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# Biomarcadores na Doença de Machado-Joseph.

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**Lista de Abreviaturas**

AD – Doença de Alzheimer (*Alzheimer's disease*)

AO – idade de início (*age of onset)*

ADAS-Cog – *Alzheimer's Disease Assessment Scale - Cognitive Subscale*

BDNF – Proteína Fator Neurotrófico Derivado do Cérebro (*Brain-derived neurotrophic fator*)

BMR – taxa metabólica basal (Basal metabolic rate)

CCFS – *Composite-Cerebellar-Functional-Score*

CTNF – fator neurotrófico ciliar (*Ciliary neurotrophic factor*)

DRPLA – Atrofia dentatorublopalidolusiana

GSH-Px – Atividade da glutationa peroxidase (*glutathione peroxidase activity*)

GSK3β - glicogênio sintase cinase 3 beta (*Glycogen Synthase Kinase 3 Beta*)

HD – Doença de Huntington (*Huntington Disease*)

HDAC6 – histona desacetilase 6 (*Histone Deacetylase 6*)

HOMA2 – Avaliação do modelo homeostático (*homeostatic model assessment*)

ICARS – *International Cooperative ataxia rating Scale*

IMC – Índice de Massa Corporal ou BMI – Body mass index

INAS – *Inventory of Non-Ataxia Symptoms*

MJD – Doença de Machado Joseph (*Machado-Joseph Disease*)

MRI – Ressonância Magnética

NESSCA – *Neurological Examination Score for Spinocerebellar Ataxia*

NGF – fator de crescimento de nervoso (*nerve growth factor*)

NSE – enolase específica de neurônio (*neuron specific enolase*)

PD – Doença de Parkinson (*Parkinson's disease*)

PET – Tomografia Computadorizada por Emissão de Pósitrons

SARA – *Scale for the assessment and rating of ataxia*

SCA – Ataxia Espinocerebelar (*Spinocerebellar Ataxia*)

SCA3 – Ataxia Espinocerebelar tipo 3 (*Spinocerebellar Ataxia type 3*)

SD – Desvio padrão (*standart deviation*)

SPI – Sensibilidade periférica à insulina ou PSI – Peripheral sensitivity to insulin

TTG – Teste de tolerância à glicose ou GTT – glucose tolerance test

TtoDd – tempo para começar/duração da doença (*Time to onset/disease duration*)

UHDRS – *Unified Huntington's Disease Rating Scale*

UPDRS – *Unified Parkinson's Disease Rating Scale*

VOR – Reflexo vestíbulo ocular (*vestibulo-ocular reflex*)

**Resumo**

A doença de Machado-Joseph (MJD), também conhecida como ataxia espinocerebelar do tipo 3 (SCA3), é uma doença neurodegenerativa de início tardio. SCA3/MJD é a mais comum dentre as ataxias espinocerebelares no mundo, tendo uma alta prevalência no sul do Brasil (seis casos em cada 100.000 indivíduos). SCA3/MJD é caracterizada por degeneração do cerebelo e o principal sintoma é a ataxia; no entanto, os sintomas são variados. A doença inicia normalmente entre os 30-40 anos de idade, podendo se manifestar em qualquer idade até na infância e apresenta uma progressão lenta. A doença é causada por uma mutação no gene *ATXN3*, onde uma expansão de repetições CAG (acima de 52 repetições) leva o indivíduo a manifestar a doença em algum momento da vida, sendo que o número de repetições CAG está associado à idade de início e gravidade da doença. No entanto, o fato das repetições CAG não explicarem completamente essas características e a progressão da doença ser lenta, se faz necessário o estudo de biomarcadores para identificação do início da doença e sua progressão. Nesta tese, serão abordados possíveis biomarcadores de estado e progressão na SCA3/MJD. Através da revisão de estudos com biomarcadores em SCA3/MJD, identificou-se que a proteína NSE, a glutationa peroxidase, o reflexo vestíbulo-ocular e vídeo-oculografia são bons candidatos a biomarcadores de estado da doença e as escalas clínicas ainda são os melhores biomarcadores de progressão. Analisando a composição corporal, foi identificado que o IMC está associado a repetições CAG expandidas. No entanto, não achamos associação com a doença, provavelmente devido à seleção dos nossos casos. Além disso, os indivíduos com SCA3/MJD apresentaram maior sensibilidade periférica à insulina quando comparados aos controles. Essa sensibilidade não está associada ao índice de massa corporal (IMC) e se correlaciona com a idade e gravidade da doença. O estudo de proteínas apresentou alteração em algumas neurotrofinas, onde NGF mostrou níveis mais baixos em pacientes com SCA3/MJD do que em controles, além de diminuir os níveis ao longo de 48 semanas. Uma correlação positiva foi observada entre NGF e *Neurological Examination Score for Spinocerebellar Ataxia* (NESSCA), além de uma correlação inversa entre BDNF e

*Scale for the assessment and rating of ataxia* (SARA). Além disso, confirmamos níveis mais elevados de NSE. O CTNF diminui com a idade em controles, mas correlação semelhante em SCA3/MJD não atingiu significância estatistica. A análise de mRNA mostrou que pacientes com SCA3/MJD apresentam expressão reduzida de *HDAC6* e que o uso de lítio aumenta a expressão dos genes *HDAC6* e de *GSK3β*. O presente trabalho inclui o estudo de um amplo espectro de possíveis biomarcadores e é um dos poucos estudos que inclui análises prospectivas. Candidatos a biomarcadores de estado e progressão foram apresentados nesse estudo, contribuindo para a compreensão da fisiopatologia da doença e para possível uso em futuros ensaios clínico.

**Abstract**

Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a late-onset neurodegenerative disease. SCA3/MJD is the most common spinocerebellar ataxias worldwide, being highly prevalent in South Brazil, where it reaches six cases per 100,000 individuals. SCA3/MJD is characterized by cerebellum degeneration and ataxia is the main symptom of the disease. However, symptoms are diverse and progression is slow. The onset of disease usually begins at 30-40 years old, but onset can be seen at any age, even during childhood. SCA3/MJD is caused by a mutation at the *ATXN3* gene, where an expansion of CAG repeats (above 52 repeats) leads to disease onset at some point in life. CAG repeat length is inversely correlated with age of onset and severity. The fact that CAG length does not explain all disease characteristics and the disease presents a slow disease progression; biomarkers studies are required for identification of disease onset and progression. In this thesis, it will be shown some candidates to state and progression biomarkers in SCA3/MJD. Through review of studies on biomarkers, it was identified NSE protein, glutathione peroxidase, vestibulo-ocular reflex and video-oculography as good candidates to biomarkers of disease state and clinical scales are the best progression biomarkers to date. Analyzing body composition, it was identified an association between BMI and CAG expanded repeats. However, no association was found with the disease, probably due to the selection of our cases. In addition, individuals with SCA3/MJD had higher peripheral sensitivity to insulin when compared to controls. This sensitivity was not associated with body mass index (BMI), and correlated with age and disease severity. Alterations in neurotrophins were demonstrated, where lower levels of NGF were shown in SCA3/MJD patients than in controls, with further reduction during 48 weeks long period. A positive correlation was seen between NGF and Neurological Examination Score for Spinocerebellar Ataxia (NESSCA), and an inverse correlation was seen between BDNF and Scale for the assessment and rating of ataxia (SARA) in SCA3/MJD at baseline. In addition, higher NSE levels were confirmed. CTNF was shown to decrease with age in controls, and this correlation was not observed in SCA3/MJD. mRNA analyses showed that

SCA3/MJD patients have reduced expression of *HDAC6,* and that treatment with lithium increases *HDAC6* and *GSK3β* genes expression. We have studied a broad spectrum of possible biomarkers, and this is one of few studies including prospective analyses. Candidates to state and progression biomarkers were presented in this study, contributing to the understanding of disease physiopathology, and for a possible application in future clinical trials.

# 1 Introdução

## 1.1 Ataxias Espinocerebelares

As ataxias espinocerebelares (“Spinocerebellar Ataxia” – SCA) são um grupo de doenças neurodegenerativas hereditárias de início tardio com sintomas heterogêneos, sendo principalmente caracterizadas por uma lenta e progressiva disfunção cerebelar, resultando em uma marcha instável (ataxia), perda da coordenação dos movimentos dos membros superiores e problemas na fala (disartria). Elas também podem estar associadas com outros sinais neurológicos, tais como sinais piramidais ou extrapiramidais, comprometimento cognitivo e oftalmoplegia.

Os sintomas citados acima se fazem presentes no grupo como um todo, sendo que algumas delas podem apresentar sintomas específicos. Um exemplo é na SCA7 onde indivíduos veem a apresentar perda visual com retinopatia. Por serem doenças progressivas, os diferentes sintomas tendem a aparecer alguns anos após surgir o primeiro sintoma (este normalmente sendo a ataxia), tornando o diagnóstico baseado apenas na história clínica muitas vezes difícil. Isso faz com que a análise molecular dos genes associados às SCAs para a confirmação das suspeitas clínicas e um correto diagnóstico dos pacientes necessário.

Atualmente, mais de quarenta *loci* distintos associados às formas mendelianas de SCAs são conhecidos (Tabela 1). Todas elas apresentam modo de herança dominante e podem ser subdivididos em três grupos dependendo o tipo de mutação: 1) As ataxias de poliglutaminas, as quais são causadas por uma expansão de CAG nos éxons dos genes correspondentes (também conhecida como doenças de poliglutaminas); 2) ataxias de repetições em regiões não codificantes, e 3) ataxias causadas por mutações convencionais, como deleção, inserção, mutação de ponto e mutação em sítios de splicing (Soong & Paulson, 2007).

Tabela 1 – Genética molecular das ataxias espinocerebelares (adaptado de Bird, 2018)

| Doença | Gene ou lócus cromossômico | Mutação | Referência |
|---|---|---|---|
| SCA1 | *ATXN1* | Repetições CAG em exon | Subramony & Ashizawa, 2011 |
| SCA2 | *ATXN2* | Repetições CAG em exon | Pulst, 2010 |
| SCA3 | *ATXN3* | Repetições CAG em exon | Paulson, 2015 |
| SCA4 | *16q22.1* | --- | Edener et al., 2011 |
| SCA5 | *SPTBN2* | Mutações de ponto ou deleção | Ikeda et al., 2006 |
| SCA6 | *CACNA1A* | Repetições CAG em exon | Gomez, 2008 |
| SCA7 | *ATXN7* | Repetições CAG em exon | Garden, 2012 |
| SCA8 | *ATXN8/ATXN80S* | Repetições CAG-CTG | Ikeda et al., 2007 |
| SCA9[1] | ----- | --- | |
| SCA10 | *ATXN10* | Repetições ATTCT | Matsuura & Ashizawa., 2012 |
| SCA11 | *TTBK2* | Inserção ou deleção (mudança no quadro de leitura) | Houlden, 2008 |
| SCA12 | *PPP2R2B* | Repetições CAG | Margolis et al., 2011 |
| SCA13 | *KCNC3* | Mutação de ponto | Pulst, 2012 |
| SCA14 | *PRKCG* | Mutação de ponto | Chen et al., 2010 |
| SCA15[2] | *ITPR1* | Mutação de ponto ou deleção da região 5’ do gene | Storey, 2011 |
| SCA16[2] | *ITPR1* | --- | Gardner, 2008 |
| SCA17 | *TBP* | Repetições CAG-CAA em exon | Toyoshima et al., 2012 |
| SCA18 | *IFRD1* | Mutação de ponto (c.514 A>G) | Brkanac et al., 2009 |
| SCA19[3] | *KCND3* | Deleção de trinucleotídeos | Duarri et al., 2012 |

Continuação Tabela 1 – Genética molecular das ataxias espinocerebelares.

| Doença | Gene ou lócus cromossômico | Mutação | Referência |
|---|---|---|---|
| SCA20 | *11q12.2-11q12.3* | Duplicação de 260kb | Storey, 2012 |
| SCA21 | *SCA21* | Mutação de ponto (c.509C>T) | Vuillaume et al., 2002 |
| SCA22[3] | *1p21-q21* | --- | Chung et al., 2003. |
| SCA23 | *PDYN* | Mutação de ponto | Bakalkin et al., 2010 |
| SCA24[4] | *1p36* | --- | Swartz et al., 2002 |
| SCA25 | *SCA25* | --- | Stevanin et al., 2005 |
| SCA26 | *EEF2* | Mutação de não repetição | Hekman et al., 2012 |
| SCA27 | *FGF14* | Mutação de ponto ou Inserção (mudança no quadro de leitura) | Van Swieten et al., 2003 |
| SCA28 | *AFG3L2* | Mutação de ponto ou deleção dos exons 14-16 | Mariotti et al., 2008 |
| SCA29 | *ITPR1* | Mutação de ponto ou deleção ou mutação em sítio de splicing | Shadrina et al., 2016 |
| SCA30 | *4q34.3-q35.1* | --- | Storey et al., 2009 |
| SCA31 | *BEAN1* | Repetições TGGAA | Sato et al., 2009 |
| SCA32 | *7q32-q33* | --- | Jiang et al., 2010 |
| SCA34 | *ELOVL4* | Mutação de ponto | Cadieux-Dion et al., 2014 |
| SCA35 | *TGM6* | Mutação de ponto | Wang et al., 2010 |
| SCA36 | *NOP56* | Repetições GGCCTG | Kobayashi et al., 2011 |
| SCA37 | *DAB1* | Repetições ATTTC | Serrano-Munuera et al., 2013 |
| SCA38 | *ELOVL5* | Mutação de ponto | Di Gregorio et al., 2014 |
| SCA40 | *CCDC88C* | Mutação de ponto | Tsoi et al., 2014 |

Continuação Tabela 1 – Genética molecular das ataxias espinocerebelares.

| Doença | Gene ou lócus cromossômico | Mutação | Referência |
|---|---|---|---|
| SCA41 | *TRPC3* | Mutação de ponto | Fogel et al. 2015 |
| SCA42 | *CACNA1G* | Mutação de ponto | Coutelier et al., 2015 |
| SCA43 | *MME* | Mutação de ponto | Depondt et al., 2016 |
| SCA44 | *GRM1* | Mutação de ponto | Watson et al., 2017 |
| SCA45 | *FAT2* | Mutação de ponto | Nibbeling et al., 2017 |
| SCA46 | *PLD3* | Mutação de ponto | Nibbeling et al., 2017 |
| SCA47 | *PUM1* | Mutação de ponto | Gennarino et al., 2018 |
| DRPLA | *16q22.1* | Repetições CAG | Edener et al., 2011. |

[1]SCA9 foi reservado, no entanto não existe nenhum dado clínico ou molecular da doença. [2]SCA16 e SCA15 são as mesmas. [3]SCA19 e SCA22 são prováveis formas alélicas no mesmo *locus*. [4]SCA24 tem padrão de herança autossômico recessivo.

Normalmente as SCAs são denominadas como ataxias espinocerebelares do tipo “N”, onde “N” é um número dado em ordem numérica seguindo a ordem de descobrimento, um caso que foge a essa regra é a atrofia dentatorublo palidolusiana (DRPLA).

A prevalência estimada para as SCAs varia muito entre as regiões, por exemplo 1,6 para cada 100.000 em Singapura (Zhao et al., 2002) e de 5,6:100.000 indivíduos foi encontrada em Portugal (Coutinho et al., 2013), um estudo de meta-análise de ataxias cerebelares hereditárias autossômicas dominantes estimou que a prevalência mundial estimada seria de 2,7 para cada 100.000 indivíduos (Ruano et al., 2014). As SCAs mais comuns são as causadas por expansões CAG em éxons, sendo que a mais comum mundialmente é a SCA3/MJD, seguida por SCA2, SCA1 e SCA6. Apesar disso, em torno de 45% dos pacientes com quadro

clínico de ataxia espinocerebelar ainda permanecem sem diagnóstico (Durr, 2010; Marelli et al., 2011).

### 1.1.1 Doença de Machado-Joseph ou Ataxia Espinocerebelar tipo 3 (SCA3/MJD)

A doença de Machado-Joseph (*Machado-Joseph Disease* – MJD) foi descrita pela primeira vez em descendentes de famílias de origem açoriana nos Estados Unidos: a família Machado (Nakano et al., 1972), proveniente da ilha de São Miguel, e a família Joseph, proveniente da ilha de Flores (Rosenberg et al., 1976). Em 1994 quando foram realizados os estudos para identificar o gene causador da doença foi descoberto que o gene causador de MJD estava localizado no cromossomo 14, sendo que essa região estava associada à Ataxia espinocerebelar do tipo 3 (SCA3), que até então seria uma ataxia não relacionada com MJD. Depois de identificada a causa específica das duas doenças, ficou comprovada que a mesma mutação que causava MJD era a que causava SCA3, definido as duas como uma mesma doença.

#### 1.1.1.1 Aspectos Clínicos

A SCA3/MJD é uma doença de início tardio, sendo que os pacientes tendem a começar a apresentar sintomas perto dos 40 anos (Sequeiros e Coutinho, 1993). No entanto, a população brasileira mostrou ter um início mais precoce, sendo a idade de início média entre 32 e 34 anos (Lopes-Cendes et al., 1997; Jardim et al., 2001a). Entretanto, casos em pacientes com início aos 4 anos de idade já foram documentados (Carvalho et al., 2008). Após apresentarem os primeiros sintomas os pacientes com SCA3/MJD tem um tempo médio de sobrevida de 21 anos (Kieling et al., 2007).

Por pertencer ao grupo das SCAs, a SCA3/MJD tem sintomas semelhantes às demais doenças pertencentes a esse grupo. Os sintomas começam com instabilidade ao virar, seguido por desequilíbrio de marcha (ataxia). Com o passar dos anos, este progride para um andar de base alargada acompanhada por

incoordenação sutil dos membros superiores e a fala intercortada e mal articulada (disartria). Eventualmente, ataxia da marcha torna-se tão severa que o paciente precisa usar muletas, culminando com o uso de cadeira de rodas. Muitos pacientes também desenvolvem distúrbios oculomotores (nistagmo), espasticidade dos membros inferiores e em alguns casos sinais parkinsonianos. A ataxia de marcha é o primeiro sintoma a ser descrito na grande maioria dos pacientes.

Escalas foram criadas para possibilitar uma melhor avaliação neurológica e assim conseguir uma medida para a gravidade das SCAs. As mais utilizadas são a ICARS (*International Cooperative Ataxia Rating Scale*) e a SARA (*Scale for the Assessment and Rating of Ataxia*) (Schmitz-Hubsch et al., 2006; Trouillas et al., 1997). Ambas as escalas visam especificamente a gravidade da ataxia em relação aos componentes axial, membros, ocular e da fala. A escala NESSCA (*Neurological Examination Score for Spinocerebellar Ataxia*) foi desenvolvida para uma avaliação mais abrangente avaliando 18 itens sendo que 13 deles correspondem a um exame neurológico padrão, analisando achados piramidais, ataxia, ataxia de limbo, nistagmo, retração palpebral, oftalmoparesia, disartria, fasciculação, movimentos distônicos, blefarospasmo, rigidez extrapiramidal, bradicinesia, amiotrofia distal e atrofia óptica e cinco itens dependem das informações relatadas pelos pacientes, mas que representam sintomas comuns apresentados por estes e que trazem desconforto para eles, que são a disfagia, perda sensorial, câimbras, vertigem e disfunção do esfíncter (Kielling et al., 2008).

Um estudo prospectivo de 10 anos de duração utilizando a escala NESSCA demonstrou que os pacientes apresentam uma piora 1,26 pontos por ano (em uma escala que vai de 0 a 40) e que cada CAG adicional na repetição expandida corresponde a uma piora de 0,15 pontos naquela taxa (Jardim et al., 2010), mostrando ser uma doença de avanço clínico lento e progressivo.

Não existe nenhum tratamento específico para as SCA. Porém, sintomas como distônia, câimbras musculares e espasticidade podem ser minimizados com medicamentos e acompanhamento de fisioterapia e fonoaudiologia também são recomendados (Manto & Marmolino, 2009).

#### 1.1.1.2 Epidemiologia

A SCA3/MJD é a SCA mais comum de uma forma geral, sendo responsável por 15% a 45% dos casos (Paulson, 2007; Schöls et al., 2004). Os primeiros indivíduos descritos com SCA3/MJD compartilhavam a origem ancestral açoriana, assim como alguns indivíduos diagnosticados posteriormente. Um pouco mais tarde, com o início dos testes moleculares, novos casos foram identificados em indivíduos com outras etnias.

