence of *SV2C* and *SLC6A3* genetic variability on levodopa dose in PD patients. The model proposed predicted 23% of levodopa dose variability considering besides genes, comedication and clinical factors. This work suggests the first pharmacogenetic algorithm for levodopa dose prediction. However, predicting the optimal dose and response of a drug in a patient is still a target distant from application in routine clinical practice. There may be clinical, biological and genetic factors that contribute to the pharmacokinetic and pharmacodynamic variability of levodopa dose within and between individuals remaining to be elucidated. In Parkinson's disease, levodopa-induced adverse effects are very disabling and of great importance to be prevented. The present results could be considered as a first attempt to understand the factors that influence levodopa dose, more studies with larger samples in independent cohorts are warranted to validate the present results and increase the proportion of the variance that remained unexplained by our model.

## Future perspective

Although a good prediction model was obtained, it still needs to be tested in an independent sample to be validated for possible clinical use. Moreover new factors should be investigated to improve the variability explained by the algorithm proposed. Prospective validation of algorithms to evaluate the clinical efficacy and safety is also highly warranted before it could be introduced in clinical use.


### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.


### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.


### Executive summary

**Background**

- Variability in levodopa dose response in Parkinson's disease impacts clinical practice however its determinants are not fully established.
- Polymorphisms in genes related to dopamine transport as *SV2C* and *SLC6A3* might have a role on levodopa dose variability.

**Patients & methods**

- A total of 224 Parkinson's disease patients with a mean age of 66.8 years were enrolled. Clinical and demographic data from all patients were also assessed.
- Patients were genotyped for rs30196 and rs10214163 polymorphisms at *SV2C* gene and rs2836371 polymorphism at *SLC6A3* gene.
- The association between genetic, clinical and demographic data with levodopa dose was assessed by multiple linear regression.

**Results**

- The levodopa dose for best motor effect was determined clinically to be 715.4 ± 297.5 mg/day.
- At the univariate analyses a pooled genetic variable including *SV2C* and *SLC6A3* genotypes was created.
- The model for levodopa dose prediction which included the pooled genetic variable, explained 23% of dose variation ($F = 11.54$; $p < 0.000001$).
- The correlation between the predicted and observed dose was statistically significant ($r_s = 0.51$; $p < 0.0001$).

**Conclusion**

- Although a good prediction model was obtained, it still needs to be tested in an independent sample to be validated for possible clinical use.

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# CAPÍTULO VIII

## *Discussão*

Discussões referentes aos resultados específicos obtidos nesta tese foram realizadas nos capítulos III a VII. Neste capítulo, discutiremos os aspectos mais gerais relacionados aos estudos de suscetibilidade, interação gene-ambiente e farmacogenética da DP.

Vários estudos epidemiológicos identificaram fatores ambientais de risco e de proteção para DP. No entanto poucas investigações sobre as interações desses fatores com genes foram realizadas. Os estudos de interação gene-ambiente já realizados mostraram diversos genes como possíveis moduladores do efeito do ambiente na DP. O presente trabalho evidenciou mais três genes com papel importante nas interações com o ambiente para a susceptibilidade a DP: ABCB1 x fumo, SV2C x exposição a tóxicos e NOS1 x cafeína, ainda não descritos. Algumas interações gene-ambiente já tinham sido descritas na literatura tais como MAOB x fumo (Checkoway et al., 1998), CYP1A2 e CYP1A1 x cafeína (Amin et al., 2012), ABCB1 x exposição à pesticidas (Dutheil et al., 2010) e PON1 x exposição a pesticidas organofosfatos (Lee et al., 2013). Assim como em outras pesquisas (Hernán et al., 2002; Tan et al., 2003; Fong et al., 2005; Dick et al., 2007; Gu et al., 2010; De Palma et al., 2010; Hill-Burns et al., 2011; Kiyohara et al., 2013), as associações antes mencionadas não foram encontradas neste trabalho. Os resultados dos estudos gene-ambiente na DP até o momento ainda são controversos, impossibilitando conclusões definitivas sobre o efeito dos genes e suas interações com variáveis ambientais já identificadas com a suscetibilidade a DP. Várias limitações desses trabalhos poderiam ser apontadas para a inexistência de resultados conclusivos tais como amostras pequenas, com diferentes composições étnicas que, impossibilitam uma comparação direta entre elas ou um agrupamento dos dados para aumentar o poder estatístico. Uma solução para esse tipo de problema seria aumentar o tamanho amostral dos estudos, incluindo uma amostra independente para replicação. A formação de parcerias entre grupos é o mais indicado para alcançar o número amostral adequado, juntamente com uma padronização dos questionários clínicos e ambientais aplicados aos pacientes e controles. Além disso, a