A SCA3/MJD é encontrada em incidência elevada em diversos países, como Portugal (58%) (Vale et al., 2010), Singapura (53%) (Zhao et al., 2002), China (49%) (Jiang et al., 2005), Holanda (44%) (van de Warrenburg et al., 2002) e Alemanha (42%) (Schols et al., 1997). Em outros países, a frequência de SCA3/MJD é mais baixa, como no Canadá (24%) (Kraft et al., 2005), nos EUA (21%) (Moseley et al., 1998), no México (12%) (Alonso et al., 2007) e na Austrália (12%) (Storey et al., 2000), sendo raramente encontrada na Índia (3%) (Faruq et al., 2009), na África do Sul (4%) (Bryer et al., 2003) e na Itália (1%) (Brusco et al., 2004).

A primeira publicação da SCA3/MJD no Brasil foi a descrição de cinco pacientes de uma mesma família (Teive et al., 1991). Relatos mais recentes apontam para uma frequência de 59,6% de SCA3/MJD dentre os casos de SCA no Brasil. No entanto, no estado do Rio Grande do Sul, a frequência de SCA3/MJD é estimada em 78,4% dos casos de ataxias (Castilhos et al., 2014), 2006). Baseado nessa estimativa, a prevalência mínima estimada de indivíduos afetados seria de 6:100.000 casos, refletindo um possível efeito fundador açoriano (Souza et al., 2016).

Os indivíduos com SCA3/MJD que se encontram nos Açores se concentram principalmente na ilha de Flores e na ilha de São Miguel, onde a prevalência estimada é de 835,2:100.000 e 27,1:100.000 (Coutinho et al., 1994), respectivamente. Acredita-se que essa presença elevada de pacientes com SCA3/MJD nestas ilhas pode ser explicada por um efeito fundador que teve início com o começo da colonização destas ilhas pelos portugueses em 1432 (Sequeiros, 1993). E a existência desta doença em alta frequência em países do

oriente, tais como Índia e Japão, e no Brasil pode ser explicado pelas grandes navegações feitas pelos portugueses nos séculos XV e XVI.

No entanto, existe uma diferença na média de idade de início entre as duas ilhas, onde os doentes originários da ilha de Flores começam a apresentar sintomas, em média, oito anos antes dos doentes originários da ilha de São Miguel, sugerindo uma diferença entre essas duas subpopulações.

Os estudos moleculares sobre a origem ancestral contribuíram para a elucidação de alguns detalhes sobre a origem da mutação causadora da doença. Nesse estudo foi sugerida a existência de dois eventos mutacionais na população portuguesa responsáveis por dois haplótipos distintos nas Ilhas de São Miguel e de Flores. O haplótipo GGC foi associado com os indivíduos originários da ilha de São Miguel e por isso também ficando conhecido como o haplótipo Machado. E o haplótipo ACA foi associado com os indivíduos originários da ilha de Flores e por isso ficou conhecido como haplótipo Joseph. Sendo que os dois haplótipos foram encontrados em pacientes de Portugal continental (Gaspar et al., 2001). No Brasil o principal haplótipo encontrado é o ACA, principalmente no rio grande do sul onde aproximadamente 93% apresenta esse haplótipo, isso é devido a um efeito fundador proveniente dos açores (Furtado et al., submetido para publicação).

#### 1.1.1.3 Aspectos Moleculares

A mutação causadora de SCA3/MJD foi descrita em 1994 (Kawaguchi et al., 1994), sendo identificada como uma expansão de CAG no éxon 10 do gene *ATXN3*, o qual está localizado no *locus* 14q32.1.

O gene apresenta 48.070 pares de bases (pb) e está dividido em 13 éxons (Figura 1), dois éxons (6a e 9a) foram recentemente adicionados (Bettencourt et al., 2010).

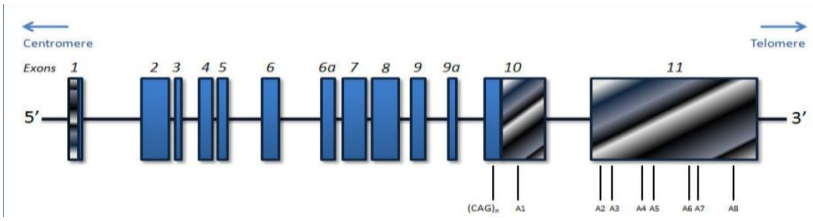


Figura 1. Esquema do gene *ATXN3* com a localização das repetições CAG (Bettencourt & Lima, 2011).

As repetições CAG são polimórficas, podendo variar em indivíduos normais de 12 a 44 repetições. Alelos com 45 a 51 repetições CAG são considerados alelos com penetrância reduzida, sendo que os portadores destes alelos podem vir a manifestar a doença em algum momento da vida. E os alelos expandidos patologicamente são aqueles com 52 ou mais repetições CAG, cujos indivíduos irão manifestar a doença em algum momento de suas vidas (Paulson, 2011).

O gene *ATXN3* codifica a proteína ataxina-3, a qual é composta por 339 aminoácidos mais o número variável de glutaminas. A forma selvagem da proteína apresenta funções de uma enzima de deubiquitinação (DUB) que atuaria na via ubiquitina proteossomo. Também foi proposto o seu envolvimento na regulação da transcrição de proteínas (Ries et al., 2008). *ATXN3* atua também na formação do agressomo junto com HDAC6, selecionando os agregados para este (Bonanomi et al., 2014). O gene *ATXN3* mutante não apresenta alteração na sua transcrição ou tradução, mas a presença anormal de poliglutaminas na proteína ataxina-3 altera a localização da mesma. A ataxina-3 é normalmente uma proteína predominantemente citoplasmática em neurônios (Pozzi et al., 2008). Entretanto, no caso da presença de um maior número de glutaminas na proteína, a mesma é encontrada concentrada no núcleo dos neurônios na forma de inclusões neuronais. Essas inclusões neuronais, que também são encontrados em outras doenças causadas por poliglutaminas, são fortemente ubiquitinadas e contêm chaperonas moleculares de choque de calor e subunidades proteossômicas, sugerindo que eles são repositórios de proteínas malformadas e agregadas (Schmidt et al., 2002). A presença de inclusões intranucleares neuronais ubiquitinadas (NIIs) tem sido reconhecida como uma característica neuropatológica destas doenças, embora sua função na patogênese continua a

ser um motivo de controvérsia (Yamada et al., 2005). Alguns estudos sugerem que as inclusões não são diretamente as estruturas responsáveis pela patogenicidade da doença e, sim, um meio da célula combater as proteínas anormais tornando-as não tóxicas através da agregação (Evert et al., 2006; Rub et al., 2006).

#### 1.1.1.4 Aspectos Genéticos

A SCA3/MJD é uma doença autossômica dominante, sendo esperado que 50% da prole herde o gene mutante. Entretanto, distorção da segregação já foi descrita nesta doença, onde ocorre uma transmissão preferencial do alelo mutante a qual é mais significativa quando o transmissor é o pai (Iughetti et al., 1998).

*Fitness* aumentado foi descrito em indivíduos afetados, onde estes tinham mais filhos do que os parentes não afetados. As mulheres com SCA3/MJD demonstraram idade mais baixa no primeiro parto e início precoce da menopausa quando comparadas com familiares não afetadas. E essas mulheres normalmente tinham os filhos antes de apresentarem qualquer sintoma, ou seja, antes de saberem que tinham a doença (Prestes et al., 2008).

Uma correlação direta entre o número de repetições CAG com a taxa de progressão da doença já foi amplamente demonstrado nessa patologia (Jardim et al., 2001b). Correlação inversa entre o número de repetições CAG e a idade de início também foi observada, assim como nas demais SCA, no entanto, o número de repetições CAG explica de 45% a 60% desta variabilidade (van de Warrenburg et al., 2002), indicando que outros fatores podem estar envolvidos como moduladores do fenótipo. O efeito da metilação do promotor do gene *ATXN3* foi sugerido recentemente (Emmel et al., 2012) e outros fatores vêm sendo investigados.

Antecipação também se está presente na SCA3/MJD pelo fato sequências com maior número de repetições são instáveis durante a meiose e tendem a se expandir nas próximas gerações. Isto leva ao início mais precoce dos sintomas e a um fenótipo mais severo em gerações subsequentes da família (Richards, 2001).

## 1.2 Biomarcadores

A revista *Nature* define que “um biomarcador é uma característica biológica que pode ser medida objetivamente e avaliada como um indicador de funções biológicas normais ou patológicas, ou de uma resposta a intervenções terapêuticas. Exemplos incluem padrões de expressão gênica, níveis de uma proteína em particular em fluídos corporais ou mudanças na atividade elétrica no cérebro” (tradução livre do autor; https://www.nature.com/subjects/biomarkers). A Organização mundial da saúde define biomarcador como “qualquer substância, estrutura ou processo que pode ser medido no corpo ou seus produtos e influência ou prevê a incidência de um resultado ou doença” (tradução livre do autor; http://www.inchem.org/documents/ehc/ehc/ehc222.htm). Apesar de existirem vários outras definições além destas, elas tendem a serem bastante similares e se sobrepõem.

Podemos dividir os biomarcadores em três grupos: biomarcadores de suscetibilidade, de estado e de progressão. Biomarcadores de suscetibilidade identifica fatores que mostram que o indivíduo irá ou tem alta probabilidade de manifestar uma doença ou característica especifica. Biomarcadores de estado indicam a presença de alguma disordem. Enquanto os biomarcadores de progressão refletem o desenvolvimento da doença.

### 1.2.1 Biomarcadores em doenças neurodegenerativas

Os principais biomarcadores usado em doenças neurodegenerativas são as escalas clínicas - ADAS-Cog para Alzheimer (Rosen et al., 1984), UPDRS para Parkinson (Fahn et al., 1987), UHDRS para Huntington (Huntington Study Group, 1996), SARA para ataxias espinocerebelares (Schmitz-Hübsch et al., 2006) - pois normalmente são simples de serem realizadas e podem ser feitas em qualquer ambiente. A utilização de mais de uma escala clínica para cada doença também não é incomum em ensaios clínicos. No entanto, essas escalas costumam ter uma evolução lenta, dificultando seu uso em ensaios clínicos ou até para o acompanhamento da história natural da doença, sendo necessários outros

marcadores que tenham uma evolução mais rápida para acompanhar os dados clínicos.

Características antropométricas são fáceis de serem medidas e, por isso, também são utilizadas. Como por exemplo, pacientes com a doença de Huntington (HD) possuem um índice de massa corporal (IMC) mais baixo, até mesmo antes de apresentarem sintomas (Tereshchenko et al., 2015). Um IMC alto está associado com maior risco para a doença de Parkinson (PD) (Sääksjärvi et al., 2014). No entanto, uma redução do IMC ao longo do tempo está associada com uma piora na escala clínica UPDRS (Wills et al., 2016). Indivíduos com a doença de Alzheimer (AD) mostraram uma redução de IMC antes da doença apesar de, após o começo da doença, não foram detectadas mudanças (Gu et al., 2014). No entanto, em um estudo se viu que quem tinha um alto IMC associado com a ausência do genótipo ε4 da APOE tinha uma progressão mais lenta da doença em comparação aos outros IMCs.

Exames de neuroimagem, como imagem por ressonância magnética (MRI) e tomografia por emissão de pósitrons (PET), fornecem dados clínicos importante para essas doenças, que os tornam fortes candidatos para biomarcadores em doenças neurodegenerativas (Szymański et al., 2010). Por exemplo, um estudo utilizando MRI viu que a intermediação (*betweenness centrality*) utilizando regiões do cérebro apresenta uma redução em indivíduos com HD que recém manifestaram sintomas ou estão próximo da idade de início estimada em comparação com controles e indivíduos mais afastados da idade de início estimada (Odish et al., 2015).

Análises de fatores moleculares também são bastante estudados, tanto para um maior entendimento da doença como para sua identificação como potenciais biomarcadores. Quantificação de RNA mensageiro é uma das possibilidades e atualmente com o desenvolvimento de sequenciadores de nova geração estudos de transcriptoma têm sido feitos, assim é selecionado alguns genes que são possíveis biomarcadores que após precisam ser confirmados em novas coortes. Essa metodologia já foi utilizada para análise de sangue de HD (Mastrokolias et al., 2015), SCA3/MJD (Raposo et al., 2015) e até mesmo em cérebros de pacientes com ataxia espinocerebelar (Bettencourt et al., 2014). MicroRNA

(miRNA) atualmente vem sendo estudados como biomarcadores em doenças neurodegenerativas (Grasso et al., 2014), e já foi relatado que a alteração nos níveis de alguns miRNA encontrados no plasma mimetizam o que acontece no cérebro de pacientes com HD (Hoss et al., 2015).

Proteínas são os fatores moleculares mais estudados para a análise de biomarcadores, as mais estudadas são as proteínas altamente associadas a causa das respectivas doenças. A doença de Alzheimer por ser uma doença que pode ter várias origens, possui um tipo de marcador específico para cada origem. Por exemplo, quando causado por beta amiloide (Aβ), os marcadores podem ser a detecção de beta amiloides no fluído cérebro espinhal ou no sangue. No entanto, a correlação de Aβ no sangue apresenta uma fraca correlação ou, até mesmo, ausência de correlação com os níveis no cérebro (Lashley et al., 2018). Na doença de Parkinson, a presença de α-sinucleína no sangue pode ser usada como biomarcador (Wang et al., 2015), e essa medição de α-sinucleína pode ser feita nas variadas formas (total, oligômeros e fosforilado) e em vários tecidos (plasma, fluido cérebro espinhal e biópsia de pele) (Khodadadian et al., 2018). O caso da doença de Huntington, por ser uma doença de herança dominante, a quantificação da proteína mutante é um bom biomarcador da doença, conseguindo diferenciar indivíduos com a doença de controles, e indivíduos em estágio tardios da doença de indivíduos que recém manifestaram a doença e/ou indivíduos que ainda vão a vir a manifestar (Weiss et al., 2012).

Outros biomarcadores normalmente estudados nessas doenças neurodegenerativas com um aspecto de agregação de proteínas, são proteínas envolvidas na degradação desses agregados, sendo do sistema ubiquitina proteassoma ou do sistema de autofagia, além de fatores importantes para o desenvolvimento e funcionamento do cérebro.

### 1.2.2 Biomarcadores na doença de Machado-Joseph

Será amplamente discutido no capítulo 1

#### 1.2.2.1 Candidatos a Biomarcadores em MJD

##### 1.2.2.1.1 Marcadores de Composição Corporal

Características antropométricas são de fácil acesso e simples de serem quantificadas. Como falado anteriormente o IMC (IMC=peso/altura$^2$) é uma medida amplamente usada, nosso grupo já demonstrou anteriormente que indivíduos com SCA3/MJD com duração de doença em torno de 10 anos apresentavam um IMC menor que controles e que isso está associado com o tamanho da CAG (Saute et al., 2012). Um estudo com pacientes com SCAs de toda a Europa (grupo EUROSCA) mostrou que o IMC declina com o tempo, e que o grupo que tinha redução de IMC apresentou uma piora na escala clínica SARA (Diallo et al., 2017).

Componentes da via da insulina podem estar envolvidos com a doença, uma vez que já foi demonstrado que pacientes com SCA3/MJD possuem mais IGFBP-1 (*insulin-like growth factor binding protein 1*) em soro, além disso apresentaram uma maior sensibilidade a insulina de acordo com HOMA2 (avaliação do modelo homeostático) e essa sensibilidade foi associada com a idade de início (Saute et al., 2011).

##### 1.2.2.1.2 Marcadores Neuronais

Quando se estuda doenças neurodegenerativas primeiramente vai se pensar em biomarcadores fortemente associados com o sistema nervoso, no caso de proteínas procuram-se principalmente as quais suas principais funções estão associadas a neurônios ou outros tipos celulares do sistema nervoso, normalmente envolvidos com neuroproteção ou dano celular.

Um dos principais alvos quando se estuda algo relacionado com sistema nervoso são as neurotrofinas, sendo a proteína fator neurotrófico derivado do cérebro (BDNF - *Brain-derived neurotrophic fator*) a neurotrofina mais pesquisada em estudos neuronais e em doenças neurológicas e/ou psiquiátricas. BDNF tem como função promover a sobrevivência e diferenciação de determinadas populações neuronais do sistema nervoso central e periférico. Além disso,

participa do crescimento axonal e da modulação da morfologia e crescimento dos dendritos. É também o principal regulador da transmissão sináptica e da plasticidade das sinapses em neurônios adultos no sistema nervoso central.

Já foram identificadas alterações de BDNF em diversas doenças neurodegenerativas, como em pacientes com doença de Alzheimer (Budni et al., 2016) e Huntington (Allen et al., 2013), onde uma redução da sua expressão é vista. Na doença de Parkinson, níveis de proteína e mRNA estão reduzidos na substância nigra. (Howells et al., 2000). Já no grupo das SCAs, foi verificado que em modelos de camundongos de SCA1 existe a redução da expressão gênica (Hourez et al., 2011) e que em cerebelo de pacientes com SCA6 a expressão gênica está suprimida e as proteínas formam grânulos anormais (Takahashi et al., 2012). Em um modelo celular de MJD, uma redução de expressão gênica foi identificada uma redução de expressão, a análise imunohistoquímica de cérebros de pacientes não demonstrou redução nos neurônios da ponte, mas uma redução significante foi vista nos neurônios do núcleo denteado (Evert et al., 2003).

Outra neurotrofina é o fator de crescimento de nervoso (NGF – *nerve growth factor*), uma proteína secretada que está envolvida na regulação do crescimento e da diferenciação de neurônios simpáticos e alguns sensoriais. Pacientes com AD apresentam menor quantidade de NGF no soro (Budni et al., 2016), assim como em pacientes com HD (Tasset et al., 2012). NGF foi utilizado em um ensaio clínico para o tratamento de AD, onde foi visto uma melhora nos indivíduos (Tuszynski et al., 2015). Em um ensaio clínico aberto em SCA3/MJD, também foi visto melhora na SARA além de melhora em outras análises clínicas (Tan et al., 2015).

O fator neurotrófico ciliar (CTNF – *Ciliary neurotrophic factor*) é uma proteína capaz de induzir diferenciação neuronal e sua sobrevivência, tendo efeito no desenvolvimento e manutenção do sistema nervoso, e pode ser relevante durante ataques inflamatórios reduzindo a destruição tecidual. O CTNF está reduzido em pacientes com PD (Chauhan et al., 2001) e foi utilizado em um ensaio clínico de HD, onde foi demonstrado melhoras em testes eletrofisiológicos (Bloch et al., 2004).

A enolase específica de neurônio (NSE – *neuron specific enolase*, também conhecida como enolase gamma, ENO2) é uma proteína com propriedade neuroprotetora e neurotrófica encontrada nos neurônios do sistema nervoso central. Um aumento nos níveis desta proteína já foi observado em cérebros de modelos de camundongos para PD, nocaute para o gene *PINK1* (Triplett et al., 2015). Em pacientes com AD, houve um aumento dos níveis de NSE em fluido cerebroespinhal (Schmidt et al., 2014). Além disso, foi mostrado aumento de NSE no soro quando comparado com controles em duas coortes de pacientes com MJD (Tort et al., 2005 e Zhou et al., 2011).

#### 1.2.2.1.3 Marcadores Gerais (sistêmicos)

Proteínas com funções sistêmicas, que normalmente são expressas ubiquamente e em grande quantidade em todos os tipos celulares, tornam-se bons candidatos a biomarcadores devido a sua fácil identificação. Além disso, podem ter sua expressão alterada facilmente. Nesse aspecto, proteínas envolvidas em rotas metabólicas, como em metabolismo de açúcares e em vias da regulação da replicação e transcrição do DNA, são bons alvos para estudo.