maioria dos estudos se baseia na análise de genes-candidato como as realizadas no presente trabalho. Uma abordagem sem hipótese *a priori* como o GWAIS (genome-wide association and interaction study) poderia revelar novos genes ainda não evidenciados em estudos com genes únicos.

Até o presente, somente dois genes foram evidenciados pela abordagem de GWAIS na DP. O *SV2C*, investigado nesta tese, e o *GRIN2A*. O *GRIN2A* foi associado à DP em interação com o consumo de cafeína (Hamza et al., 2011). O GRIN2A é um receptor glutamatérgico envolvido na neurotransmissão excitatória e dessa forma poderia estar envolvido na neurodegeneração. Três trabalhos tentaram replicar essa associação, destes, apenas um (Yamada-Fowler et al., 2014) confirmou esse resultado enquanto que essa associação não foi observada em outros dois estudos (Ahmed et al., 2014; Kim et al., 2018). Existem importantes limitações que poderiam explicar essas discrepâncias nesses estudos tais como: amostras menores que o estudo de GWAIS, diferentes exposições à cafeína entre as populações, falta de ajuste de confundidores e de pareamento das amostras. Visto que os resultados obtidos até o presente sobre o *GRIN2A* trazem muitas limitações e que esse gene possui plausibilidade biológica de associação a DP, mais estudos ainda são necessários.

Os resultados obtidos no presente trabalho bem como os descritos na literatura mostraram que o gene *SV2C* já foi associado com a dose de levodopa (capítulo VII), a exposição à tóxicos (capítulo IV) e ao fumo na DP (Hill-Burns et al., 2013). Evidências experimentais revelaram que camundongos knock-out para SV2C apresentavam menores concentrações de dopamina no estriado quando comparados aos camundongos controles (Dunn et al., 2017). Visto que o SV2C parece auxiliar no trafego vesicular, participando na captação e englobamento de neurotransmissores, um sequestro ineficaz de dopamina poderia levar ao estresse celular, aumentando a vulnerabilidade do neurônio a tóxicos, e culminando na morte celular. No entanto, até o presente, não foram realizados experimentos que comprovem variação funcional entre os alelos de *SV2C*. Os estudos de associação

mostraram que o risco para a DP poderia estar sendo modulado por alelos específicos juntamente com substâncias protetoras ou de risco como fumo e a exposição a tóxicos. Já a variação de dose de levodopa tomada pelos pacientes observada entre genótipos do *SV2C* poderia ser decorrente do número de neurônios que ainda estão produzindo a dopamina na *substantia nigra*. Chama a atenção que o mesmo alelo que está associado a menores doses de levodopa, também está associado à menor risco de DP em fumantes (Hill-Burns et al., 2013). Enquanto que o alelo que foi associado a maiores doses de levodopa (capítulo VII), parece estar associado a maior risco de DP quando exposto a tóxicos (capítulo IV).

Uma grande preocupação dos estudos de associação e de interação é a estratificação populacional. Em um país como o Brasil, onde a população é altamente miscigenada, a preocupação em corrigir efeitos da estratificação é ainda maior (Pena et al., 2011). Diferenças de ancestralidade entre amostras (ex: caso e controle) podem levar a resultados espúrios ou mascarar verdadeiras associações. Uma forma de reconhecer e corrigir a estratificação populacional nos estudos com pequenas amostras é utilizando marcadores informativos de ancestralidade (AIMs). Em estudos com grande número amostral e muitos marcadores como nos GWAS, a estratificação pode ser corrigida também com o uso de análise de componentes principais (Huckins et al., 2014).

Em uma amostra como a deste trabalho, o uso de AIMs seria o mais adequado e resultaria em correções mais precisas para diferenças de ancestralidade entre casos e controles. Devido aos altos custos para a aplicação desses marcadores essa abordagem não pôde ser realizada. No entanto, todas as análises incluíram o fenótipo “cor da pele”, definido por auto-declaração como co-variável, além disso, não foram verificadas diferenças de frequências genotípicas entre pessoas auto-declaradas brancas ou não-brancas. Foram feitas também análises secundárias apenas com indivíduos brancos, obtendo-se essencialmente o mesmo resultado, exceto para a associação entre ABCB1 e fumo (capítulo V).

A instigante observação de que o cigarro e o café são importantes fatores de neuroproteção os coloca em evidência para estudos de novos medicamentos para atuar na terapia e prevenção da DP.  Apesar de se acreditar que esses fatores atuem por vias diferentes, as duas substâncias são estimulantes e atuam no sistema dopaminérgico. Até o momento não se sabe exatamente qual das substâncias presentes no café, no chimarrão e no tabaco são determinantes para essa neuroproteção. Compostos fenólicos encontrados nessas três substâncias, como o ácido clorogênico e quínico poderiam ser bons candidatos para o inicio desta investigação (Tso et al., 1970; Filip et al., 2001; Farah and Donangelo, 2006).

Os efeitos dos compostos fenólicos, presentes também em outros alimentos, são amplos, e envolvem efeitos antioxidantes, anti-inflamatórios, antiplaquetários e antialergênicos (Miean & Mohamed, 2001). Descobrir o composto exato ou o conjunto deles que promove a neuroproteção seria importante para definir como o café, o chimarrão e o tabaco exercem esse efeito.  Processos químicos que segregam compostos do cigarro e do café poderiam permitir definir quais desses compostos participam mais ativamente da neuroproteção das células da *substantia nigra* em modelos animais. Uma vez conhecendo a(s) substância(s) e seu modo de ação seria possível investigar mais especificamente o seu papel na neuroproteção bem como quais genes e variantes de rotas metabólicas específicas estariam envolvidos. Essas abordagens permitiriam de uma maneira mais eficaz e precisa, servir de base para o desenvolvimento de fármacos para neuroproteção.

A farmacogenética da levodopa evidenciou a possibilidade de tratamento personalizado para os pacientes com DP, sem a necessidade de introduzir novos medicamentos em sua terapia. Um dos objetivos desses estudos seria otimizar as doses para reduzir os efeitos adversos da levodopa e aumentar a qualidade de vida dos pacientes. Esses estudos até o presente ainda não trouxeram resultados imediatamente aplicáveis nas decisões clínicas (Schumacher-Schuh et al., 2014; Hutz & Rieder, 2018).

A administração crônica da levodopa embora seja eficaz para reduzir os sintomas da DP, induz a uma série de fenômenos indesejados como discinesias, flutuações motoras e alucinações. Na procura por diminuir esses efeitos adversos, novos medicamentos e biocompostos estão sendo testados pela indústria farmacêutica.

A farmacogenética se baseia na variabilidade genética dos indivíduos. Porém, a resposta à medicação não depende somente da variabilidade genética, é uma característica multifatorial, envolvendo dados clínicos, farmacológicos, biológicos e de hábitos dos pacientes também. Dentre as variáveis biológicas, vem ganhando destaque nas pesquisas com DP o estudo da microbiota do sistema gastrointestinal.

A microbiota afeta a biologia do hospedeiro pela participação na digestão de alimentos, produção de vitaminas, metabolização de drogas e degradação de toxinas. Além disso, o conjunto dos genomas da microbiota, o microbioma, afeta o hospedeiro pela atividade de seus genes e pela manipulação da expressão dos genes humanos (Payami, 2017). Existem duas vias de interação entre o microbioma e as medicações. Uma em que o microbioma facilita o metabolismo dos medicamentos prescritos, e a outra em que a mediação pode afetar a composição do microbioma dos indivíduos.

O uso de inibidores da COMT e de anticolinérgicos já foi associado a diferenças quantitativas em gêneros de bactérias nos pacientes com DP em dois estudos (Scheperjans et al., 2015; Hill-Burns et al., 2017).

O microbioma pode afetar a eficácia e toxicidade das medicações ou causar efeitos adversos nos pacientes, sendo uma variável importante nos futuros estudos de farmacogenética. Além disso, determinar a melhor composição do microbioma para cada fármaco ou conjunto delas pode ajudar a desenvolver um tratamento de reposição da microbiota.