Um possível exemplo é a glicogênio sintase cinase 3 beta (GSK3β - *Glycogen Synthase Kinase 3 Beta*) é uma enzima chave no metabolismo do glicogênio, com as mais variadas funções (divisão, proliferação, motilidade e sobrevivência celular), no cérebro essa proteína está envolvida na plasticidade sináptica, provavelmente pela regulação dos receptores de NMDA. O GSK3β é o principal alvo do lítio, sendo inibidor da atividade da proteína (Stambolic et al., 1996). Na doença de Parkinson, onde o lítio já foi testado como um possível tratamento, um polimorfismo no gene de GSK3β foi implicado como modificador de risco (Yu et al., 2014). Em modelo de *drosophila* de MJD, a neurodegeneração foi parcialmente aliviada pela inibição da atividade de GSK3β (Jia et al., 2013)

A histona desacetilase 6 (HDAC6 – *Histone Deacetylase* 6), atua na desacetilação de histonas, regulando a transcrição de genes e o ciclo celular. Além disso, atua na via de degradação de proteínas mal enoveladas, mediando o transporte dessas proteínas através do agrossomo, onde interage com a proteína

ataxina 3 (Bonanomi et al., 2014), o que a torna um bom alvo para análise. Em estudos em cérebro de pacientes com doença de Parkinson, ela foi colocalizada com a α-sinucleína e ubiquitinas (Miki et al., 2011) e, em modelo de camundongo com HD, a HDAC6 induziu autofagia reduzindo a neurodegeneração causada pelas poliglutaminas (Pandey et al., 2007)

# 2 Objetivos

## 2.1 Objetivo geral:

Identificar e avaliar biomarcadores na doença de Machado-Joseph/ataxia espinocerebelar do tipo 3 (SCA3/MJD)

## 2.2 Objetivos específicos:

- Revisar o "estado da arte" de potenciais marcadores do estado de doença em SCA3/MJD, com foco em marcadores de neurofisiologia e compostos de fluidos biológicos.
- Descrever o índice de massa corporal (IMC) e sensibilidade periférica à insulina (SPI) em pacientes sintomáticos precoces com SCA3/MJD e em indivíduos pré-sintomáticos e correlacioná-los com a gravidade da doença e mutação.
- Verificar alterações de neurotrofinas (BDNF, NGF, CTNF e NSE) em pacientes com SCA3/MJD submetidos ao tratamento com lítio.
- Estudar o possível uso dos níveis de expressão de mRNA dos genes *GSK3β* e *HDAC6* em leucócitos de pacientes com SCA3/MJD como biomarcadores de intervenção de lítio.

## 3 Resultados

Os resultados do presente trabalho serão apresentados a seguir na forma de artigos:

Capitulo 1: State biomarkers for Machado Joseph disease: validation, feasibility and responsiveness to change.

Capitulo 2: Body composition and peripheral insulin sensitivity in early and pre-symptomatic stages of Machado-Joseph Disease.

Capitulo 3: Neurotrophins and markers of neuronal damage in Machado-Joseph disease

Capitulo 4: GSK3B and HDAC6 expression levels as potential biomarkers of lithium in SCA3/MJD

## 3.1 Capítulo 1: State biomarkers for Machado-Joseph disease: validation, feasibility and responsiveness to change

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# State biomarkers for Machado-Joseph disease: validation, feasibility and responsiveness to change

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**Key-Words: biomarkers; neurophysiology; Machado-Joseph disease; spinocerebellar ataxia type 3**

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**Abstract**

Machado-Joseph disease (SCA3/MJD) is the most common spinocerebellar ataxia worldwide, and particularly in Southern Brazil. Due to an expanded polyglutamine at ataxin-3, SCA3/MJD presents a relentless course with no current disease modifying treatment. Clinical scales used to measure SCA3/MJD progression present moderate effect sizes, a major drawback for their use as main outcomes in clinical trials, given the rarity and slow progression of the disease. This limitation might be overcome by finding good surrogate markers. We present here a review of studies on peripheral and neurophysiological markers in SCA3/MJD that can be candidates for state biomarkers. Data on markers already studied were summarized, giving emphasis on validation against clinical scale, and responsiveness to change. While some biological fluid compounds and neurophysiological parameters showed poor responsiveness, others seemed to be good candidates. Some potential candidates that are waiting for responsiveness studies were serum levels of neuron specific enolase, vestibulo-ocular reflex and video-oculography. Candidates evaluated by RNA and microRNA expression levels need further studies to improve their measurements. Data on peripheral levels of Beclin-1 and DNAJB1 are promising but still incipient. We conclude that several potential candidates should follow onto validating studies for surrogate state biomarkers of SCA3/MJD.

## 1. Introduction

Machado-Joseph disease, also known as spinocerebellar ataxia type 3 (SCA3/MJD), is an autosomal dominant spinocerebellar ataxia caused by an expanded CAG repeat (longer than 51 triplets) at *ATXN3* gene, giving rise to an expanded polyglutamine (polyQ) at ataxin-3 protein (Saute & Jardim, 2015). With a mean age at onset of 34-40 yo (Dürr *et al.*, 1996; Schöls *et al.*, 1997; Tang *et al*., 2000; Globas *et al.*, 2008; du Montcel *et al.*, 2014; de Castilhos *et al.*, 2014; Zhou *et al.*, 2014), SCA3/MJD involves predominantly the cerebellar, pyramidal, extrapyramidal, motor neuron, and oculomotor systems. Gait ataxia is commonly the first symptom, followed by diplopia, dysarthria, spasticity, dystonic movements, sensory losses and other findings, in different combinations (Jardim *et al.*, 2001; Saute & Jardim, 2015). SCA3/MJD is very heterogeneous and never exclusively ataxic. Currently there is no disease modifying treatment and SCA3/MJD presents a relentless progression, with an average survival of 21.18 years after onset of symptoms (Kieling *et al.*, 2007). However, several lines of pre-clinical research gave rise to good candidate treatments targeting different cellular and molecular pathways, a scenario in which robust designs of clinical trials will be paramount for the success of the therapeutic endeavor (Li et al., 2015; Duarte-Silva et al., 2018; Matos et al., 2018). Considering the very slow progression of SCA3/MJD on clinical scales and the rarity of the disease, state biomarkers might be important surrogate endpoints for these future clinical studies.

Biomarkers are "substances, structures, or processes that can be measured in the body or its products and influence or predict the incidence or outcome of disease, of treatments, or of environmental exposures" (WHO, 2001). Trait biomarkers are

present prior to start of the disease process, while state biomarkers are due to disease process or due to a therapy response, and mirror disease progression.

State biomarkers should be correlated to clinically meaningful endpoints. If state biomarkers show advantages when comparing to clinical endpoints, they can replace them in clinical trials (Aronson, 2005). This is the case of a biomarker whose changes can be measured easily and in a more sensitive way than clinical endpoints. Such surrogate markers are especially important for phase II, randomized clinical trials (phase II RCT) addressed to raise preliminary evidence of efficacy for a given drug, especially in the context of rare diseases.

Efficacy of a given treatment is most fully demonstrated when outcomes of treated versus control groups vary according to a minimal clinically important difference (MCID); and MCID were never clearly determined to SCA3/MJD. The closest to that was obtained by the Scale of Assessment and Rating of Ataxia (SARA), a validated semi-quantitative scale that progresses between 0.65 and 1.56/40 points per year (Ashizawa *et al.*, 2013; Chan *et al.*, 2011; Schmitz-Hübsch *et al.*, 2006, and 2010; Jacobi *et al.*, 2015), and where 1.5 points were noted by patients, according to the patient global impression of improvement (PGI-I). Nevertheless, disease progression is slow as measured by SARA and by all other clinical scales in use - the International Cooperative Ataxia Rating Scale (ICARS) (Trouillas *et al.,* 1997), Neurological Examination Score for Spinocerebellar Ataxias (NESSCA) (Kieling *et al.*, 2008), Composite-Cerebellar-Functional-Score (CCFS) (du Montcel *et al.*, 2008), and the Inventory of Non-Ataxia Symptoms (INAS) (Schmitz-Hübsch *et al.*, 2008). Clinical trials should be tailored to face this issue.

A drawback shared by all clinical scales is their large variability, which can reduces their effect sizes (ES) either by the Cohen´s effect size (CES) or the standardized response mean (SRM) (Streiner and Norman, 2008; Saute *et al.*, 2012). The average SRM obtained for SARA scale was 0.5 (Schmitz-Hübsch *et al.*, 2010). Considering SARA SRM with a progression of 1 point per year, between 175 and 328 subjects would be needed in each arm to show a 50% reduction in the disease progression rate in a future trial (Chan *et al.*, 2011; Schmitz-Hübsch *et al*., 2010; Saute *et al.*, 2015). For a rare disease, these numbers are actually unfeasible. This might be overcome by the discovery of a good surrogate, or a set of surrogate markers, with ES larger than those presented by current clinical scales.

Since biomarkers are much needed, we aimed to review the state of art of potential surrogate markers of disease state in SCA3/MJD, focusing on neurophysiology markers and biological fluid compounds. Candidates for state biomarkers were included, provided that some preliminary evidence in humans was already published. Validation against a meaningful clinical endpoint, feasibility, rate of change in time (progression rate), and responsiveness to change were the parameters in focus.

## 2. Methods

### 2.1 Criteria for including studies

We included studies describing biological fluid compounds and neurophysiological measures that could be candidate for state biomarkers. Case-control and

prospective studies and clinical trials were included, provided that quantitative information on their candidate markers were given.

Original studies on cellular or animal models as well as studies in humans lacking quantitative data or when specific SCA3/MJD diagnosis was missing, case reports, case series (without controls), reviews, comments, editorials, and guidelines, and studies written in other languages apart from English, were excluded. Neuroimaging studies were addressed in a recent systematic review (Klaes *et al.*, 2016), and therefore were not included in this review.

Clinical rating scales or scores for cerebellar ataxia and studies whose design was intended to identify a trait biomarker - for instance, studies searching for modifiers of age at onset - were not within the scope of this review.

### 2.2 Search methods

We performed a search in MEDLINE until November, 2017. The search terms were (Machado-Joseph disease OR spinocerebellar ataxia) AND (Biomarker* OR Biologic* Marker* OR Laboratory Marker* OR Serum Marker* OR Surrogate Endpoint* OR Biochemical Marker* OR Immune Marker* OR immunologic* marker* OR miRNA) OR (Biomarker* OR Electroencephalography* OR Evoked potentials* OR Transcranial Magnetic Stimulation* OR Quantitative Motor Features* OR Vestibular* OR Video-Oculography* OR Nerve Conduction Studies* OR Electromyography*).

In addition, a manual search for references known by authors that did not covered by the above search strategy was also performed, and such studies were included.

### 2.3 Study organization

Results were presented in two groups of candidate biomarkers: biological fluid compounds and neurophysiology characteristics. The main scientific queries were related to evidences on validation against a clinical scale, responsiveness, and clinical significance. If already estimated, sample sizes for future trials were mentioned as well.

### 2.4 Sensitivity to change

Cohen's Effect Size (CES) or the Standardized Response Mean (SRM) were provided to candidate biomarkers, when available. The following formulas were applied (1) mean score change / standard deviation (SD) of score at baseline (for CES); and (2) mean score change / SD of score change (for SRM) when data were available and CES or SRM were not determined.

## 3. Results

**3.1** Biological fluid compounds

**Table 1** summarizes data on biological fluid compounds reported on SCA3/MJD and included in the present review. Studies with positive results related to disease state, on neurotrophic/growth factors, inflammatory mediators and astrocyte

activators, markers of neuronal and glial loss, oxidative stress and protein quality control systems markers are described below. Longitudinal data was available only for eotaxin levels; the effect size of this candidate was described in **Figure 1**.

Among compounds associated to symptomatic status of SCA3/MJD carriers, just serum neuron-specific enolase (NSE) levels and glutathione peroxidase activity (GSH-Px) were found to be related to SCA3/MJD by two independent case/control studies each (Tort *et al.*, 2005; Zhou *et al.*, 2011; Pacheco *et al.*, 2013; de Assis *et al.*, 2017). NSE is a peripheral marker of neuronal dysruption, and increased levels of this protein are associated to neuronal death. However, inconsistent associations were found between NSE and clinical scales (**Table 1**). GSH-Px activity reflects antioxidant defense capacity. A moderate inverse correlation of this marker was shown with NESSCA, and differences were observed between symptomatic and presymptomatic phases of the disease (de Assis *et al.*, 2017).

Some biological fluid compounds were associated to SCA3/MJD or to disease severity by single studies using unbiased approaches. Pro-inflammatory factors were particularly prominent among them. After a transcriptome-wide gene expression profile approach, quantitative PCR (qPCR) confirmed upregulation of FCGR3B and SELPLG in SCA3/MJD, and the first one was related to disease duration (Raposo *et al.*, 2015). Another unbiased approach analyzed microRNA (miR) of peripheral blood samples. miRNA are post-transcriptional repressors that can regulate gene expression at different levels. The expression of four specific miRs were found to be up- or down-regulated in SCA3/MJD patients; some of them are involved in astrocytes proliferation. Of note, down-regulated expression pattern of miR-25 and miR125b were associated to longer disease duration (Shi *et al.*,

2014). Another unbiased approach evaluated serum cytokines levels and higher levels of serum eotaxin - a cytokine secreted by eosinophils in periphery and by astrocytes in central nervous system (CNS) - were found in asymptomatic carriers when compared to both symptomatic patients and controls. A reduction in this protein levels was demonstrated in the symptomatic period a year later (da Silva Carvalho *et al*., 2016). Eotaxin levels and SARA scores obtained simultaneously in these carriers (Saute *et al*., 2014) were both broadly dispersed, but ES of Eotaxin - 0.06 - was smaller than ES of SARA - 0.50 (**Figure 1**).

### 3.2 Neurophysiology

**Table 2** summarizes data on neurophysiological candidates found by the present review. Longitudinal data was available for one parameter of motor evoked potentials (MEP) and for one parameter of peripheral neurophysiology; the effect size could be estimated for the last one (**Figure 1)**.

#### 3.2.1 Central neurophysiology

Motor evoked potentials (MEP) evaluates pyramidal tract conductivity by MEP-derived parameters such as central motor conduction time (CMCT), amplitude and resting threshold. CMCT in SCA3/MJD was found to be prolonged and associated to clinical scales by some studies (Jhunjhunwala *et al*., 2013; Farrar *et al.*, 2016). Cortical activity related to movement preparation and execution, and signs of cortical dysfunction in resting motor threshold, short-interval intracortical inhibition (SICI), and cortical silent period duration were found by a recent study, even in presymptomatic SCA3/MJD individuals (Farrar *et al.*, 2016). These markers were

strongly correlated to ICARS. Data on SICI and ICARS progression in 18 months were given in mean and standard error of mean. Therefore, CES could not be estimated (**Figure 1**).

Among sensory evoked potentials, visual evoked potentials (VEPs), brainstem auditory-evoked response (BAER), somatosensory-EPs (SSEPs), pain-related evoked potentials, and sensory gating at hippocampus/brainstem were already studied in SCA3/MJD, and no good candidate was risen as a state biomarker (**Table 2**).

### 3.2.2 Video-oculography

Diplopia is a very common finding in patients with SCA3/MJD, and can be attributed to ophthalmoplegia or vergence abnormalities. While ophthalmoplegia is easily detected in symptomatic phases of disease, subtle findings such as gaze-evoked and rebound nystagmus, square-wave jerks, saccadic hypermetria, and impaired ocular pursuit are measurable abnormalities described not only in symptomatic (Buttner *et al*., 1998; Ghasia *et al.,* 2016), but also in presymptomatic carriers (Jacobi *et al*., 2013; Raposo *et al*., 2014). Quantitative oculomotor findings have been recently described through video-oculography (Wu *et al.*, 2016). Several parameters were studied, and most of them were shown to be significantly disturbed even in preclinical phases of disease, and to be related to DD and to SARA is later phases (**Table 2**). A stepwise worsening from pre-ataxic to symptomatic carriers were seen in the frequency and average amplitude of horizontal gaze-evoked eye movements, upward peak saccade velocity and total

antisaccadic error rates. The lowest dispersion rates in pre-ataxic and symptomatic groups were obtained when measuring the upward peak saccade velocity.

### 3.2.3 Vestibular system

Vertigo and imbalance when turning the head are frequent complaints in SCA3/MJD, pointing to involvement of the vestibular system. Measurement of myogenic potentials in the ipsilateral sternocleidomastoid muscle after loud monaural clicks, and of vestibulo-ocular reflex (VOR) after a head impulse test (HIT) were among the neurophysiological evaluations of vestibular dysfunction. VOR disturbances after HIT has been described for a long time in SCA3/MJD (Buttner *et al*., 1998; Gordon *et al*., 2003). VOR registrations were improved by using magnetic search coils (Gordon *et al*., 2014), and video-oculography (VOG) portable systems turned quantitative testing of the VOR possible at the bedside (Agrawal *et al.*, 2014). In a recent study, VOR gain in SCA3/MJD subjects was significantly lower than in controls, and correlated with SARA scores in the overall group of ataxic disorders (Luis *et al*., 2016). VOR dispersion seemed to be larger than SARA dispersion in SCA3/MJD group (**Table 2**).

### 3.2.4 Peripheral neurophysiology

SCA3/MJD has been associated with axonal neuropathy of both motor and sensory nerve fibres, detected by marked reductions of compound muscle (CMAP) and sensory nerve action potential (SNAP) amplitudes. In addition to sensory

losses, muscle cramps might be related to this process, being due to the electrical irritability of unmyelinated nerve twigs, enhanced by collateral sprouting secondary to loss of motoneurons. This electrical irritability of unmyelinated nerve twigs was studied once and further clarification in this disorder is required (Kanai *et al*., 2003).

Axonal neuropathy in SCA3/MJD is most probably a neuronopathy rather than a distal axonopathy (Kanai *et al*., 2003; Escorcio Bezerra *et al*., 2013), and CMAP and SNAP amplitudes are considered indirect measures of the number of peripheral axons. Axonal neuropathy was mainly explained by age in SCA3/MJD (Klockgether *et al*., 1999; França *et al.*, 2009a; Linnemann *et al.*, 2016). In a longitudinal observation, sural SNAP showed a significant deterioration after 13 months (França *et al*., 2009a). CES of SNAP - 0.34 - was a little higher than CES of ICARS - 0.20 - obtained in the same period (França *et al*., 2009b) (**Figure 1**).

## 4. Discussion

Several biological fluid compounds and neurophysiological parameters described in SCA3/MJD subjects seemed to be good candidates, but are far from being validated as surrogate state markers for this condition. Most publications described case-control observations where cases were already symptomatic; in contrast, altered results of the peripheral levels of eotaxin and on the video-oculography were already found in pre-symptomatic states. Some candidates were associated with disease duration after symptoms onset. The oxidative stress marker GSH-Px, movement-evoked potentials, vestibulo-ocular reflex (VOR), and several video-oculography parameters correlated reasonably and significantly with clinical scales,

at this same stage. Only three studies presented a longitudinal design, while none candidate marker was tested in the context of a clinical trial. Validation against a meaningful clinical endpoint was done in some studies. Rate of change in time was obtained for peripheral eotaxin measurements, SICI, and SNAP amplitudes. Although responsiveness to change was not evaluated by the original studies, published parameters let us roughly estimate CES for eotaxin and SNAP. Those values were worse than those obtained for the clinical scales (ICARS, SARA and NESSCA) applied simultaneously. It is worth to emphasize that the number of studies that have been designed with the specific aim of identifying biomarkers is extremely limited, in this disorder. We could have added other inclusion criteria to our review, such as sample size, existence of technical validation and of a validation cohort, and statistical adjustments in relation to age or gender. Since these additional inclusion criteria would narrow our results, we chose to summarize these and other characteristics on Tables 1 and 2, letting the reader to judge about the candidates´ value for future studies.

SCA3/MJD is a disease essentially confined to the central nervous system. Biological fluid compounds might theoretically reflect the burden of damage related to the disease if they either cross the Blood-Brain barrier or be activated both in CNS and in the periphery. In any case, the search for peripheral compounds is justified by their feasibility in the clinical setting. Although SCA3/MJD pathogenesis is not thoroughly understood and pitfalls might occur in choosing candidates for biomarkers (Aronson, 2005), several clues were already established and are prone to be followed by laboratory studies. Three unbiased surveys aimed to find upregulated genes (Raposo *et al.*, 2015), microRNAs differentially expressed (Shi

*et al*., 2014), and cytokine patterns (da Silva Carvalho *et al*., 2016) in SCA3/MJD carriers. Preliminary evidence of the first two studies associated overexpression of pro-inflammatory factors FCGR3B and TNFSF14 and protein coded by *CLC* to SCA3/MJD, a pattern that subsides with late phases of disease; and downregulation of microRNAs associated with activation of astrocytes miR-25 and miR-125b that got even worse in late phases of disease. Accuracy and reproducibility have not been established to date for mRNA and miRNA expression analyses; data were present as fold change or expression ratios. Moreover, potential superiority of effect sizes cannot be inferred since dispersion measurements (SE, SEM or SD) and relation to clinical scales were not available.