Como amplamente discutido os resultados aqui apresentados ainda não são conclusivos, na perspectiva de continuar a pesquisa desenvolvida nessa tese, seria necessário primeiramente aumentar a amostra, para que se possam obter dados mais robustos principalmente nos estudos de interação gene-ambiente.

A avaliação de mais polimorfismos em cada gene também poderia contribuir para um panorama mais claro dos significados das associações encontradas. Por exemplo, a interação da cafeína e *NOS1* (capítulo VI) poderia ser melhor avaliada se os dois polimorfismos da *NOS1* (rs1047735 e rs2682826), localizados em éxons fossem também genotipados. Como discutido nesse capítulo, a maioria dos estudos não mostraram esses polimorfismos como fator de suscetibilidade para a DP. Porém o papel dessas variantes modulado pela quantidade de cafeína ainda não foi investigado.

No capítulo III abordou-se o estudo de um conjunto de genes derivados de GWAS na suscetibilidade a DP. Foi visto que com a contribuição de oito polimorfismos, chegou-se próximo do poder de um teste de triagem para a DP. O modelo obtido no presente trabalho (AUC = 0,744) não difere em poder discriminatório do modelo observado por Nalls et al. (2016), com trinta variantes genéticas (AUC = 0,748). Dessa forma, a questão que permanece em aberto é se o efeito seria maior apenas incluindo um número maior de polimorfismos. O ideal seria buscar novas variantes para que se possa desenvolver um teste de diagnóstico considerando também as variáveis clínicas e demográficas. Apesar de não estar associado em estudos de GWAS, o polimorfismo L55M da *PON1* poderia também ser inserido neste modelo, uma vez que foi associado à DP na nossa população (capítulo IV).

A fim de explorar o efeito dos genótipos do *SV2C* na dose de levodopa, pretendemos realizar o desafio oral de levodopa. O desafio consiste em grupos dos genótipos de extremos de dose do SV2C que seriam avaliados em relação à resposta motora após tomar uma dose da levodopa. A hipótese é de que pacientes que tem genótipo associado a

menores doses de levodopa (CC), possuiria melhor resposta à medicação e por maior tempo, comparado ao genótipo AA.

A DP possui uma alta prevalência e incidência entre os idosos (Pringsheim et al., 2014; Hirsch et al., 2016). Muitos pacientes recebem o diagnóstico errado ou demoram muito para serem diagnosticados com DP. O diagnóstico precoce e o tratamento personalizado poderia diminuir os efeitos adversos e aumentar a qualidade de vida dos pacientes.

Este trabalho enriqueceu o conhecimento da variabilidade da DP tanto em aspectos de suscetibilidade quanto farmacogenética. Os dados obtidos serão importantes na continuação de pesquisas genéticas em relação à doença. É importante salientar que abordagens que cruzam os dados da genômica à transcriptômica, proteômica, metagenômica e metabolômica irão fornecer um melhor panorama da etiologia e patofisiologia da DP para auxiliar no tratamento e diagnóstico da DP.

# CAPÍTULO IX

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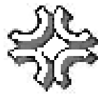
# *Anexos*



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

**COMISSÃO CIENTÍFICA**

A Comissão Científica do Hospital de Clínicas de Porto Alegre analisou o projeto:

**Projeto:** 140554
**Data da Versão do Projeto:** 30/09/2014

**Pesquisadores:**
CARLOS ROBERTO DE M RIEDER
VIVIAN ALTMANN

Título: A CONTRIBUIÇÃO DE VARIANTES DO DNA MITOCONDRIAL COMO FATOR DE SUSCEPTIBILIDADE PARA A DOENÇA DE PARKINSON

Este projeto foi APROVADO em seus aspectos éticos, metodológicos, logísticos e financeiros para ser realizado no Hospital de Clínicas de Porto Alegre.
Esta aprovação está baseada nos pareceres dos respectivos Comitês de Ética e do Serviço de Gestão em Pesquisa.

- Os pesquisadores vinculados ao projeto não participaram de qualquer etapa do processo de avalição de seus projetos.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao Grupo de Pesquisa e Pós-Graduação (GPPG)

Porto Alegre, 02 de dezembro de 2014.