At least three serum measurements showed interesting characteristics: the already mentioned eotaxin, and NSE and GSH-Px (Tort *et al.*, 2005; Zhou *et al*., 2011; da Silva Carvalho *et al*., 2016; de Assis *et al.*, 2017). Eotaxin is a peptide secreted not only in peripheral tissues by T lymphocytes, but also by astrocytes in the CNS (da Silva Carvalho *et al*., 2016). In the unbiased study on cytokines in SCA3/MJD, eotaxin levels were significantly higher in asymptomatic than in symptomatic carriers or in controls; although neither correlated to clinical scales nor to disease duration at baseline, eotaxin levels reduced after 360 days in symptomatic carriers. Eotaxin patterns were in line with results of the microRNA study (Shi *et al.*, 2014): both unbiased studies raised the hypothesis of astrocyte activation in SCA3/MJD, possibly present in pre-clinical phases, and evolving to exhaustion as disease progresses. Although eotaxin effect size was small in symptomatic carriers (**Figure 1**), effect size on preclinical phases remains unknown. The peripheral indicator of ongoing neuronal damage NSE has been evaluated by two different studies on

SCA3/MJD (Tort *et al*., 2005; Zhou *et al.*, 2011). Increased serum levels of NSE were described by both publications and the larger study was able to associate NSE to disease duration. In contrast, NSE levels were inversely related to the Extended Disability Status Scale of Kurtzke (EDSS) in the older, and directly related to ICARS and SARA in the more recent study. While this discrepancy remains unsolved, the application of NSE as a potential biomarker is precluded. The activity of the antioxidant enzyme glutathione peroxidase (GSH-Px) was low in SCA3/MJD symptomatic individuals in two studies (Pacheco *et al.*, 2013; de Assis *et al.*, 2017). GSH-Px differences from symptomatic to presymptomatic phases of the disease suggested a temporal association of lower GSH-Px activity to more advanced disease stages, sustaining some expectation in this candidate marker.

Neurophysiological studies have been done based on the hypothesis that the underlying neurological function under study is relevant for SCA3/MJD symptomatology. However, important findings associated to this disease are related to cerebellum and cerebellar-brainstem connections. There is no bedside tool to measure electrophysiological manifestations of cerebellar dysfunction. In spite of that, promising markers emerged from neurophysiology. Among parameters obtained from MEP, central motor conduction time and SICI were significantly changed and related to ICARS in symptomatic carriers (**Figure 1**). SICI variability was very large, suggesting that potential CES would be small, for future trials addressed to pyramidal involvement in this disease. VOR is affected in SCA3/MJD symptomatic carriers, and showed a moderate association to SARA, with similar measures of dispersion. Peripheral nerve studies have been performed as well, and sural SNAP showed a significant deterioration after 13 months (França

*et al.*, 2009a, 2009b). We were able to estimate CES of both SNAP and ICARS: 0.34 and 0.20, respectively (**Figure 1**). SARA CES was superior to both - 0.50.

Since they portray brainstem dysfunction, neurophysiological measurements of eye movement abnormalities are very interesting candidate biomarkers. A promising case-control study reported that frequency and amplitude of gaze evoked nystagmus, smooth pursuit eye movements (gain), upward peak velocity and accuracy of saccades, and error rates of antisaccades were already affected in pre-clinical phases of the disease, and were all related to SARA scores and to disease duration, in symptomatic carriers (Wu *et al*., 2016). This results scenario suggests that these manifestations decline in SCA3/MJD in a progressive manner. Although SD of SARA scores was not presented, other observations described SD being equivalent to 40% to 60% of SARA average results (Jacobi *et al.*, 2011, 2015; Ashizawa *et al.,* 2013; Saute *et al*., 2014). Some video-oculographic parameters obtained in SCA3/MJD subjects showed proportionally smaller SDs than these figures, like horizontal mean pursuit gain and upward saccadic accuracy (**Table 2**).

Although evidence levels remains preliminary, paragraphs below address promising additional biomarkers due to their direct roles in the SCA3/MJD pathophysiology. Molecules associated to quality control systems might play a very relevant role in SCA3/MJD, and we can highlight here two promising ones: beclin-1 and DNAJB1. Beclin-1 is a marker of protein quality control systems, and low protein as well as mRNA levels were found in fibroblasts from symptomatic SCA3/MJD individuals (Nascimento-Ferreira *et al.,* 2011; Onofre *et al.*, 2016). DNAJB1 is a molecular chaperone that stimulates the ATPase activity of Hsp70

heat-shock proteins in order to promote protein folding and prevent misfolded protein aggregation. High DNAJB1 levels were associated with earlier ages at onset than those predicted by the CAG repeat length (Zijlstra *et al.*, 2010). Both compounds should be further evaluated using larger sample sizes and by performing longitudinal observations.

Soluble mutant ataxin-3 levels were measured by time-resolved Forster resonance energy transfer (TR-FRET) immunoassay in human cell lines and brain samples of transgenic SCA3/MJD mice model (Nguyen *et al.*, 2013); properties of soluble ataxin-3 as a disease biomarker were not addressed up to date. Soluble mutant protein levels have been measured in other neurodegenerative disorders, such as in in Huntington disease (HD), and associated to clinical features (Moscovitch-Lopatin *et al*., 2013). Soluble huntingtin is currently being evaluated as an outcome in recent HD clinical trials (Süssmuth *et al*., 2015; Huntington Study Group Reach2HD Investigators, 2015). Likewise measurements of soluble mutant ataxin-3 should be evaluated in future longitudinal studies on SCA3/MJD.

Finally, it is worth to stress that biomarkers are mostly needed for the pre-clinical phases of SCA3/MJD. The pathological process is already onway before the onset of gait ataxia and future therapies will probably be more effective if starting early. Studies on pre-symptomatic carriers face more difficulties than others, such as lack of adherence and ethical issues. Fortunately, the time burden measured by the concept “disease duration” since the onset of symptoms and useful for symptomatic studies, can be solved by equations that predict the age at onset and that have recently appeared in the literature (Tezenas du Montcel et al., 2014;

Mattos et al., 2018). They will help validating biomarkers for the pre-symptomatic phases.

In conclusion, several potential candidates as state biomarkers have been preliminarily described, albeit through a majority of studies without good sample sizes and rigorous designs for validation of biomarkers. Candidates for surrogate biomarkers of the pre-symptomatic state were even more scarcely described in the literature. Studies on pre-clinical phases, such as those performed on cytokines and on neurophysiological measurements of eye movement abnormalities, are even more important, since most clinical scales give normal scores in this period. Prospective evaluations are required for all of them, together with measurements of clinical scales and of PGIs. Validation against a MCID, rate of change in time, and responsiveness to change should be established. We are aware that several barriers can delay this goal, including restrains that goes beyond scientist efforts and patients goodwill. For example, neurophysiology, molecular and neuroimages data depends upon technology companies, where planned obsolescence is intrinsic to the production lines. The constant change in platforms turns all knowledge acquisition longer and harder than expected. Hence, solutions for these dilemmas have to be searched for, and future carefully planned as well as all-embracing, multi-center studies can be the answer.

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## 6. Conflict of Interest

The authors declare no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

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## 9. List of tables

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## 10. List of Figures

Figure 1 – Candidate biomarkers that have been followed longitudinally in SCA3/MJD subjects. (A) Summary on the longitudinal data obtained for eotaxin and Scale for Assessment and Rating of Ataxia (SARA); sensory nerve action potential (SNAP) amplitudes of sural nerves and International Cooperative Ataxia Rating Scale (ICARS); and short-interval intracortical inhibition (SICI) of motor evoked potentials and ICARS. (B) Cohen effect sizes, when available or when estimation was possible.

Figure 1

A

| Variable | N | Change in 12 months | Standard Deviation at baseline | Standard Error of Mean at baseline | Study |
|---|---|---|---|---|---|
| Eotaxin, Log of results in pg/ml) | 66 | 0.032 | 0.52 | - | Da Silva Carvalho et al., 2016 |
| SARA | 66 | 2.5 | 4.4 | - | Da Silva Carvalho et al., 2016 |
| SNAP amplitudes, in µV | 48 | 3.4 | 10 | - | França et al., 2009a and b |
| ICARS | 48 | 4 | 20 | - | França et al., 2009a and b |
| SICI, in % | 3 | 0.6 | - | 3.6 | Farrar et al., 2016 |
| ICARS | 3 | 5.9 | - | 3.6 | Farrar et al., 2016 |

B

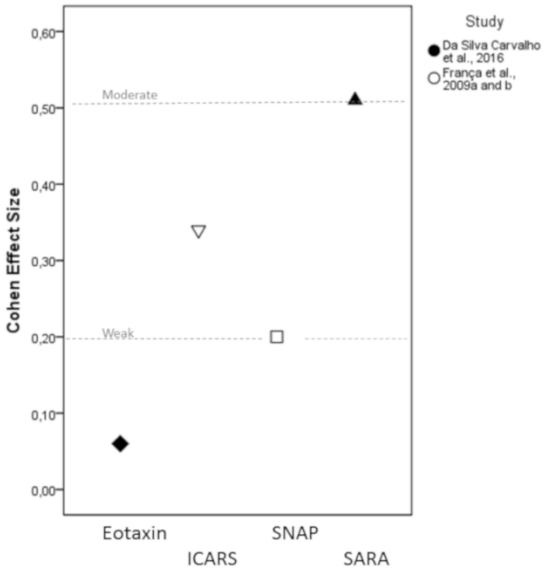

**Table 1. Peripheral compounds studied in spinocerebellar ataxia type 3/Machado-Joseph disease (SCA3/MJD) carriers, and prone to be candidates for state biomarkers of this disease. Compounds are presented according to area of metabolism.**

| Candidate Marker | Reference | Sample Size | | Sample | Comparison with controls | | Correlations were found among SCA3/MJD subjects? | |
|---|---|---|---|---|---|---|---|---|
| | | SCA3/MJD Cases | Controls | | SCA3/MJD Cases | Controls | With clinical scales | With disease duration |
| ***Neurotrophic/Growth factors*** | | | | | | | | |
| Insulin | Saute *et al.*, 2011 | 46 | 42 | Serum | Insulin levels: 6.2(3.5) uIU/mL* | Insulin levels: 9.5(6) uIU/mL | No | No |
| | | | | Serum | HOMA2-%B: 83.9(35) | HOMA2-%B: 92.9(50.5) | No | No |
| | | | | Serum | Log(HOMA2-%S): 4.8(0.55)** | Log(HOMA2-%S): 4.35(0.63) | No | No |
| IGF-1 | Saute *et al*., 2011 | 46 | 42 | Serum | Total IGF-1: 114.5(32.2) ng/mL | Total IGF-1: 117.4(36.3) ng/mL | No | No |
| | | | | Serum | Free IGF-1 (IGF-1/IGFBP-3 molar ratio): 0.36(0.24)* | 0.23(0.12) | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| IGFBP-1 | Saute *et al.*, 2011 | 46 | 42 | Serum | 2.67(1.8) ng/mL ** | 1.32(0.98) | No | No |
| IGFBP-3 | Saute *et al.*, 2011 | 46 | 42 | Serum | 1.4(0.8) ug/mL** | 2.01(0.36) | No | No |
| ***Activation of pro-inflammatory factors*** | | | | | | | | |
| *FCGR3B* gene | Raposo *et al.*, 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 2.597*; SD not informed | NA | ND | Yes*<br>FC and SD not informed |
| *TNFSF14* gene | Raposo *et al.*, 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 1.687; SD not informed | NA | ND | Yes (short disease duration only)*<br>FC and SEM not informed |
| *SELPLG* gene | Raposo *et al.*, 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 1.324*; SD not informed | NA | ND | No |

| ***Activation of astrocytes*** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| miR-34b | Shi *et al*., 2014 | 9 (DC)<br>35 (VC) | 7 (DC)<br>25 (VC) | Serum | Up-regulated:<br>Ratio Cases/Controls: 4.79***<br>SD not informed | NA | No | No |
| miR-29a | Shi *et al*., 2014 | 9 (DC)<br>35 (VC) | 7 (DC)<br>25 (VC) | Serum | Down-regulated:<br>Ratio Controls/Cases: 4.7* | NA | No | No |
| miR-25 | Shi *et al*., 2014 | 9 (DC)<br>35 (VC) | 7 (DC)<br>25 (VC) | Serum | Down-regulated:<br>Ratio Controls/Cases: 2.04* | NA | No | Yes (longer disease duration only)*<br>Ratio and SEM not informed |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| miR-125b | Shi *et al*., 2014 | 9 (DC)<br>35 (VC) | 7 (DC)<br>25 (VC) | Serum | Down-regulated:<br>Ratio Controls/Cases: 2.1* | NA | No | Yes (longer disease duration only)*<br>Ratio and SEM not informed |
| GFAP | Shi *et al*., 2015 | 136 | 151 | Serum | 8.86(4.33) ng/mL ** | 3.93±2.38 | No | No |
| Eotaxin | Da Silva Carvalho *et al.,* 2016<br>Saute *et al*., 2014 | 66 (Symptomatic)<br>13 (Asymptomatic) | 43 | Serum | Symptomatic carriers logEotaxin: 1.3 (SE=0.1) (SD: 0.50724).<br>Asymptomatic carriers logEotaxin: 2.3 (SE=0.2) *** | Controls: 1.33 (SE=0.09) | No | No |
| ***G-protein coupled receptors*** | | | | | | | | |
| *P2RY13* gene | Raposo *et al.*, 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 1.665*; SD not informed | NA | ND | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Enzyme*** | | | | | | | | |
| *CLC* gene | Raposo *et al.,* 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 2.041<br>SD not informed | NA | ND | Yes*<br>FC and SD were not informed |
| ***Others*** | | | | | | | | |
| *SLA* gene | Raposo *et al.*, 2015 | 12 (DC)<br>42 (CC) | 12 (DC)<br>35 (CC) | RNA from peripheral blood | FC: 1.333<br>SD not informed; | NA | ND | Yes (short disease duration only)*<br>FC and SD not informed |
| ***Markers of neuronal/glial loss*** | | | | | | | | |
| NSE | Tort *et al.*, 2005 | 22 | 22 | Serum | 8.05(4.2) ng/mL *** | 4.65 (1.80) ng/mL | EDSS (R=-0.729*) | No |
| | Zhou *et al.*, 2011 | 102 | 100 | Serum | 6.95(2.83) ng/mL*** | 4.83 (1.70) ng/mL | ICARS R=0.242*<br>SARA R=0.248*<br>ICARS = 26.68 (13.37)<br>SARA = | R=0.259** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 9.98 (4.65) | |
| S100B | Tort *et al.*, 2005 | 22 | 22 | Serum | 0.108(0.073) ug/l | 0.082 (0.042) ug/l | No | R=0.452* |
| | Zhou *et al.*, 2011 | 102 | 100 | Serum | 0.07(0.06) ng/ml *** | 0.05 (0.02) ng/ml | No | No |
| Neurofilament | Wilke *et al.,* 2018 | 8 | 16 | Serum | 70 pg/ml (range: 40 to 105) *** | 22 pg/ml (8 to 35) | No | No |
| ***Oxidative Stress Markers*** | | | | | | | | |
| DCFH-DA | de Assis *et al.*, 2017 | 58 (Symptomatic)<br>12 (Presymptomatic ) | 47 | Serum | Symptomatic SCA3/MJD: 335.7 nmol/mg of protein (SE 21.2)***<br>Presymptomatic individuals: 91.8 nmol/mg of protein (SE 42.2) | Controls: 182.8 nmol/mg of protein (SE 20.3) | No | No |
| | Pacheco *et al.,* 2013 | 7 | 7 | Serum | 172.126(66.49) nmol/mg of protein | 171.606(20.395) nmol/mg of protein | NA | NA |
| SOD | de Assis *et al.*, 2017 | 58 (Symptomatic )<br>12 (Presymptomatic) | 47 | Serum | Symptomatic: 9.3 (SE 0.5) U/mg of protein *<br>Presymptomatic: | Controls: 10.8 (SE 0.5) U/mg of protein | No | No |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 12.3 (SE 1.1) U/mg of protein | | | |
| GSH-Px | De Assis *et al.*, 2017 | 58 (Symptomatic )<br><br>12 (Presymptomatic) | 47 | Serum | Symptomatic: 56.3 (SE 2.4) U/mg of protein ***<br><br>Presymptomatic: 76.8 U/mg of protein (SE 5.2) | 70.3 (SE 2.3) U/mg of protein | NESSCA R=-0.309*<br><br>NESSCA = 14.27 (4.7)<br><br>SE = 0.598 | No |
| Thiol groups | Pacheco *et al.*, 2013 | 7 | 7 | Serum | 0.112 nmol/mL of erythrocytes (0.032)*** | 0.275 nmol/mL of erythrocytes (0.047) | NA | NA |
| Catalase | Pacheco *et al.*, 2013 | 7 | 7 | Serum | 40.7(10.1) mol of $H_2O_2$/mL of erythrocytes/min* | 27.67(10.01) mol of $H_2O_2$/mL of erythrocytes/min | NA | NA |
| DNA damage index (comet assay) | Pacheco *et al.*, 2013 | 7 | 7 | Lymphocytes | Higher level of DNA damage in SCA3/MJD individuals* (raw values were not presented) | Higher level of DNA damage in SCA3/MJD individuals* (raw values were not presented) | NA | NA |
| Others (total polypheno, protein carbonyl, | Pacheco *et al.*, 2013 | 7 | 7 | Serum/Plasma | Total polyphenols: 0.632 (0.498) mg/mL<br><br>Protein carbonyl: | Total polyphenols: 1.029 (0.770) mg/mL<br><br>Protein carbonyl: | NA | NA |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TBARS) | | | | | 2.751 (0.181) nmol/mg protein<br><br>TBARS: 44.534 (33.01) nmol/mL of erythrocytes | 2.665 (0.471) nmol/mg protein<br><br>TBARS: 31.786 (32.312) nmol/mL of erythrocytes | | |
| ***Protein quality control systems*** | | | | | | | | |
| Beclin-1 | Nascimento-Ferreira *et al.*, 2011 | 2 | 1 | Fibroblast (protein ) | Case 1 – 0.86 (0.087)<br><br>Case 2 – 0.69 (0.05) | 1.15 (0.038) | NA | NA |
| | Onofre *et al.,* 2016 | 5 | 4 | Fibroblast- (protein and mRNA) | Lower Beclin-1 levels in cases .*<br>Raw values were not presented | Lower Beclin-1 levels in cases.*<br>Raw values were not presented | NA | NA |
| DNAJB1 | Zijlstra *et al.*, 2010 | 22 | 6 | Fibroblast | No.<br><br>Raw values were not presented | NA | NA | NA |
| HSPB1 | Zijlstra *et al.*, 2010 | 22 | 6 | Fibroblast | Higher levels in cases*.<br><br>Raw values were not presented | NA | NA | NA |
| HSPA1A and HSPA8 | Zijlstra *et al.*, 2010 | 22 | 6 | Fibroblast | No.<br><br>Raw values were not presented | NA | NA | NA |

* p <0.05; ** p <0.01; *** p < 0.001

IGF-1, insulin-like growth factor 1; IGFBP, insulin-like growth factor binding protein; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; DCFH-DA , 2′,7′-dichlorofluorescein diacetate; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; TBARS, thiobarbituric acid reactive substances; DC, discovery cohort; CC, confirmation cohort; HOMA, Homeostasis Model Assessment; HOMA2-%B, HOMA2 - steady-state β-cell function; HOMA2-%S, HOMA2 - peripheral insulin sensitivity; EDSS, Expanded Disability Status Scale; ICARS, international cooperative ataxia rating scale; SARA scale for the assessment and rating of ataxia; NESSCA, Neurological Examination Score for Spinocerebellar Ataxias; IQ, interquartile; NA, not available; ND, not done; SD, standard deviation; SE, standard error; FC, fold change; SEM, standard error of mean.