Prof. José Roberto Goldim
Coordenador CEP/HCPA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

**Projeto de pesquisa:** Farmacogenética da Doença de Parkinson: associação entre polimorfismos nos genes DRD1, DRD2, DAT1, AADC, COMT e MAO-B com complicações do uso de levodopa.

**Pesquisadores**: Artur Schuh, Raquel Townsend, Mariana Socal, Daniele Fricke, Thaís Monte, Carlos Rieder e Mara Hutz.

**Pesquisador Responsável:** Dr. Carlos Rieder
Serviços de Neurologia - Hospital de Clínicas de Porto Alegre.
Telefones para contato: 2101-8182 e 2101-8520.

O Serviço de Neurologia deste hospital e o Departamento de Genética da UFRGS estão promovendo o projeto de pesquisa "Farmacogenética da Doença de Parkinson". A levodopa é das drogas mais freqüentemente utilizadas no tratamento da doença de Parkinson e é a que melhor controla os sintomas motores desta doença. Algumas pessoas toleram muito bem a levodopa, outras apresentam alguns efeitos adversos. Os efeitos adversos mais comuns são os movimentos involuntários, tipo balanceios do corpo, chamados de discinesias. Outro efeito indesejável que alguns pacientes apresentam com o tempo de uso é o encurtamento da ação. Isso significa que algumas pessoas tomam o remédio porém ele não dura o tempo suficiente no organismo. A resposta ao tratamento e os efeitos indesejáveis não são os mesmos em todas as pessoas que usam levodopa.

Este estudo quer identificar possíveis causas genéticas para as diferenças na resposta ao uso de levodopa na doença de Parkinson. Encontrar um papel genético na resposta de cada pessoa é importante para entendermos melhor a doença e o seu tratamento.

O estudo envolverá pacientes em atendimento neste hospital e consistirá em uma avaliação clínica na consulta e na realização de um exame físico neurológico. Será, ainda, feita uma avaliação com testes de memória e testes para avaliar sintomas depressivos. Toda a consulta levará em torno de 40 minutos.

**Em seguida os pacientes serão encaminhados para coleta de sangue (para extração do DNA).**

O material genético que sobrar poderá ser conservado (armazenado) ou não, conforme a decisão da cada paciente. O que ficar armazenado poderá ser utilizado em novos exames: estudo de outros genes em novas pesquisas. No caso de serem propostas novas pesquisas com este material, elas serão avaliadas pelos Comitês de Ética em Pesquisa local e nacional, e somente serão realizadas mediante nova autorização do paciente para aquele estudo específico.

Toda a participação neste estudo é absolutamente confidencial (os dados serão utilizados sem identificação do paciente), bem como os resultados da avaliação clínica e dos exames genéticos. É permitida a desistência em qualquer fase da avaliação, sem qualquer tipo de problema para o participante. O estudo será financiado por recursos já aprovados para estudos de farmacogenômica pelo CNPq (Processo nº 47.256/2006-4) e dos Institutos do Milênio para equipe do Departamento de Genética da UFRGS e outras agências de fomento, sendo que não haverá

custo algum para o paciente ou seus familiares. O presente projeto foi avaliado e aprovado pelo Grupo de Pesquisa e Pós-Graduação e pelo Comitê de Ética deste hospital. Os pacientes e familiares serão informados dos resultados da pesquisa.

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – PROJETO: Farmacogenética da Doença de Parkinson: associação entre polimorfismos nos genes DRD1, DRD2, DRD3, DAT1, AADC, COMT e MAO-B com complicações do uso de levodopa.**

Eu, ___________________________________, declaro que fui informado de que participarei do projeto de pesquisa "Farmacogenética da Doença de Parkinson" acima citado. Fui informado de que minha decisão em participar não comprometerá meu tratamento neste hospital, sendo meus dados e resultados de meus testes absolutamente confidenciais. Além disso, fui informado de que a qualquer momento posso desistir do estudo, sem qualquer problema para meu tratamento. Declaro que aceito participar do estudo e que meus dados sejam incluídos na análise coletiva dos resultados sem identificação.

( ) **SIM**: autorizo manter meu material genético excedente (DNA) armazenado, sabendo que poderá ser usado em meu benefício diagnóstico direto, no futuro, ou para novas pesquisas, das quais serei informado e poderei novamente optar em participar ou não

( ) **NÃO**: não autorizo armazenar meu material genético após este exame.