**Table 2. Neurophysiological findings obtained in spinocerebellar ataxia type 3/Machado-Joseph disease (SCA3/MJD) carriers, and prone to be candidates for state biomarkers of this disease.**

| Candidate Marker | Reference | Sample size | | Comparison with controls | | Correlations were found among SCA3/MJD subjects? | |
|---|---|---|---|---|---|---|---|
| | | SCA3/MJD Cases | Controls | SCA3/MJD Cases | Controls | With clinical scales | With disease duration |
| **Polysomnography** | | | | | | | |
| Sleep efficiency (%) | Chi et al., 2013 | 15 | 16 | 68.4 (15.7)** | 82.8 (9.3) | ICARS: r = -0.786*** | No |
| REM sleep percentage (%) | | | | 6.8 (6.1)*** | 15.0 (4.9) | ICARS: r = -0.595* | No |
| **Central neurophysiology** | | | | | | | |
| Movement-evoked potentials triggered by transcranial magnetic stimulation (MEP): central motor conduction time | Yokota et al., 1998 | 10 | 16 | 4.5(0.8) | 4.8(1.1) | ND | No |
| | Schwenkreis et al., 2002 | 12 | 14 | 6.9 (0.9) | 6.6 (1.1) | ND | ND |
| | Jhunjhunwala et al., 2013 | 6 | 32 | 6.8 (1.5)*** | 4.8 (0.6) | No | ND |
| | Farrar et al., 2016 | 11 (2 pre-ataxic) | 62 | 7.5 (0.4)*** | 5.3 (0.2) | ICARS: r = 0.81 ** | ND |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| MEP amplitude | Yokota et al., 1998 | 10 | 16 | 0.70 (0.19)** | 0.39 (0.13) | ND | No |
| MEP: resting motor threshold | Schwenkreis et al., 2002 | 12 | 14 | 48.3 (7.6) | 49.4 (10.3) | ND | ND |
| | Jhunjhunwala et al., 2013 | 6 | 32 | 49.8 (8.8)** | 41.5 (6.6) | ICARS: No | ND |
| | Farrar et al., 2016 | 11 (2 pre-ataxic) | 62 | 62.9 (3.2) | 59.5 (1.0) | ND | ND |
| MEP: intracortical facilitation | Schwenkreis et al., 2002 | 12 | 14 | 101.4 (29.2)*** | 157.5(26.5) | ND | ND |
| Threshold tracking paired-pulse transcranial magnetic stimulation : short intracortical inhibition (SICI) (in %) | Farrar et al., 2016 | 11 (2 pre-ataxic) | 62 | −1.3 (1.4)*** | 10.3 (0.7) | ICARS: r = −0.78** | ND |
| Movement-evoked potentials: late BP with dominant (right) hand movements | Lu et al., 2008 | 9 | 8 | 0.37 (0.75)** | 2.40 (1.38) | ND | ND |
| Suppression of the auditory evoked potential P50 (hippocampus and brainstem) | Ghisolfi et al., 2004 | 12 | 24 | 76.2 (7.3) *** | 42.1 (4.4) | ND | No |

| Candidate marker | Reference | Sample size | | Comparison with controls | | Correlations were found among SCA3/MJD subjects? | |
|---|---|---|---|---|---|---|---|
| | | SCA3/MJD Cases | Controls | SCA3/MJD Cases | Controls | With clinical scales | With disease duration |
| **Vestibular system** | | | | | | | |
| Ocular Vestibular Evoked Myogenic Potentials (oVEMP, n10) | Ribeiro et al., 2015 | 14 | 20 | 10.6 (1.4) | 10.5 (0.9) | ND | ND |
| Vestibulo-ocular reflex (VOR) by search coils; gain | Gordon et al., 2014 | 10 | 7 | 0.35 to 0.76 (mean=0.56(15) | 0.73 to 0.97 | SARA: No | ND |
| Vestibulo-ocular reflex (VOR) by Video-oculography. Head velocity to eye velocity linear regression (VORr) | Luis et al., 2016 | 15 | 40 | 0.50 (0.30)** | 0.94 (0.08) | SARA: r = –0.4** | ND |
| **Video-oculography** | | | | | | | |
| Gaze-evoked eye movements (GEEM), horizontal. Frequency (Hz) | Wu et al., 2016 | 44 symptomatic 12 pre-ataxic | 40 | 1.65(0.75 (symptomatic)*** 0.83 (0.5 (pre-ataxic)** | 0.09 (0.15) | SARA: r=0.593** | r=0.550** |
| Average amplitude of horizontal GEEM | | | | 3.40(2.30)*** 1.60(0.66)*** | 0.31 (0.55) | SARA: r = 0.760** | r = 0.526** |
| Horizontal mean pursuit gain (%) | | | | 69.4(10.8)*** 81.3 (8.0) | 87.9 (4.1) | SARA: r = −0.642** | r = −0.470** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Upward peak saccade velocity (°/seconds) | | | | 338(109.3)***<br>424(81.6)*** | 563 (100.5) | SARA:<br>r = −0.397** | r = −0.282* |
| Upward saccadic accuracy (%) | | | | 85.1(16.0)*<br>93.0(9.0) | 94.4 (7.4) | SARA:<br>r = −0.547** | r = −0.471** |
| Total antisaccadic error rate (%) | | | | 66.8(22.9)***<br>36.4(24.1)*** | 19.2 (14.0) | SARA:<br>r = 0.330** | r = 0.360** |

| Candidate marker | Reference | Sample size | | Comparison with controls | | Correlations were found among SCA3/MJD subjects? | |
|---|---|---|---|---|---|---|---|
| | | SCA3/MJD Cases | Controls | SCA3/MJD Cases | Controls | With clinical scales | With disease duration |
| **Peripheral neurophysiology** | | | | | | | |
| Compound muscle action potential (CMAP) amplitudes (mV) (tibial) | Klockgether et al., 1999 | 58 | 91 | 16.4 (7.6) | 23.0 (6.9) | ND | No |
| | França et al., 2009a | 48 | 20 | 9.6 (4.2) | 9.0 (1.7) | ND | ND |
| | Suga et al., 2014 | 17 | 80 | 9.2 (4.3)** | 12.6 (3.3) | ND | ND |
| Sensory nerve action potential (SNAP) amplitudes (μV) (sural) | Klockgether et al., 1999 | 58 | 91 | 6.7 (4.7)# | 17.8 (7.5) | ND | No |
| | França et al., 2009a<br>França et al., 2009b | 48 | 20 | 12.1 (9.9)** | 24.1 (6.3) | ND | No |
| | Suga et al., 2014 | 16 | 80 | 11.1 (8.2)** | 19.3 (9.7) | ND | ND |

| Candidate | Reference | Sample size | | Comparison with controls | | Correlations were found among SCA3/MJD subjects? | |
|---|---|---|---|---|---|---|---|
| | | SCA3/MJD Cases | Controls | SCA3/MJD Cases | Controls | With clinical scales | With disease duration |
| Motor nerve conduction velocity, tibial nerve (m/s) | Klockgether et al., 1999 | 58 | 91 | 45.1 (4.4) | 46.7 (3) | ND | No |
| | França et al., 2009a | 48 | 20 | 44.8 (8.0)** | 49.3 (2.3) | ND | ND |
| | Suga et al., 2014 | 18 | 80 | 42.7 (3.8)** | 47.0 (4.0) | ND | ND |
| Sensory nerve conduction velocity, sural nerve (m/s) | Klockgether et al., 1999 | 58 | 91 | 44.7 (5.2) | 49.0 (4.1) | ND | No |
| | França et al., 2009a | 48 | 20 | 45.1 (12.5)** | 52.0 (3.0) | ND | ND |
| | Suga et al., 2014 | 15 | 80 | 47.5 (6.0) | 49.6 (4.1) | ND | ND |
| Motor axon strength-duration time constant | Kanai et al., 2003 | 20 | 32 | 0.48 (0.02)* | 0.39 (0.01) | ND | ND |

* p <0.05; ** p <0.01; *** p < 0.001; # not tested.

BP, Bereitschaftspotential; CES, Cohen effect size; GEEM, gaze-evoked eye movements; ICARS, international cooperative ataxia international rate scale; MEP: Movement-evoked potentials  triggered by transcranial magnetic stimulation; ND, not done; NA, no data available; SARA, scale for the assessment and ration of atacia ; SICI: short intracortical inhibition, SRM: standardized response mean.

## 3.2 Capítulo 2: Body composition and peripheral insulin sensitivity in early and pre-symptomatic stages of Machado-Joseph Disease

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**Body composition and peripheral insulin sensitivity in early and pre-symptomatic stages of Machado-Joseph Disease**

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**Abstract**

**Background:** spinocerebellar ataxia type 3/Machado Joseph disease (SCA3/MJD) is due to an expanded CAG (CAGexp) repeat at *ATXN3.* Reduced body mass index (BMI) and increased peripheral sensitivity to insulin (PSI) were reported in moderate/advanced disease.

**Objectives:** To describe BMI and PSI in early symptomatic and presymptomatic SCA3/MJD individuals and to correlate them with disease and mutation severity.

**Methods:** 68 symptomatic and 12 pre-symptomatic SCA3/MJD subjects and 48 controls were recruited. BMI, lean mass index (LnBMI), %lean mass (%LM), basal metabolic ratio/weight (BMR), and glucose tolerance test (GTT) were studied. Correlations with age, CAGexp, time to onset of the pre-symptomatic, disease duration of the symptomatic groups (TtoDd), and the Neurologic Examination Score for Spinocerebellar Ataxia (NESSCA) were then tested.

**Results:** LnBMI was inversely correlated with CAGexp ($R=-0.541$, $p=0.001$) in all SCA3/MJD carriers. BMR and %LM were higher in subjects with age of onset (AO) earlier than expected from their CAGexp ($p=0.05$). GTT was lower in SCA3/MJD symptomatic individuals than in controls ($p<0.01$). GTT was inversely correlated with NESSCA ($R=-0.377$, $p=0.021$). No associations were found with TtoDd.

**Conclusions:** We found a relative independence between BMI and PSI roles and alterations in SCA3/MJD. The mutation severity was related to BMI, while factors related to age and independent from CAGexp were related to BMR. PSI was directly associated to the neurological burden as measured by NESSCA. If BMI and PSI change in time, the speed is independent from TtoDd.

**Key Words:** Spinocerebellar ataxia type 3, Machado-Joseph Disease, BMI, Insulin sensibility.

## INTRODUCTION

Spinocerebellar ataxia type 3, also known as Machado–Joseph disease (SCA3/MJD), is an autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion (CAGexp) at *ATXN3*.[1] SCA3/MJD is the most common form of SCA worldwide,[2] reaching a minimal prevalence of 6:100,000 in Rio Grande do Sul, Brazil.[3]

SCA3/MJD starts usually around 32–40 years[4-6] with cerebellar ataxia affecting gait and later limb, speech and swallowing coordination. Variable degrees of pyramidal, extrapyramidal, and peripheral nerve dysfunction occur during disease course resulting in heterogeneous phenotypic presentations.[1,7]

The CAGexp leads to elongation of the polyglutamine(polyQ) tract within the ataxin-3 protein, a feature shared with other polyQ diseases such as other SCA and Huntington's disease.[8,9] The polyQ repeat expansion is thought to confer a toxic gain-of-function to the various polyQ-encoding proteins promoting their misfolding and aggregation, but the precise mechanism of disease pathogenesis remains unknown.[1,10]

We have previously shown that SCA3/MJD patients with mean disease duration of 10 years presented lower body mass index (BMI) than age, sex and socioeconomically matched controls. CAGexp length was the only independent factor associated with BMI, independent from age, disease severity and duration;[11] similar to what was shown in symptomatic and presymptomatic individuals with Huntington´s disease (HD).[12,13] A longitudinal study on BMI progression in SCA1, SCA2, SCA3/MJD and SCA6 patients from the EUROSCA showed that BMI declined over time in general, and that the subgroup with decreasing BMI showed a faster deterioration of SARA.[14]

Moreover, SCA3/MJD patients presented higher peripheral sensitivity to insulin (PSI) according to the homeostatic model assessment (HOMA2) and higher peripheral levels of the insulin-like growth factor binding protein 1 (IGFBP-1), findings that might be related to BMI.[15] Insulin sensitivity (IS) was considered as a potential disease modifier, because it was inversely associated with age of onset (AO), independently from its main determinant, CAGexp length.

Almost all the attention on SCA3/MJD and other polyQ diseases research has been given to the pathology on central and peripheral nervous systems. However, studies on transgenic animal model of HD[16,17] showed a wide range of non-CNS tissues polyglutamine inclusions, and post mortem studies in infantile onset SCA7 showed ataxin-7 nuclear inclusions in endothelial, cardiac, skeletal muscle, exocrine pancreas, and duodenal epithelial cells.[18] These data together with our findings related to peripheral IS and BMI of SCA3/MJD patients indicated that systemic alterations, not directly related to the neuronal dysfunction, might also play a significant role in the pathogenesis of polyQ diseases.

Our main objective was to evaluate the body composition and the insulin sensitivity through glucose tolerance test (GTT) in early disease SCA3/MJD patients and in presymptomatic carriers of SCA3/MJD mutation, trying to deepen and further validate our previous findings.

## METHODS

### Population, design and eligibility criteria

Symptomatic patients with molecular diagnosis of SCA3/MJD or asymptomatic individuals with normal neurological examination at 50% risk of SCA3/MJD on the basis of an affected first-degree relative who were seeking for presymptomatic testing were recruited for this case-control study at the Neurogenetics outpatients clinic, Hospital de Clinicas de Porto Alegre, from May 2011 to July 2013.

Symptomatic patient's data were collected during the baseline assessments of a randomized clinical trial (RCT) where disease duration of more than 10 years and inability to walk independently (canes, sticks or walkers were allowed) were exclusion criteria.[19] The control group consisted of previously at-risk individuals who did not carry CAGexp at *ATXN3* plus healthy unrelated individuals with age, gender, and environmental characteristics similar to symptomatic individuals. Thyroid, renal or hepatic disorders, or history of other significant neurological or systemic medical disorder were also exclusion criteria.

## Molecular and clinical evaluations

The *ATXN3* expanded region was analyzed as previously described.[20] Neurological Examination Score for Spinocerebellar Ataxias (NESSCA)[21] and Scale for the Assessment and Rating of Ataxia (SARA)[22] were performed in all symptomatic individuals. Disease duration and age at disease onset were informed by patients and/or relatives. Mean estimated age at onset of the pre-symptomatic group was calculated with individuals' current age and CAGexp length in *ATXN3*, using the calculate table reported and available at de Mattos et al 2018.[23]

## Body composition

Weight and height were measured always using the same devices and BMI was calculated with the formula [weight/(height)$^2$]. Lean [LMI, lean mass weight/(height)$^2$] and fat mass index [FMI, fat mass weight/(height)$^2$], %lean mass (%LM) and basal metabolic ratio/weight (BMR) were determined using bioimpedance (Biodynamics BIA 450).

## Sample Collection

Biological material collection was performed between 8 a.m. and 12 p.m. after 12 hours fasting. Serum was obtained by blood centrifugation at 6000g for 5 min, and tested for glucose tolerance test (GTT), measuring glucose (mg/dL) 2h post intake of 75g of glucose to estimate the PSI.

## Statistical Analysis

The time remaining for disease onset (TTO) was estimated for each pre-symptomatic subject, based on the difference between the predicted age of onset and the current age of the person. TTO was presented in negative numbers. Then TTO and DD were merged into a unique variable of time, called time to onset/disease duration (TtoDd).

Variables that did not present normal distribution on Shapiro-Wilk test were transformed into natural logarithm (Ln). Baseline characteristics among groups were compared with ANCOVA (controlling for age), unpaired student t-test or chi-square test. Correlations were performed with Pearson and Spearman correlation tests. Statistical significance was defined as $p<0.05$.

### Ethics

The study protocol was approved by the ethics committee of our institution (registered as CAAE: 49486015.9.0000.5327 at the Brazilian National platform, Plataforma Brasil). All patients gave informed consent to participate in the study.

## RESULTS

Sixty-eight individuals were included in the early/moderate stage symptomatic SCA3/MJD group; 12 the asymptomatic SCA3/MJD; and 48 the healthy control group (9 related and 39 unrelated to SCA3/MJD individuals). Clinical and demographic characteristics are described in **Table 1**. Gender distribution was similar among the three groups.

**Table 1. Overall characteristics of subjects under study**

| | Controls | SCA3/MJD Symptomatic carrier | SCA3/MJD Pre-symptomatic carrier | p |
|---|---|---|---|---|
| N (females) | 48 (28) | 68 (34) | 12 (7) | 0.646[a] |
| Age at evaluation mean (SD) | 40.4 (13.35) | 40.9 (9.5) | 32.4 (8.1) | 0.49[a,b] |
| CAGn at normal alleles | 22.9 (4.25) | 22.07 (4.99) | 21.25 (3.65) | 0.587[a] |
| CAGn at expanded alleles | NA | 75.2 (3) | 73.5 (3) | 0.73[c] |
| Age at onset | NA | 34.9 (9) | NA | NA |
| Time after onset of symptoms | NA | 5.99 (2.5) | NA | NA |
| Predicted time to disease onset | NA | NA | -11.8 (6.9) | NA |

a - ANOVA
b - Symptomatic against pre-symptomatic (p=0.041); controls vs pre-symptomatic (p=0.066). Tukey
c - t-test

Since time after onset of symptoms was limited to ten years (in fact it was 6+-2.5 years), there was a strong correlation between age and AO (R=0.965, p=0.0001), and between age and CAGexp (R=-0.650, p=0.0001, all carriers; R=-0.811, p=0.0001, symptomatic carriers). I.e., AO was directly dependent and confounded with the age of symptomatic individuals. In order to reduce confounding bias, AO were only studied through the following approach. Predicted AO according to CAGexp was estimated to all SCA3/MJD carriers. Ages of pre-symptomatic carriers at the time of the study were all equal to or younger than the predicted AO for their CAGexp (at birth as well as corrected for their actual ages) (**Figure 1**). Symptomatic carriers were classified as those with AO earlier (AO-), equal (AOn) to and later (AO+) than expected, if their AO were inside the 95%

confidence interval predicted by their CAGexp. Six, sixty-one and one symptomatic carriers were classified as AO-, AOn and AO+, respectively. This stratification allowed some comparisons between AO- and AOn subjects, independently from their CAGexp.

**Body composition**

Body composition was firstly compared among groups. Since lnBMI was similar between males and females, only age was controlled as a covariate. LnBMI, lnLMI, lnFMI, %LM, and BMR were similar among groups ($p>0.05$; ANCOVA; **Table 2**). A subgroup analysis on symptomatic individuals and controls under 38 years-old (median age of control group) was performed and, in this subgroup, SCA3/MJD patients (n=29) presented lower LnBMI, %LM, BMR, LnLMI, and LnFMI than 24 healthy controls ($p<0.001$).

In order to explore the role of parameters of body composition in SCA3/MJD, potential relations with age, CAGexp, TtoDd, symptomatic state, NESSCA and SARA scores were studied. LnBMI was inversely correlated with CAGexp ($R=-0.445$, $p=0.001$, **Figure 2A**) and directly correlated with age ($R=0.443$, $p=0.001$, **Figure 2B**) only. Both age and CAGexp improved the strength of association, on regression analysis ($R^2=0.240$, $p=0.0001$). LnLMI was associated with CAGexp ($R=-0.303$, $p=0.05$) but not with age or TtoDd, in both symptomatic and pre-symptomatic subjects. Although CAGexp and age correlated independently with lnFMI, %LM and BMR, just age explained the variability of these parameters on regression analysis.

Extremes in AO were related to some parameters of body composition: %LM were of 82.7% (6.5) versus 73.8 (8) ($p=0.05$), and BMR were of 25.8 (2) versus 23 (2.5) ($p=0.05$) in AO- and AOn symptomatic subjects, respectively (**Figures 2C** and **2D**). Regression analysis confirmed that both %LM and BMR ($R=0.68$, $p<0.0001$ for both analyses) were associated to age and to the extreme phenotype of AO. Neither time to/time after onset (all CAGexp carriers) nor time after onset (symptomatic group) was related to changes in the parameters of body composition, when age was controlled.

**Peripheral Sensitivity to Insulin**

Natural logarithm of glucose levels after GTT (lnGTT) were 4.61 (SEM 0.07), 4.43 (0.05) and 4.6 (0.04) mg/dL on pre-symptomatic and symptomatic carriers, and controls, respectively (**Table 2**). Differences were significant between symptomatic carriers and controls ($p<0.01$, ANCOVA), and showed a trend for significance between pre-symptomatic and symptomatic carriers ($P=0.06$, ANCOVA).

**Table 2 Overall body composition of studied groups**

| | **Controls** | **SCA3/MJD Symptomatic carrier** | **SCA3/MJD Pre-symptomatic carrier** | **p** |
|---|---|---|---|---|
| N | 48 | 65 | 12 | |
| LnBMI (kg/m²) [raw values] | 3.25 (0.03) [26.47 (0.83)] | 3.2 (0.02) [25.05 (0.61)] | 3.19 (0.05) [24.68 (1.16)] | 0.283 |
| %LM (%) | 72.27 (8.5) | 74.34 (8.5) | 72.25 (8.6) | 0.476 |
| BMR (kcal/kg) | 22.55 (2.65) | 23.19 (2.66) | 22.54 (2.68) | 0.475 |
| LnLMI (kg/m²) [raw values] | 2.92 (0.02) [18.74 (0.4)] | 2.87 (0.02) [17.86 (0.38)] | 2.87 (0.03) [17.73 (0.47)] | 0.213 |
| LnFMI (kg/m²) [raw values] | 1.9 (0.07) [7.49 (0.55) | 1.75 (0.08) [6.45 (0.44)] | 1.85 (0.14) [7.07 (1.02)] | 0.307 |
| LnGTT(mg/dL) [raw values] | 4.6 (0.04) [103.56 (4.34)] | 4.43 (0.05) [88.30 (4.28)] | 4.61 (0.07) [103.08 (7.1)] | 0.014* |

normal values are within brackets, not transformed into natural logarithm

*p value among all groups (ANCOVA)

In order to explore the role of lnGTT on SCA3/MJD, potential relations with age, CAGexp, time to/after disease onset, symptomatic state, NESSCA and SARA were studied. Extremes in AO were not related to GTT (**Figure 3A**).

When all SCA3/MJD carriers were analyzed, lnGTT correlated with CAGexp (R=-0.342, p=0.011), age (R=0.339, p=0.011), and NESSCA (R=-0.377, p=0.021) (**Figure 3B**). On regression analysis, only age and NESSCA remained significant ($R^2$=0.269, p=0.015). When only symptomatic subjects were studied, age (R=0.44, p=0.003) and NESCCA (R=-0.377, p=0.021) correlated with lnGTT. LnGTT did not correlate with disease duration or with predicted time to onset.

In the overall group, as expected, lnGTT correlated with lnFMI (R=0.310, p=0.01), %LM (R=-0.307, p=0.01), and BMR (R=-0.308, p=0.01). On regression analysis, the association with BMR was maintained (R=-0.308, p=0.01). Among CAGexp carriers, lnGTT correlations were in the same direction, and were even stronger than in the overall group: lnGTT correlated with lnBMI (R=0.316, p=0.05), with lnFMI (R=0.510, p=0.0001), %LM (R=-0.556, p=0.0001), and BMR (R=-0.556, p=0.0001). When age, group (symptomatic and asymptomatic carriers), and %LM or BMR were considered causal variables in regression analysis, associations with group and %LM or BMR were maintained (R=0.606, p=0.001).

We speculated whether lnGTT could be a biomarker of an independent, causal factor affecting neurological manifestations in SCA3/MJD, due to its correlation with NESSCA (**Figure 3B**). When lnGTT, age, and CAGexp were used as independent variables, lnGTT and CAGexp can predict 41% of NESSCA variability, on regression analysis (R=0.653, p=0.0001). In spite of NESSCA to be a measure of disease burden, lnGTT was not related to TtoDd in the overall group not to time after onset, in the symptomatic group (**Figure 3C**).

## DISCUSSION

BMI and PSI are covariates and change together in health as well as in SCA3/MJD. Weight loss improves PSI in human beings.[24] Moreover, weight gain and a reduced PSI are common events related to ageing. However, covariance and ageing do not explain all the relationship between these biomarkers and SCA3/MJD. In the present study, we were able to show that BMI is inversely related to CAGexp, and that BMR is related to additional factors associated with earlier ages at onset. We have also showed that PSI was directly related to the neurological burden as measured by NESSCA. All the associations were robust. The fact that BMI and PSI were not related to TtoDd suggests that metabolic processes underlying BMI and PSI progress at different rates from the progression rate of neurologic manifestations. Moreover, present findings favor a relative independence between BMI and PSI, in the scope of SCA3/MJD: (1) larger the CAGexp, lower was the BMI; however, BMI was not related to neurological severity; (2) stronger the PSI measured by GTT, worse was NESSCA; however, PSI was not related to CAGexp.

SCA3/MJD and other polyQ diseases have been considered for a long time as exclusively neurological disorders. Several lines of evidence from patients and transgenic animal models refuted this idea;[11,15-18] although, the characterization and understanding of the role of systemic alterations in polyQ diseases remains unknown. In a previous study, we have shown that moderate to advanced disease stages SCA3/MJD patients presented lower BMI than matched controls and that longer $CAG_{exp}$ tracts were the only independent predictor of lower BMI, linking the disease causal mutation with body composition.[11] We also found that these patients presented higher peripheral sensitivity to insulin and that insulin sensitivity could be a potential disease modifier. Now, we deepen the characterization of body composition and IS in SCA3/MJD studying a different cohort of early/moderate stage symptomatic and presymptomatic SCA3/MJD individuals.

A direct association between a reduced BMI and SCA3/MJD was not found in the present study, mostly because we recruited subjects at early phases of the disease. As a matter of fact, a subgroup analysis showed that in early stages of SCA3/MJD, low BMI is present only in younger individuals, with longer CAGexp.

Therefore, the present data shows that body composition, as measured by BMI, is affected by the CAGexp in SCA3/MJD. BMI loss occurs, at least in more advanced cases,[14,15] but in a different speed from the progression rate of the neurologic deterioration, since BMI was not related to TtoDd or to clinical scales. The pattern of BMI loss is hard to be followed in longitudinal observations, due to the opposite trend - increases in BMI - seen in ageing populations. In the recent longitudinal study from Eurosca consortium, there were actually three groups of patients according to BMI longitudinal changes: those with increased BMI, stable BMI and decreased BMI. All alternatives can happen.

The relationship between BMI and CAGexp, and of BMR and extremes in AO, in SCA3/MJD, raises a number of potential mechanistic explanations (effectors). Although dysphagia may be an important cause of weight loss, this relation was actually absent in different SCA3/MJD cohorts.[14,15,25] Hypermetabolism might be due to movement disorders like lower motor neuron degeneration and dystonia.[14] However, these findings occur in moderate/late stages of the SCA3/MJD, subtypes 3 and 1,[1] respectively, and cannot explain our present findings, obtained from early stages of disease. The association between BMR and the extremes in AO independent from the CAGexp suggest that hypermetabolic states can be a risk factor for more severe manifestations. Due to that, the most plausible explanation lies in a potential increased catabolic state in the presence of a polyQ, as already described in patients with SCA1.[26]. Authough autonomic dysfunction might be the underlying cause, we speculate whether the increased energy consumption that we propose is present in SCA3/MJD, could be related to the generation of systemic intracellular aggregates. Non-neuronal tissues might get rid of aggregates by promoting asymmetrical mitosis[27,28] - maybe at expenses of an increased energy consumption and weight loss.

We confirmed data previously published by our group on moderate/advanced SCA3/MJD of higher PSI[15] in this present, different cohort of presymptomatic subjects, and of patients with early disease stages, using a different method (previously higher HOMA2-%S and now lower glucose levels after GTT).

Both BMI and PSI correlated with age, as expected. Our previous study showed that PSI correlated with AO, independently from the CAGexp. In the present cohort, AO was tightly related to age, due to the recruitment of subjects with up to 10 years of disease duration, and age is a strong confounder for PSI and BMI. We decided to explore the effect of the AO independent from the CAGexp by stratifying subjects according to their prediction of the AO. This stratification is very useful to study AO determinants independent from the CAGexp. Quite a few symptomatic subjects fell outside the confidence interval for their predicted AO. The number of very early onset (3) and very late onset (1) subjects might have precluded the finding of significant differences in PSI among groups (**Figure 3A**). Therefore, we were not able to confirm inverse association of PSI with age at disease onset, independently from its main determinant, CAGexp length.

However, we detected a moderate association between PSI and neurological burden, as measured by NESSCA and by SARA (not shown). In this relationship, what comes before? We can foresee two alternatives: (a) neuronal dysfunction and death in central nervous system comes before, producing an "inflammatory cascade" that would induce an increase in the PSI; (b) an increased PSI, determined by a hidden, unknown factor, is present in SCA3/MJD and worsens neurologic progression (worse NESSCA, worse SARA; maybe worse AO). Weight loss would potentiate this effect. We speculate that these unknown factors operate in the insulin signalling pathway, and that the mutant ataxin-3 itself might be one of these factors.

PSI has been linked to both neuroprotection and neurodegeneration in context of age related disorders. An increase in PSI activates DAF-2/IRS and will lead to DAF-16/FOXO inhibition. The inhibition of the PSI, on the other hand, lead to DAF-16/FOXO activation with increased expression of chaperones and stress resistance, and increase C. elegans life span.[29] PSI inhibition also activates HSF-1, which increase the expression of heat shock proteins that may reduce the proteotoxicity of expanded polyQ. Still, some studies pointed that Akt and autophagy activation through IRS-2 are protective in Huntington´s disease and in SCA1 transgenic models.[30-32] Recent data, however, showed that Akt did not have significant effect in SCA1 cerebellar tissues, and that ataxin-1 phosphorylation in

cerebellum on serine 776 is dependent on other kinase.[33] Altogether, these data illustrate the insulin paradox related to proteotoxicity and neurodegeneration.

Huntington disease and SCA1 patients present impaired peripheral insulin sensitivity when compared to controls.[12,34] In HD impaired insulin sensitivity was strongly associated with longer CAG repeats.[12] This data could favor both a reactive neuroprotective role that impaired insulin sensitivity or that impaired insulin sensitivity could worsen the disease burden. Differently from ataxin-1 and huntingtin, ataxin-3 function has a more intimate relation with PSI. CDC-48 regulates the UPS mobilizing Ub substrates to proteasome 26S. Ataxin-3 is a deubiquitinating (DUB) protein that interacts with CDC-48. C. elegans deficient in both CDC-48 and ataxin-3 showed an increased life span of 50% that was mediated through the IIS, probably due to editing ubiquitination of IIS substrates. CDC-48 and ataxin-3 deficiency do not add additional increase in life span of partial deficient DAF-2 in C. elegans and inhibition of DAF-16/FOXO abolish the gain in lifespan of CDC-48 and ataxin-3 deficiency. Therefore, lower levels of ataxin-3 would be associated with decrease in PSI through DAF-2 and increase in DAF-16/FOXO.[35] PSI signaling reduction in C. elegans transgenic SCA3/MJD lead to increase HSF-1 and reduced neurodegeneration. DAF-2 and AGE-1 reduction and DAF-16/FOXO increase neuroprotective actions were not associated with changes in ataxin-3 expression.[36] Since we have found that increased insulin sensitivity in SCA3/MJD patients is associated with worsened NESSCA and SARA scores, a possible hypothesis would be that ataxin-3 with expanded polyQ tract would presents higher affinity to interact with insulin signaling than wild type, increasing insulin sensitivity. Considering that increase PSI activity reduces FOXO and HSF-1 activities, this would enhance proteotoxicity, with premature ageing, and premature disease onset. Mutant ataxin-3 DUB activity was already shown to be higher than wild type form in proteins like parkin and CHIP.[37,38]

Since PSI tends to reduce as we grow older, longitudinal studies on changes in PSI will face several difficulties in raising strong evidences relating PSI with progression rate of neurological findings in SCAs in general and in SCA3/MJD in particular. Association studies linking phenotypes and molecular markers, or

functional studies with cellular models might be better suited to shed light into causal pathways, in future.

In spite of several strengths, our study has some limitations. This study did not have a single, primary outcome and, therefore, we did not perform sample size calculation and study power determinations. However, since we are dealing with a rare disease and our sample size is one of largest on biomarkers studies of SCA3/MJD, the number of recruited individuals turned to be one of the study's merits.

In conclusion, our data strength the evidence of systemic alterations in SCA3/MJD, and links disease mutation severity with both lean and fat mass reduction. We confirmed an association between BMI and CAGexp; moreover, we raised evidence relating PSI to worsened neurologic manifestations. Although, we cannot assume a cause/consequence relationship of PSI and neurological burden, it is possible that a dysfunction in insulin signaling might worse the neurodegeneration. Understanding the interaction of the insulin system components, on one side, and of the energy consumption/body components, on the other, with the expanded ataxin-3 in pathophysiology of SCA3/MJD should be targeted in future studies, with potential therapeutic implications.

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**Contributors:** GVF, JAMS, MLS-P and LBJ: designed the study. JAMS, GNS, AR, TLM, ASS, RDA, KCD, RMC and CRMR: acquisition of clinical data. GVF and TCG: acquisition of laboratorial data. GVF, VBL, SC and LBJ: performed statistical analysis. GVF, JAMS, MLS-P and LBJ: wrote the manuscript. All authors: drafting and revising the article.

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**Competing interests:** None declared.

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**Figure 1. Age of pre-symptomatic carriers and age of onset of symptomatic against age of onset predicted for the size of CAGexp.** (predicted using the calculate table reported and available at de Mattos et al 2018[23])

**Figure 2. Body composition.** a) Ln Body mass index (lnBMI) x CAGexp; b) LnBMI x Age; c) % of loan mass (%LM) at different groups of actual age of onset compared to age of onset predicted for the size of CAGexp; and d) Basal metabolic ratio/weight at different groups of actual age of onset compared to age of onset predicted for the size of CAGexp.

**Figure 3. Peripheral sensitivity to insulin.** a) Ln of the blood glucose level after 120min of ingestion of 75g of glucose (LnGTT) at different groups of actual age of onset compared to age of onset predicted for the size of CAGexp; b) NESSCA x LnGTT; and c) LnGTT x time to onset/disease duration (TtoDd).

Figure 1.

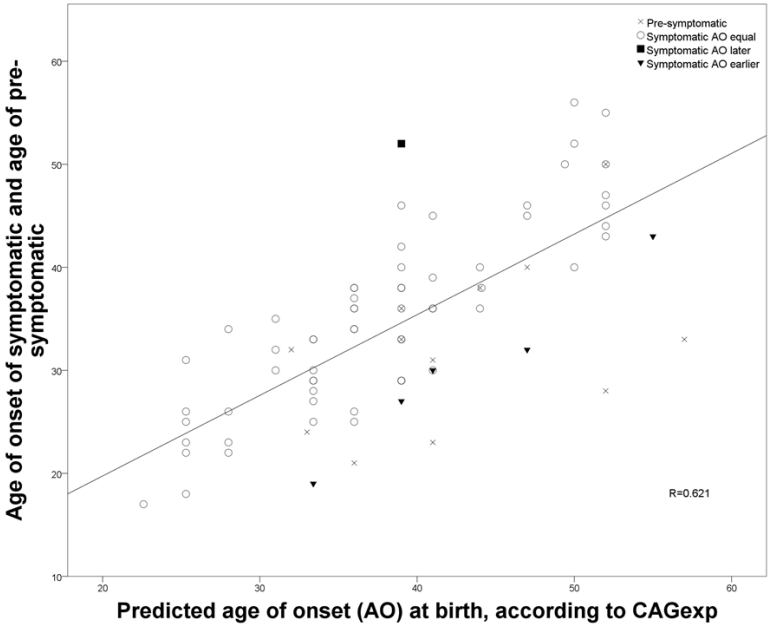

Pre-symptomatic
Symptomatic AO equal
Symptomatic AO later
Symptomatic AO earlier
R=0.621
Age of onset of symptomatic and age of pre-symptomatic
Predicted age of onset (AO) at birth, according to CAGexp
10
20
30
40
50
60

Figure 2.

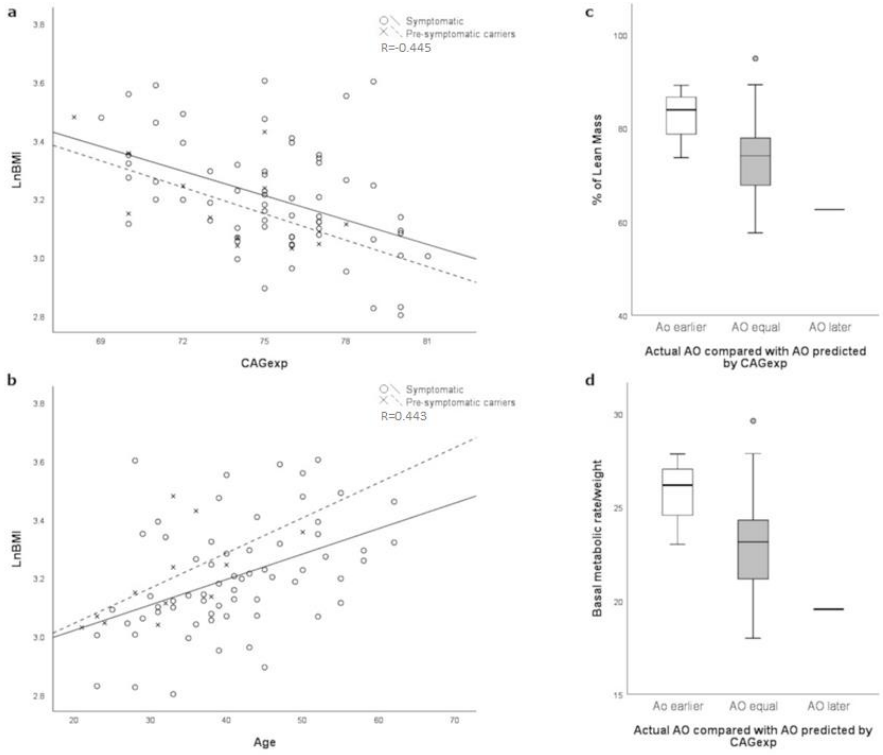
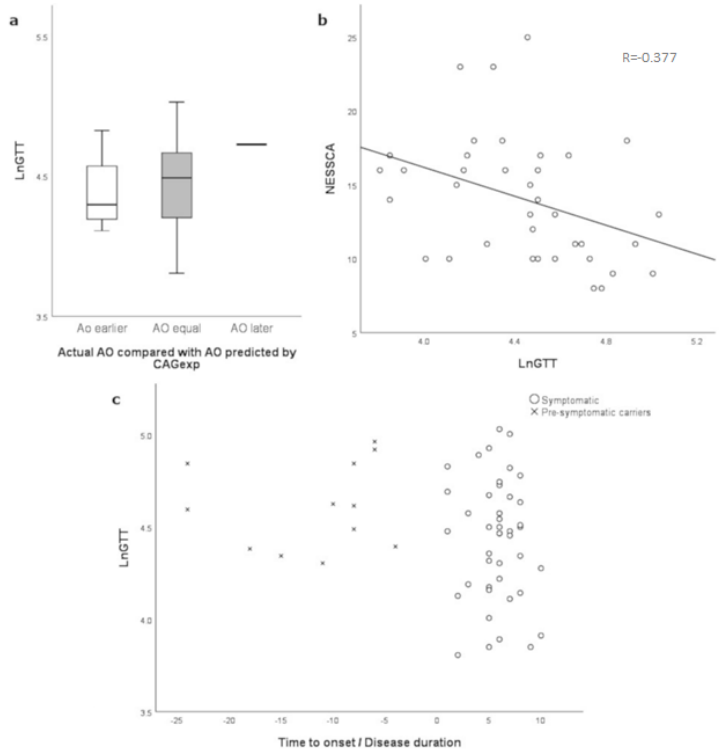

a
Symptomatic
Pre-symptomatic carriers
R=-0.445
LnBMI
CAGexp
b
Symptomatic
Pre-symptomatic carriers
R=0.443
LnBMI
Age
c
% of Lean Mass
Ao earlier
AO equal
AO later
Actual AO compared with AO predicted by CAGexp
d
Basal metabolic rate/weight
Ao earlier
AO equal
AO later
Actual AO compared with AO predicted by CAGexp

Figure 3.


a
LnGTT
Ao earlier
AO equal
AO later
Actual AO compared with AO predicted by CAGexp
b
R=-0.377
NESSCA
LnGTT
c
Symptomatic
Pre-symptomatic carriers
LnGTT
Time to onset / Disease duration

## 3.3 Capítulo 3: Neurotrophins and markers of neuronal damage in Machado-Joseph disease

A ser submetido no Journal of the Neurological Sciences.

**Neurotrophins and markers of neuronal damage in Machado-Joseph disease**

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**Abstract**

Neurotrophins are important regulators of neural survival, function, plasticity, development, and apoptosis. BDNF and NGF are the most studied while CTNF and NSE are markers of neuronal damage. Spinocerebellar ataxia type 3 (SCA3/MJD) is a neurodegenerative disease where these proteins were never studied, excepting NSE. Our objective was to analyze levels of these proteins in SCA3/MJD patients up to 48-weeks intervention with lithium and compare to untreated patients. Blood samples were collected from 61 SCA3/MJD patients, divided into two groups in a randomized manner, at four different stages (baseline, 12, 24 and 48-weeks). In addition, samples from 38 controls were also collect at baseline. Serum was isolated, and levels of proteins were determined through enzyme immunoassay. Levels of BDNF and CTNF were similar between groups. SCA3/MJD group showed lower NGF levels, and higher NSE in serum when compared to control. It was also observed a gradual decrease in NGF levels over 48-weeks in patients. A direct correlation was observed between NGF and NESSCA in contrast to an inverse correlation between BDNF and SARA. High NSE seric levels demonstrated here denotes this protein as a good marker of neuronal damage in SCA3/MJD. NGF seems to be a good biomarker candidate. However, further investigation is required.

Keywords: SCA3, MJD, Neurotrophins, NGF, NSE

## Introduction

Neurotrophins are important regulators of neural survival, function, plasticity, development, and apoptosis. The most studied is brain-derived neurotrophic factor (BDNF), which during development promotes the survival and differentiation of selected neuronal populations. BDNF also participates in axonal growth, pathfinding and in modulating dendritic growth and morphology, and is a major regulator of synaptic transmission and plasticity in adult synapses [1]. Nerve growth factor (NGF) is another neurotrophin involved in the regulation of growth and the differentiation of sympathetic and certain sensory neurons [2]. Ciliary neurotrophic factor (CNTF) is a neurotrophic factor capable of inducing neuronal differentiation and survival, which has effects on the development and maintenance of nervous system [3]. Neuron specific enolase (NSE) could be a potentially useful biomarker for assessing neuronal damage, having neurotrophic and neuroprotective properties on a broad spectrum of central nervous system (CNS) neurons [4].