Porto Alegre, ___ de __________________ de 200_.

Ass: ____________________________________________________

( ) paciente ( ) familiar responsável – nome:__________________________

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Projeto de pesquisa: A CONTRIBUIÇÃO DE VARIANTES DO DNA MITOCONDRIAL COMO FATOR DE SUSCEPTIBILIDADE PARA A DOENÇA DE PARKINSON

TERMO PARA CONTROLES

Pesquisadores: Vivian Altmann, Dr. Artur Schuh, Dr. Emilio Moriguchi, Dr. Carlos Rieder e Dra. Mara Hutz.

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Serviços de Neurologia - Hospital de Clínicas de Porto Alegre.
Telefones para contato: 3359-8182 e 3359-8520.

Você está sendo convidado (a) a participar em um projeto de pesquisa envolvendo pacientes e famílias de indivíduos com doença de Parkinson e indivíduos sem esta doença. O objetivo do estudo é identificar a alteração genética que causa a doença de Parkinson.

A participação neste estudo envolve responder a um questionário com perguntas sociais e demográficas (idade, sexo, escolaridade, cor) e uma avaliação clínica e, também uma coleta de 4 ml de sangue venoso será efetuada. Os riscos associados a esse procedimento são mínimos, podendo ocorrer dor e manchas roxas (hematoma) no local da coleta do sangue. O desconforto será mínimo, pois se trata de uma coleta de sangue geralmente da veia do braço que será realizada por profissional treinado e habilitado para realizar esse procedimento. Com a amostra de sangue será possível extrair o DNA e, com isso, realizar testes para verificar variações entre os indivíduos.

Para realizar este estudo é necessário comparar um grupo de pessoas que possuem a doença de Parkinson com um grupo de pessoas que não possuem a doença, caso aceite participar, você será incluído (a) no grupo de pessoas sem a doença de Parkinson.

A participação nesse estudo não trará nenhuma vantagem direta a você. Contudo, os resultados desse estudo podem, a longo prazo, auxiliar em um melhor diagnóstico e tratamento da doença de Parkinson.

A participação nesse estudo é voluntária, podendo se recusar a participar, assim como você poderá desistir de participar em qualquer momento, sem prejuízo do atendimento recebido na instituição. Toda a participação neste estudo é absolutamente confidencial (os

dados serão utilizados sem identificação do participante), bem como os resultados da avaliação clínica e dos exames genéticos.

A participação no estudo não envolve custos e você não receberá nenhum tipo de pagamento por participar. Em caso de dúvidas você poderá contatar o pesquisador responsável: Dr. Carlos Rieder (Tel: 3359-8182), no serviço de Neurologia do Hospital de Clínicas de Porto Alegre, no 2° andar, ou a pesquisadora Vivian Altmann (Tel: 3308-6735). Você poderá também contatar o Comitê de Ética em pesquisa do Hospital de Clínicas de Porto Alegre, no 2" andar, sala 2227 ou pelo telefone 3359-7640, de segunda a sexta, das 8h às 17h.

Eu, _____________________________________________, declaro que fui informado de que participarei do projeto de pesquisa "A contribuição de variantes do DNA mitocondrial como fator de susceptibilidade para a doença de Parkinson" acima citado. Fui informado de que minha decisão em participar não comprometerá meu atendimento neste hospital, sendo meus dados e resultados de meus testes absolutamente confidenciais. Além disso, fui informado de que a qualquer momento posso desistir do estudo, sem qualquer problema para meu atendimento na instituição. Declaro que aceito participar do estudo e que meus dados sejam incluídos na análise coletiva dos resultados sem identificação.

Este termo é assinado em 2 vias, uma para o participante e outra para o pesquisador.

( ) SIM: autorizo manter meu material genético excedente (DNA) armazenado, sabendo que poderá ser usado em meu benefício diagnóstico direto, no futuro, ou para novas pesquisas, das quais serei informado e poderei novamente optar em participar ou não

( ) NÃO: não autorizo armazenar meu material genético após este exame.

Porto Alegre, ___ de ___________________ de 20__.

Assinatura: _______________________________________________________

Pesquisador: ______________________________________________

Assinatura: ________________________________________________