These proteins play important roles in the maintenance of nervous system and have been associated with neurodegenerative diseases. BDNF is decreased in Huntington disease (HD) [5], in Alzheimer disease (AD) [6], and in Parkinson disease (PD) [7]. Levels of NGF are lower in HD [8] and in AD [6]. NGF was used as a treatment in AD [9] and an improvement was observed. Therefore, NGF could be a good treatment candidate for trials in other neurodegenerative disease. Reduced levels of CTNF were shown in PD [10] and this protein was used as an intervation drug in a clinical trial for HD [11]. Higher levels of NSE were demonstrated in peripheral blood in both HD [12] and AD [13].

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is a late onset genetic neurodegenerative disease characterized by ataxia, followed by diplopia, dysarthria, spasticity, and other findings. SCA3/MJD is due to an expanded CAG tract in the *ATXN3* gene, and length of expanded repeat is, in general, inversely correlated to age of onset of the disease. To date, there is no effective treatment available. NSE levels were evaluated in just two studies in SCA3/MJD patients [14,15], and higher NSE levels were shown in peripheral blood

in SCA3/MJD patients than in controls. Lower levels of BDNF were demonstrated in a cellular model of SCA3/MJD [16]. Our objective was to analyze protein levels of BDNF, NGF, CTNF and NSE in SCA3/MJD patients up to 48-weeks intervention with lithium and compare to untreated patients.

## Methods

### Material and methods

#### Subjects

A total of 61 SCA3/MJD patients were included in a clinical trial previously performed at Hospital de Clínicas de Porto Alegre (HCPA), in Rio Grande do Sul, Brazil, by our group. Intervention was based on an oral administration of lithium carbonate (Carbolitium; Eurofarma, São Paulo, Brazil), and details can be found elsewhere [17].

In brief, patients were randomly assigned to lithium group (n=30) or placebo group (n=31) and followed for 48 weeks. Neurological scales NESSCA and SARA were performed at baseline and at 24 and 48 weeks of intervention. Peripheral blood samples were collected at baseline, 12, 24, and 48-weeks from patients' groups. Length of CAGexp was already available. In addition, a control group composed by 38 healthy controls was selected, and blood samples were collected in the beginning of the trial.

The study protocol was approved by the ethics committee of our institution (registered as CAAE: 49486015.9.0000.5327 at the Brazilian National platform, Plataforma Brasil). All patients gave informed consent to participate in the study.

#### Protein Measurements

For the biochemical analyses, 10 mL of peripheral blood were collected. Blood was immediately centrifuged at 6,000 × g for 5 min, and serum was immediately frozen at -80°C, and kept until analysis.

Serum BDNF levels were measured with a sandwich-ELISA using an available immunoassay kit according to the manufacturer's instructions (DuoSetELISA Development, R&D Systems, Inc., Minneapolis, MN, USA). Serum NGF levels was measured using a commercially available enzyme immunoassay kit. The amounts of NGF were determined by an immunoassay kit (DuoSet ELISA Development, R&D Systems, Inc., USA). Serum CTNF was measured using a Quantikine®ELISA immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). Serum NSE was measured using an eletrochemiluminescent assay provided by Roche Diagnostic®, Indianapolis, IN. The reaction and quantification were performed by Elecsys-2010 (Roche). Since NSE is also present in blood cells, no hemolyzed sample was used. All samples and standards were measured in duplicate, and the coefficient of variation was less than 5%. The serum BDNF and NSE levels are expressed as ng/ml, NGF and CTNF is expressed as pg/mL.

Statistical Analysis

All analyses were carried out using the SPSS 18 statistical software package (SPSS Inc., Chicago, IL). Normality of distribution was tested by Kolmogorov-Smirnov test, Independent-sample Mann-Whitney U test was used to see difference between groups at baseline, Spearman correlation to correlate mRNA levels with clinical characteristics, and Wald chi-square was used to analyze difference between treatments among time. All tests were two-tailed, performed at the significance level $\alpha = 0.05$.

## Results

Gender and age distribution did not differ between SCA3/MJD group (30 female (49.2%); Mean age: 40.77, SD±9.10) and control group (11 female (55.0%); Mean age: 40.30, SD±12.67). No difference of clinical characteristics was observed at baseline between lithium and placebo.

Protein experiments at baseline did not show difference of CTNF between SCA3/MJD patients and controls (p=0.246, Fig. 1A). Considering BDNF, slightly higher levels were seen in SCA3/MJD patients (p=0.051, Fig. 1B). We have also seen higher NSE levels in patients when compared to controls (p=0.026, Fig. 1C). On the other hand, lower NGF levels were seen in patients (p=0.010, Fig. 1D).

When we correlate protein levels with clinical features, we found a straight correlation between NGF and NESSCA (rho:0.298; p=0.046) (Fig. 2A) and an inverse correlation between BDNF and SARA (rho:-0.276; p=0.36) (Fig. 2B) in patients at baseline. CTNF correlate inversely with age in controls (rho:-0.369; *p*=0.038), in SCA3/MJD patients groups age (rho:-0.258; p=0.063) and age of onset (rho:-0.268; p=0.052) show a trend to correlate inversely. Correlation with any other parameters was found on these three proteins, and none correlation was found with NSE.

A gradual decrease of NGF levels was observed along 48 weeks in patients (Fig. 3A). None of the other proteins showed significant difference in expression at any time of the study (Fig. 3B,C,D). Therefore, no difference was seen in protein levels when lithium group was compared to placebo group.

**Discussion**

Neurotrophins are proteins identified as growth factors for development, survival, and function of neurons. However, it has been shown that those proteins have functions in reproductive and immune systems [18]. Although, they have an important role in nervous system, and have been studied in neurodegenerative disease such HD, AD and PD. Neurotrophins have never been studied as possible biomarkers of spinocerebellar ataxia patients.

Our study shows that protein levels of some neurotrophins (BDNF and CTNF) are similar between SCA3/MJD patients and controls. This lack of difference in neurotrophins in blood can be due to the fact that the main effect of these proteins is in the nervous system. Lower BDNF mRNA leves and immunoreactive granules were previously demonstrated in cerebellum of SCA6 patients [19].

We confirm higher protein levels of neural damage marker (NSE) [14,15], and this increase in serum could be result of neural damage that goes to peripheral blood.

A correlation of CTNF and age observed in controls was previously showed in rats, along with a strong correlation between CTNF and age-related changes in muscle mass [20]. The trend to correlation in SCA3/MJD patients of CTNF with age collaborate with the idea that CTNF decrease with aging, the possible correlation with age of onset can be because the fact that CTNF is a potent survival factor for neurons and oligodendrocytes, although we can confirm that this correlation is real, one time that in our study age and age of onset is strongly correlated (data not shown).

Lower seric levels of NGF in SCA3/MJD patients can be an effect of dysregulation on NGF system, once NGF is a secreted protein [2]. We found a directed correlation with NESSCA (disease severity) that was also previously demonstrated in Huntington disease where HD patients have lower NGF levels; those levels increase with disease severity [8].

No effect was seen of lithium intervention in these four proteins, although it was described previously that lithium treatment increase BDNF in serum of AD patients [21] and in serum of bipolar patients [22]. It is relevant to point out that BDNF levels of those patients were lower than controls [23,24], differently of what we found in SCA3/MJD patients included in our study. NGF increase in brain of rats with lithium [25] and lithium does not modify NSE levels in bipolar disease [26]. No study analyzed the effect of lithium in CTNF.

This was the first study in SCA3/MJD patients looking for neurotrophins in peripheral blood, and one of very few studies analyzing possible biomarkers along time. We have shown here correlation between specific neurotrophins and clinical scales and generate preliminar data indicanting NGF as a good candidate to state and progression biomarker in SCA3/MJD.

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Competing interests: None declared.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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**Fig. 1 – Mean neurotrophin levels in baseline from SCA3/MJD patients and controls.** a) CTNF; b) BDNF; c) NSE; and d) NGF. *p<0.050.

**Fig. 2 – Correlation proteins and clinical features in SCA3/MJD patients**. a) NGF X NESSCA; b) BDNF X SARA.

**Fig. 3 – Variation of protein levels among time.** a) NGF; b) BDNF; c) CTNF; and d) NSE in baseline (white), 12 (grey), 24 (striped) and 48 weeks (grid). *p<0.050.

Fig. 1

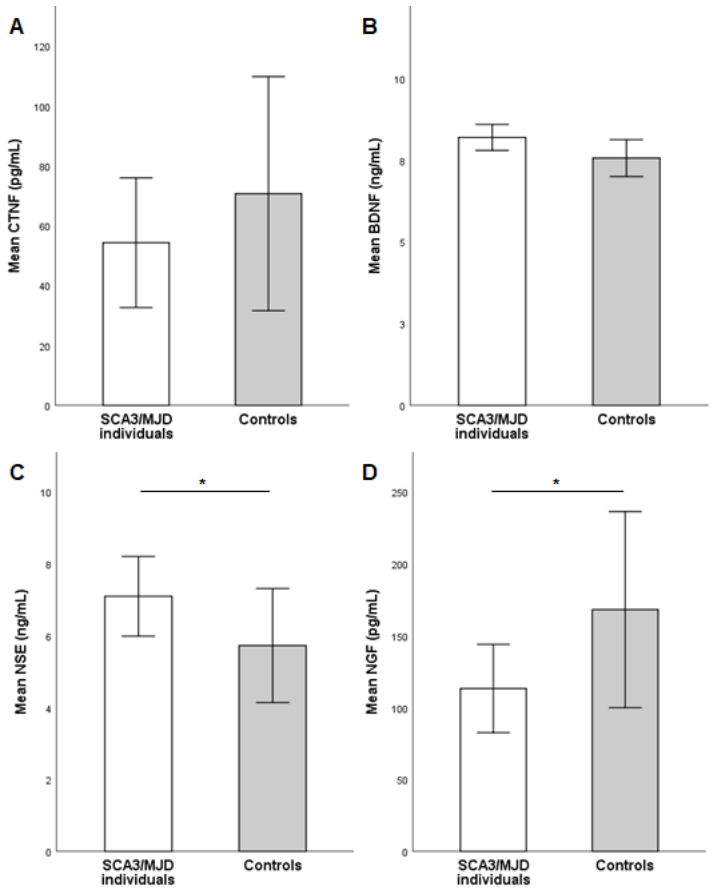

A
Mean CTNF (pg/mL)
120
100
80
60
40
20
0
SCA3/MJD individuals
Controls
B
Mean BDNF (ng/mL)
10
8
5
3
0
SCA3/MJD individuals
Controls
C
Mean NSE (ng/mL)
*
10
8
6
4
2
0
SCA3/MJD individuals
Controls
D
Mean NGF (pg/mL)
*
250
200
150
100
50
0
SCA3/MJD individuals
Controls

Fig. 2

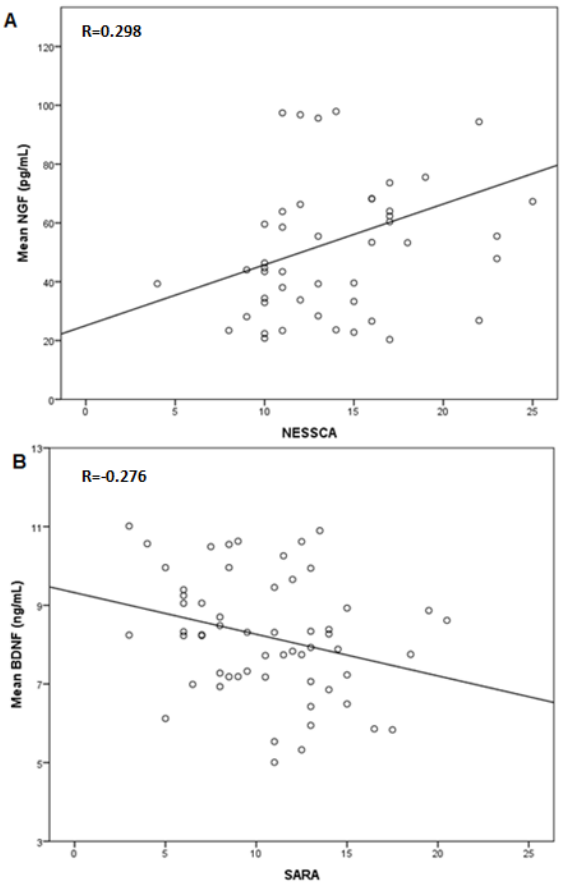
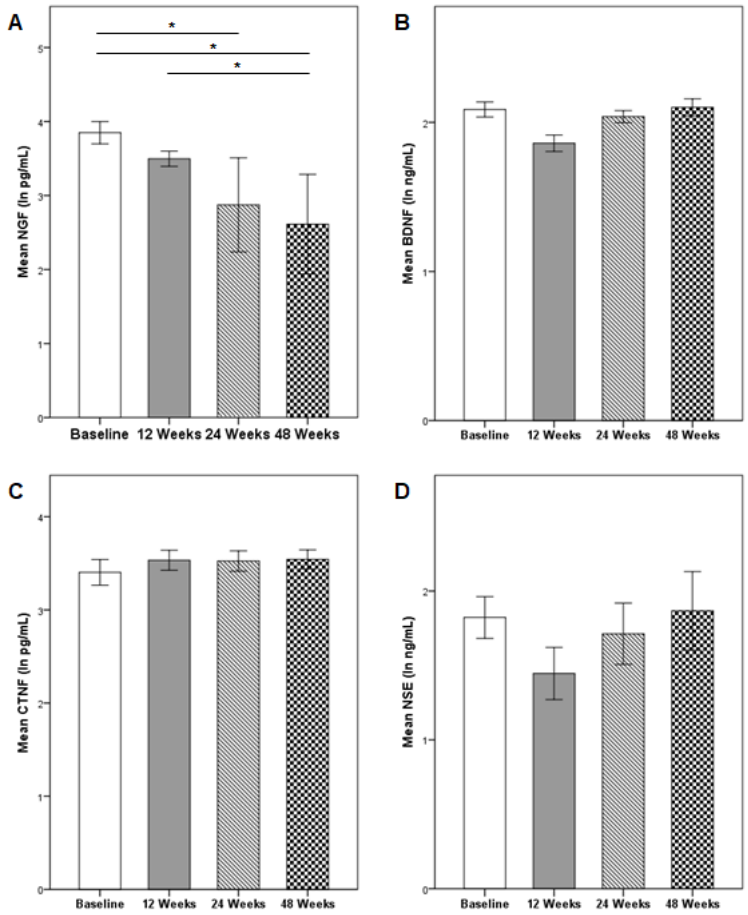


Fig. 3


A
Mean NGF (ln pg/mL)
Baseline
12 Weeks
24 Weeks
48 Weeks
B
Mean BDNF (ln ng/mL)
C
Mean CTNF (ln pg/mL)
D
Mean NSE (ln ng/mL)

## 3.4 Capítulo 4: *GSK3β* and *HDAC6* expression levels as potential biomarkers of lithium in SCA3/MJD

A ser submetido no *NeuroMolecular Medicine*

## *GSK3β* and *HDAC6* expression levels as potential biomarkers of lithium in SCA3/MJD

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**Abstract**

Spinocerebellar ataxia type 3 (SCA3/MJD) is a neurodegenerative disease that has high frequency in the south part of Brazil, with no effective treatment available. A double blinded clinical trial was performed by our group to evaluate safety and efficacy of lithium in a group SCA3/MJD patients, but no statistically difference on efficacy was observed when using clinical scales. We present here data generated on selected candidates to be biomarkers of lithium intervention based on mRNA expression levels of *GSK3β* and *HDAC6* genes. In total, 61 SCA3/MJD patients were included, divided into two groups (lithium and placebo), and followed by 24 weeks. Blood samples collect at baseline and following a 24-weeks intervention. Samples from 20 controls were also collected at baseline. mRNA expression levels were performed by standard methods, and lower *HDAC6* expression levels were shown in patients when compared to controls. Higher expression levels of both *GSK3β* and *HDAC6* were seen in lithium when compared to placebo group. A correlation was established between GSK3β variation and NESSCA variation, especially in untreated SCA3/MJD patients. This study showed novel insight into two candidate genes and pathology of SCA3/MJD.

**Key Words:** SCA3, MJD. *GSK3β, HDAC6,* Biomarkers

## Introduction

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is a late onset neurodegenerative disease, inherited as an autosomal dominant trait, caused by an expansion of a CAG tract (CAGexp) at the *ATXN3* gene (Kawaguchi et al. 1994). SCA3/MJD is the most common spinocerebellar ataxias (SCA) worldwide (Paulson 2007), and high prevalence of the disease was also seen in Brazil (de Castilhos et al. 2014). The minimal prevalence of SCA3/MJD in our region (Rio Grande do Sul, Brazil) was updated to be 6:100,000 inhabitants (Souza et al. 2016). To date, no effective treatment is available.

As neuroprotective effects of lithium were demonstrated in other neurodegenerative disorders, a double blinded clinical trial was performed by our group to evaluate safety and efficacy of lithium in a group SCA3/MJD patients (Saute et al. 2014). The Neurological Examination Score of Assessment of Spinocerebellar Ataxia - NESSCA (Kieling et al. 2008) was used to evaluate efficacy as a primary outcome. Additional clinical scales, such as Scale for the Assessment of Spinocerebellar Ataxia - SARA (Schmitz-Hubsch et al. 2006), Spinocerebellar Ataxia Functional Index - SCAFI (Schmitz-Hubsch et al. 2008) and Composite Cerebellar Functional Score - CCFS (Du Montcel et al. 2008), were also applied to evaluate efficacy as a secondary outcome. In the end of the trial, lithium was considered safe, but no statistically difference on efficacy was observed when the main clinical scales used to measure progression of ataxia, NESSCA and SARA, were considered. However, SCAFI and CCFS detected minor progression, suggesting that lithium may have efficacy against ataxic manifestations. Nevertheless, these scales present a slow progression in the SCA3/MJD along a year. Therefore, a large number of patients and/or more time of follow up is required in order to observe some effect using these scales as outcomes (Saute et al. 2015).

Hence, molecular biomarkers can be good candidates to be employed instead of clinical scales to measure disease progression. Ideally, a good biomarker has to be easy to measure, and should be able to translate even small clinical changes. We present here data generated on selected candidates to be biomarkers of lithium intervention based on mRNA expression levels of glycogen synthase kinase 3 beta (*GSK3β*) and histone deacetylase 6 (*HDAC6*) genes. These candidates were chosen based on the fact that *GSK3β* expression can be modified by lithium intake (Malhi & Outhred. 2016), and that expression of *HDAC6* plays a role on neuroprotection, taking part into aggresome formation in cellular model of SCA3/MJD (Burnett & Pittman. 2005).

**Material and methods**

Subjects

A total of 61 SCA3/MJD patients were included in a clinical trial previously performed at Hospital de Clínicas de Porto Alegre (HCPA), in Rio Grande do Sul, Brazil, by our group. Intervention was based on an oral administration of lithium carbonate (Carbolitium; Eurofarma, São Paulo, Brazil), and details can be found at Saute et al 2014.

In brief, patients were randomly assigned to lithium group (n=30) or placebo group (n=31), and followed for 24 weeks. Neurological scales NESSCA and SARA were performed at baseline and at 24 weeks of intervention. Peripheral blood samples were collected at both baseline and 24 weeks from patients groups. Length of CAGexp was already available. In addition, a control group composed by 20 healthy controls was selected, and blood samples were collected in the beginning of the trial.

The study protocol was approved by the ethics committee of our institution (registered as CAAE: 49486015.9.0000.5327 at the Brazilian National platform, Plataforma Brasil). All patients gave informed consent to participate in the study.

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated from 10 mL blood sample using LeukoLOCK™ Total RNA Isolation System (Ambion), following manufacturer's instructions. Two hundred nanograms of RNA was then converted to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), also following manufacturer's instructions. mRNA levels were measured by quantitative real-time PCR (qPCR) using gene-specific TaqMan® assays (for *GSK3β* gene - Hs01047719_m1 and for *HDAC6* gene Hs00997416_g1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control (Hs99999905_m1). Reactions were performed in a total volume of 12 µL containing 1 µL of diluted cDNA solution, 1X of gene-specific TaqMan® assay, 1X of endogenous control TaqMan® assay, and 1X PCR Master Mix (Applied Biosystems, USA). Cycling program was 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions of each sample were performed in triplicate and samples from 24 weeks were performed with a control from baseline in an ABI Prism 7500 Fast Sequence Detector System (Applied Biosystems, USA).

Quantification cycle (Cq) values were determined using ABI Prism Sequence Detection Software v1.3.1 (Applied Biosystems, USA), and relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen. 2001), using GAPDH as an endogenous control, and relative to one sample as calibrator (equal to 1).

Statistical Analysis

All analyses were carried out using the SPSS 18 statistical software package (SPSS Inc., Chicago, IL). Normality of distribution was tested by Kolmogorov-Smirnov test, Independent-sample Mann-Whitney U test was used to see difference between groups at baseline, Spearman correlation to correlate mRNA levels with clinical characteristics, and Wald chi-square was used to

analyze difference between treatments among time. All tests were two-tailed, performed at the significance level α = 0.05.

**Results**

Four patients left the study after baseline. No difference was observed in age and gender distribution between SCA3/MJD group (mean age: 40.77, SD±9.10; 30 female [49.2%]) and control group (mean age: 40.30, SD±12.67; 11 female [55.0%]). When patients groups were compared (lithium and placebo), no difference of clinical characteristics was observed at baseline (lithium group: CAGnor 21.27 [SD ± 4.63]; CAGexp 75.20 [SD ± 3.25]; age of onset 35.1 [SD ± 8.35]; disease duration 6 [SD ± 2.68]; 14 female [46.7%]; placebo group: CAGnor 22.90 [SD ± 5.42]; CAGexp 75.45 [SD ± 2.84]; age of onset 34.3 [SD ± 8.99]; disease duration 6.13 [SD ± 2.53]; 16 female [51.6%]).

GSK3β mRNA expression levels at baseline show no difference between SCA3/MJD patients and controls (Independent-sample Mann-Whitney U test, $p$=0.961) (Fig. 1a). However, HDAC6 mRNA levels showed statistical difference between SCA3/MJD patients and controls, where lower levels of expression was observed in SCA3/MJD patients (Independent-sample Mann-Whitney U test, $p$=0.001) (Fig. 1b).

SCA3/MJD group did not show correlation between mRNA expression levels of GSK3β or HDAC6 when compared to length of normal CAG or CAGexp, age of onset, age, disease duration, NESSCA or SARA at baseline (Spearman correlation, $p$>0.05). No difference in mRNA levels were seen between lithium and placebo groups at baseline (Independent-sample Mann-Whitney U test, $p$=0.961).

Nevertheless, when considering GSK3β mRNA expression levels after intervention, an increase of 3.68 times and 2.85 times was observed in lithium group and in placebo group, respectively. Lithium group increased 1.29 times more than placebo group with statistically significant difference (Wald chi-square,

*p*>0.001) (Fig. 2a). Similar pattern was also observed in HDAC6 mRNA expression levels. Higher expression was seen in both, lithium (2.22 times) and placebo groups (1.69 times). The increment observed in patients of lithium group was statistically significant (1.31 times higher than in patients of placebo group) (Wald chi-square, *p*=0.042) (Fig. 2b).

A weak inverse correlation of variation in GSK3β expression levels and NESSCA variation over time was detected when patients from both groups were placed together (Spearman correlation, rho= -0.279; *p*=0.038) (Fig. 3a). However, this correlation disappears when patients were analyzed as follows: in lithium group (Spearman correlation, rho= -0.176; *p*=0.390) (Fig. 3b) and in placebo group (Spearman correlation, rho= -0.351; *p*=0.057). As this significance is near the limit, this can be the effect of low number of individuals in this group (Fig. 3c).

**Discussion**

SCA3/MJD is a neurodegenerative disease with numerous of possible targets to therapy, such as targeting mRNA or protein aggregation, increasing ubiquitin-proteasome system or autophagy system, among others (Matos et al. 2018). However, having several targets do not exist a treatment only a few palliative cares for specific symptoms. Open labels studies with SCA3/MJD patients were already performed with valproic acid (Lei et al. 2016) and NGF (Tan et al. 2015), and effective outcomes were shown. One double blinded trial was performed using trimethoprim and sulfamethoxazole (Schulte et al. 2001), but no improvement was reported. Outcomes of those studies were based on improvement of clinical parameters. Therefore, as part of a double blind clinical trial conducted by our group (Saute et al. 2011), we evaluate selected candidates as potential peripheral biomarkers in the trial condition.

Lithium is a well-known drug used for treating bipolar disorder, and its most known mechanism is inhibition of GSK3β protein. Lithium treatment has been associated with neuroprotection against neurodegenerative conditions such as

Alzheimer, Parkinson, and Huntington diseases (Lazzara & Kim, 2015), as well as autophagy induction (Motoi et al. 2014). Lithium treatment has been associated with reduction of toxicity in cellular (Lopes et al. 2016) and *drosophila* models (Jia et al. 2013) of SCA3/MJD. Considering lithium intervention in our study, we evaluated the effect of treatment in mRNA expression levels of *GSK3β* in SCA3/MJD patients.

We confirm that there is no difference in GSK3β mRNA expression levels between SCA3/MJD patients and controls. This was an expected outcome considering that GSK3β has no correlation with SCA3/MJD pathology. But, after 24 weeks treatment, an increase of GSK3β mRNA expression was observed at both, lithium or placebo group, although the increment in patients of lithium group was slightly higher than those of placebo group. This moderate effect can be a metabolic response to produce higher amounts of mRNA in order to compensate inhibition of GSK3β protein for long period, since GSK3β play an important role in several pathways. However, a peripheral system (leukocytes) was examined where the effect of mutant ataxin-3 was undetectable. Reduced levels of GSK3β mRNA was previously detected in brain of murine model of Alzheimer disease treated with lithium for 35 days, but no difference was seen in leukocytes (Mendes et al. 2009). Therefore, the effect observed here has to be further investigated.

We have also detected an inverse correlation of GSK3β variation with NESSCA variation after 6 months in SCA3/MJD patients as a whole. Although this correlation is not seen when patients were placed into two groups, placebo group has shown a trend to keep this correlation, and associated with disease severity. Still, as correlation was not present in patients from lithium group, this can indicate that lithium was able to raise *GSK3β* expression in our cohort, disrupting correlation with disease severity.

We have also evaluated mRNA expression levels of *HDAC6* gene, which codes for a protein that interacts with ataxin-3 to form the aggresome (Ouyang et al. 2012). Findings presented here show lower *HDAC6* mRNA expression levels in

SCA3/MJD patients than in controls that can indicate disruption of aggresome formation, and consequently an impairment of autophagy. Previous published data reported impair of autophagy in fibroblast of SCA3/MJD patients (Onofre et al. 2016). Treatment with lithium can induces autophagy (Motoi et al. 2014), and an increase of *HDAC6* expression such as that seen in lithium group can enhance aggresome formation, or even lithium by itself can augment HDAC6 protein activity (Beurel 2011).

Although higher mRNA expression levels were observed in both groups, we cannot rule out altogether these candidates as biomarkers. As this approach was novel, data reported here can be used as a guide for designing other studies, including more patients, other candidate genes and/or other. Further, planning of additional control groups can be essential for evaluating technical issues, such as sample collection and methodology.

Nevertheless, we have shown lower levels of *HDAC6* mRNA expression in patients when compared to controls. Moreover, a statistically significative increase in mRNA expression levels of both *GSK3β* and *HDAC6* was detected as a result of a 24-weeks lithium treatment. Data reported here have never been reported earlier, and validation is required in other cohorts.

Finally, as far as we are aware, this is the first study looking into molecular biomarkers in the setting of SCA3/MJD clinical trial, and one of few studies using mRNA levels in disease progression. Those results were generated by analyzing lymphocytes, and cannot be assumed to show similar pattern in the central nervous system. However, the role of HDAC6 can be further studied in the pathology of SCA3/MJD, and maybe a new therapeutic target in this disease.

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**Fig.1** mRNA expression levels at baseline from SCA3/MJD patients and controls. Relative quantification levels of a) GSK3β and b) HDAC6, normalized by GAPDH. *outliers; [a]$p<0.05$.

**Fig.2** mRNA expression levels in lithium and placebo group at different times. Relative quantification (log scale) of a) GSK3β and b) HDAC6, normalized by GAPDH, in lithium and placebo groups at baseline (in white) and 24 weeks (in grey). [Δ]outliers.

**Fig.3** Correlation of GSK3β variation and NESSCA variation. Difference of GSK3β expression levels and NESSCA score between 24 weeks and baseline (log scale) a) all SCA3/MJD patients, b) lithium group, and c) placebo group.

Fig.1

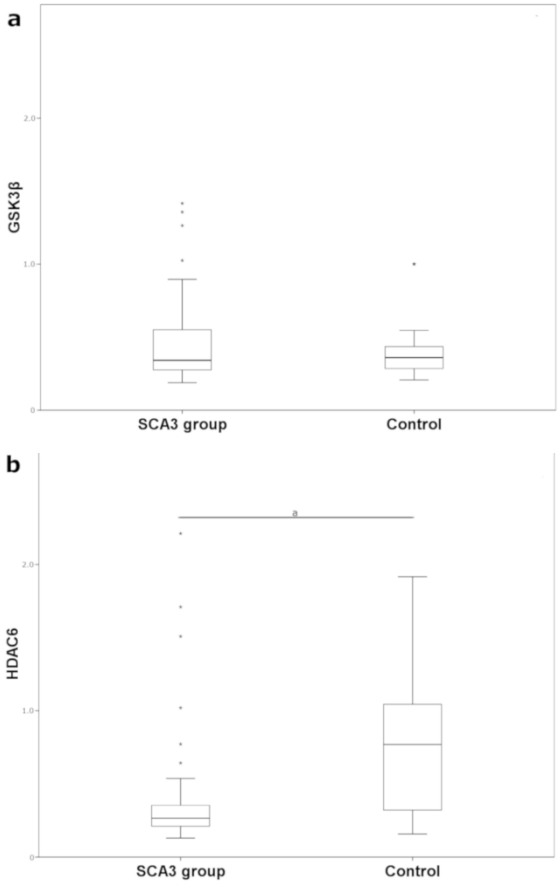

a
GSK3β
2.0
1.0
0
SCA3 group
Control
b
HDAC6
a
2.0
1.0
0
SCA3 group
Control

Fig.2

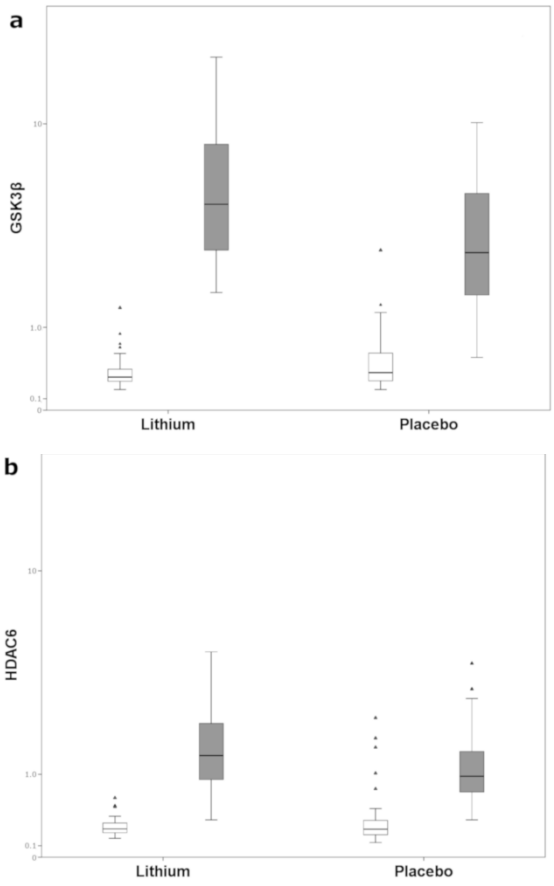
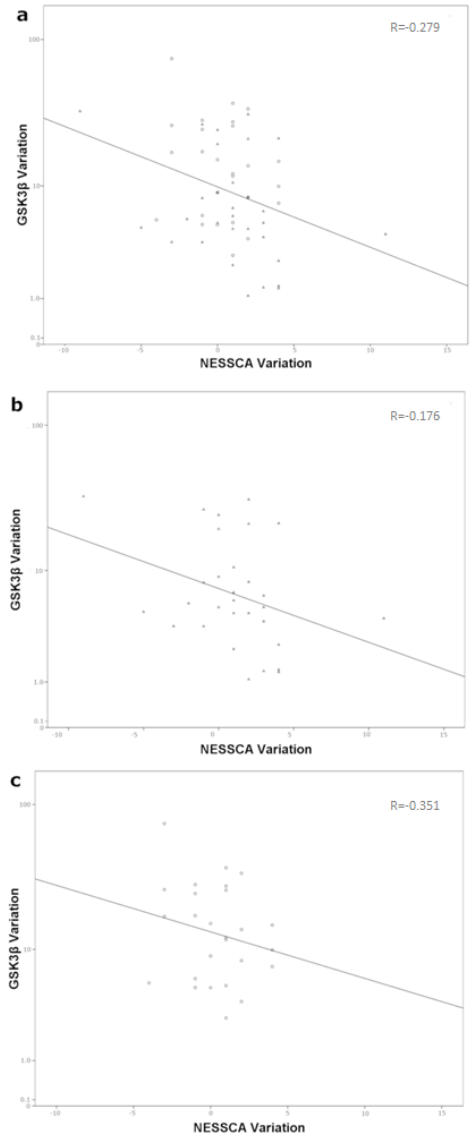

a
GSK3β
10
1.0
0.1
0
Lithium
Placebo
b
HDAC6
10
1.0
0.1
0
Lithium
Placebo

Fig.3


a
R=-0.279
GSK3β Variation
NESSCA Variation
b
R=-0.176
GSK3β Variation
NESSCA Variation
c
R=-0.351
GSK3β Variation
NESSCA Variation

# 4 Discussão

Vários estudos procurando biomarcadores de estado em SCA3/MJD já foram realizados. No entanto, a maioria são estudos de caso-controle, tendo muito poucos estudos longitudinais e nenhum no contexto de ensaio clínico. Os componentes mais diferenciados já foram analisados, sendo que NSE (Tort et al., 2005, Zhou et al., 2011), GSH-Px (Pacheco et al., 2013; de Assis et al., 2017), VOR (Luis et al., 2016) e vídeo-oculografia (Agrawal et al., 2014) seriam bons marcadores para serem validados como biomarcadores de estado dos sintomas de SCA3/MJD. Para estudos prospectivos, o uso de escalas clínicas deve sempre ser feita. O avanço das tecnologias torna possível o acesso a muito mais informações. No entanto, a mudança constante de plataformas torna a aquisição de conhecimento mais demorada e difícil que o esperado.

O IMC não apresentou associação com SCA3/MJD no nosso estudo, possivelmente devido ao efeito de seleção dos indivíduos em estudo, pois foi selecionado indivíduos em início de doença e já foi demonstrado em casos avançados que uma redução de IMC ocorre (Diallo et al., 2017; Saute et al., 2011). E o IMC foi associado ao CAG expandido. A análise de indivíduos que iniciavam a doença antes da idade prevista pela CAG mostrou que eles apresentavam uma taxa metabólica basal maior que o grupo com a idade de início conforme prevista pelo CAG. A mais plausível explicação para esse achado é o aumento do estado catabólico na presença das poliglutaminas, que já foi descrita em indivíduos com SCA1 (Mahler et al., 2014).

Nós confirmamos uma maior sensibilidade periférica à insulina (SPI) em indivíduos com SCA3/MJD, em uma coorte com menos tempo de doença e utilizando outro método (teste de tolerância à glicose - TTG). O estudo anterior foi realizado em indivíduos com maior tempo de doença e utilizando a metodologia de HOMA2-%S (Modelo de Avaliação da Homeostase) (Saute et al., 2011). No estudo anterior, a SPI foi correlacionada com a idade de início; nesse estudo não conseguimos afirmar essa correlação, pois a idade ao exame e a idade de início estão muito associados, não sendo possível distinguir qual das duas ou se

somente uma está associada à SPI. Associação direta entre a idade e a SPI já foi descrita anteriomente (Goodpaster et al., 1999). Contudo, o TTG em SCA3/MJD não tem influência do IMC e apresenta uma correlação inversa com a NESSCA, sem ter influência do CAG e da idade, mostrando um possível efeito da via da insulina na piora neurológica.

Quando estudamos marcadores neuronais, não encontramos nenhuma diferença de BDNF ou CTNF em soro de indivíduos com SCA3/MJD quando comparado com controles ou ao longo do tempo de 48 semanas. Esses valores semelhantes possivelmente se devem ao fato desses marcadores serem principalmente encontrados no sistema nervoso. No entanto, vimos que NGF está reduzido em indivíduos com SCA3/MJD quando comparado com controles, e que esses níveis diminuem ao longo de 48 semanas. Também encontramos uma correlação direta dos níveis de NGF com a NESSCA, sendo que esse menor nível de NGF e uma correlação com uma escala clínica já foi encontrada em indivíduos com doença de Huntington (Tasset et al., 2012). Além disso, reproduzimos em uma nova coorte que os níveis proteicos de NSE em soro de indivíduos com SCA3/MJD são mais elevados quando comparado a controles, como visto anteriormente (Tort et al., 2005 e Zhou et al., 2011).

A análise dos níveis de mRNA de GSK3β vimos que indivíduos com SCA3/MJD não apresentam diferença de controles. No entanto, o uso de lítio aumentou os níveis de mRNA nos indivíduos com SCA3/MJD quando comparado com placebo. Isso pode ser uma resposta periférica ao uso do lítio, uma vez que ele é um conhecido inibidor da atividade proteica da GSK3β. Ademais, uma fraca correlação inversa foi encontrada entre a variação de GSK3β em seis meses e de NESSCA nesse mesmo tempo. Essa correlação desaparece quando analisamos os grupos lítio e placebo separadamente, apesar de que, no grupo placebo, existe uma fraca tendência a correlação que deve se confirmar, caso fosse aumentado o número amostral do grupo, e no grupo lítio desaparece completamente uma vez que vimos que o lítio altera os níveis de mRNA.

HDAC6 demonstrou possuir menor expressão de mRNA nos indivíduos com SCA3/MJD do que em controles, possivelmente é efeito de uma

desregulação no sistema de degradação de proteínas, mais especificamente da autofagia, pois HDAC6 está envolvida na formação do agregossomo juntamente com a ATXN3 selvagem (Ouyang et al., 2012) e a autofagia está comprometida em indivíduos com SCA3/MJD até mesmo em tecidos que a princípio não estariam sendo afetados como fibroblastos (Onofre et al., 2016). Quando os pacientes foram tratados com lítio, aumento na expressão de mRNA foi observado. Esse fato pode ser devido a dois efeitos: (1) que o lítio leva a um aumento da autofagia (Motoi et al., 2015) e a indução de autofagia pode levar a um aumento do HDAC6 e (2) que o lítio aumenta a atividade da HDAC6 (Beurel, 2011), e por este estar em baixos níveis de mRNA o sistema tenta suprir essa ausência para se ter uma maior atividade.

A SCA3/MJD é uma doença com a causa definida, no entanto seu completo funcionamento fisiopatológico ainda não é conhecido isso necessário o estudo de biomarcadores para um melhor entendimento da doença, e por ser uma doença neurodegenerativa de progresso lento somente o uso de escalas clínicas torna difícil os estudos de progressão sendo necessário achar outros biomarcadores. Os nossos estudos indicaram que TTG e mRNA de HDAC6 como novos biomarcadores da doença. Demonstramos que GSK3β e HDAC6 podem ser usados como biomarcadores para o uso de lítio, além de reproduzir os resultados já apresentados sobre NSE. Apesar de não acharmos um bom marcador de progressão de doença que possa substituir a escala clínica identificamos, podemos compreender um pouco melhor a doença e identificar que seja uma doença que não afeta exclusivamente o sistema nervoso, e sim tendo um efeito em todo o organismo que é pouco percebido devido ao efeito principal é causado no cerebelo.

Esse foi um dos poucos estudos procurando biomarcadores na doença de Machado-Joseph, e principalmente biomarcadores de progressão. Confirmamos alguns biomarcadores, como NSE e a sensibilidade à insulina, e descobrimos novos potenciais biomarcadores como HDAC6 e o NGF. Estes precisam de uma análise em outras populações e/ou em outros modelos de estudo para poderem ser usados como biomarcadores. No entanto, escalas clínicas ainda são as

melhores opções para o estudo de progressão da doença. Os dados apresentados nessa tese ajudaram a aumentar o conhecimento e as possibilidades de estudos na SCA3/MJD.

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