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RAFAEL CAESAR GOMES GONÇALVES

EFEITO DE VARIANTE DE NUCLEOTÍDEO ÚNICO EM GENE CANDIDATO COMO POTENCIAL MODIFICADOR DA IDADE DE INÍCIO EM PACIENTES COM DOENÇA DE MACHADO-JOSEPH

Porto Alegre

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Bacharel em Biomedicina.

Orientadora: Prof.ª Dr.ª Maria Luiza Saraiva Pereira

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INSTITUIÇÕES E AGÊNCIAS DE FOMENTO

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"— *Você precisa entender que nossa civilização é tão vasta que não podemos permitir que nossas minorias sejam transtornadas e agitadas. Pergunte a si mesmo: O que queremos neste país, acima de tudo? As pessoas querem ser felizes, não é certo? [...]*

— Os negros não gostam de Little Black Sambo. Queime-o. Os brancos não se sentem bem em relação à Cabana do pai Tomás. Queime-o. Alguém escreveu um livro sobre o fumo e o câncer de pulmão? As pessoas que fumam lamentam? Queimemos o Livro. [...] Leve sua briga lá para fora. Melhor ainda, leve o incinerador. Os enterros são tristes e pagãos? Elimine-os também. Cinco minutos depois que uma pessoa morreu, ela está a caminho do Grande Crematório [...]. Dez minutos depois da morte, um homem é um grão de poeira negra. Não vamos ficar arengando os in memoriam para os indivíduos. Esqueça-os. Queime tudo, queime tudo. O fogo é luminoso e o fogo é limpo. [...]

— [...] Não se pode construir uma casa sem pregos e madeira. Se você não quiser que se construa uma casa, esconda os pregos e a madeira. Se não quiser um homem politicamente infeliz, não lhe dê os dois lados de uma questão para resolver; dê-lhe apenas um. Melhor ainda, não lhe dê nenhum. [...] Se o governo é ineficiente, despótico e ávido por impostos, melhor que ele seja tudo isso do que as pessoas se preocuparem com isso. [...] Encha as pessoas com dados incombustíveis, entupa-as tanto com "fatos" que elas se sintam empanzinadas, mas absolutamente "brilhantes" quanto a informações. Assim, elas imaginarão que estão pensando, terão uma sensação de movimento sem sair do lugar. E ficarão felizes, porque fatos dessa ordem não mudam. Não as coloque em terreno movediço, como filosofia e sociologia, com que comparar suas experiências. Aí reside a um telão de tevê e montá-lo novamente, e a maioria consegue, hoje em dia está mais feliz do que qualquer homem que tenta usar a régua de cálculo, medir e comparar o universo, que simplesmente não será medido ou comparado sem que o homem se sinta bestial e solitário. [...]"

Fahrenheit 451, Ray Bradbury, 1953

RESUMO

Ataxia espinocerebelar tipo 3 ou doença de Machado-Joseph (SCA3/MJD) é uma doença neurodegenerativa causada por uma expansão de repetições CAG. Essa expansão é inversamente correlacionada com a idade de início dos sintomas (AO). Em média, cerca de 55,2% da variação na AO é explicada pelas repetições CAG. Isso indica que outros moduladores, genéticos ou ambientais, podem afetar o início dos sintomas. Dados recentes mostraram que a variante intrônica no gene *DLGAP2* (rs2293909) estava associada com uma antecipação na AO em um grupo de pacientes brasileiros com SCA3/MJD. No estudo atual, a frequência genotípica da variante rs2293909 foi demonstrada num grupo de pacientes de SCA3/MJD da região Sul do Brasil. A distribuição das frequências alélicas foi de 0,207 para o alelo C e 0,793 para o alelo T em pacientes com SCA3/MJD, enquanto nos controles locais foi 0,300 para o alelo C e 0,700 para o alelo T. Não houve diferença significativa nas frequências alélicas ($p=0.048$) ou genotípicas ($p=0.149$) entre pacientes e controles, apesar de ter sido observada uma tendência na frequência alélica. Com isso, estabelecemos as frequências alélicas e genotípicas da variante rs2293909 num grupo brasileiro de pacientes com SCA3/MJD (n=184) e de indivíduos da população normal (n=50). Neste estudo, foi observada uma indicação de que o alelo C na variante rs2293909 está associada com início precoce da doença. Dessa forma, essa variante pode ser um fator adicional que modula a AO na SCA3/MJD.

Palavras-chave: Ataxia espinocerebelar tipo 3; Doença de Machado-Joseph; PolyQ; gene *DLGAP2*; rs2293909.

ABSTRACT

Spinocerebellar ataxia type 3, or Machado-Joseph disease (SCA3/MJD), is a neurodegenerative disorder caused by an expansion of CAG repeats. This expansion is inversely correlated to age of onset (AO) of symptoms. However, on average, just up to 55.2% of variation in AO can be explained by CAG length. Then, additional modulators, either genetic or environmental, can play a role in modulating disease onset. Recent data demonstrated that an intronic variant at *DLGAP2* gene (rs2293909) was associated with an anticipation of AO in a Brazilian group of SCA3/MJD patients. In the present study, genotype frequency of rs2293909 was demonstrated in a group of SCA3/MJD patients from South Brazil. Allele frequency distribution was 0.207 for C allele and 0.793 for T allele in SCA3/MJD patients, and 0.300 for C allele and 0.700 for T allele in local controls. There was no statistically significant difference in allele ($p=0.048$) nor genotype ($p=0.149$) frequencies between patients and controls, although a tendency was seen in the allele frequency. Therefore, we established allele and genotype frequencies of rs2293909 in a group of Brazilian SCA3/MJD patients (n=184) as well as controls (n=50). In this study, we have an indication that the C allele of rs2293909 is associated with early onset of the disease. Therefore, this variant can be an additional factor to modulate AO in SCA3/MJD. As previously stated, combined effects are very likely to be involved in disease modulation.

Keywords: Spinocerebellar ataxia type 3; Machado-Joseph disease; PolyQ; *DLGAP2* gene; rs2293909.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

AO — Idade de início (dos sintomas) | *age of onset (of symptoms)*

AOA1 — Ataxia com Apraxia Oculomotora tipo 1 | *Ataxia Oculomotor Apraxia type 1*

AOA2 — Ataxia com Apraxia Oculomotora tipo 2 | *Ataxia Oculomotor Apraxia type 2*

A–T — Ataxia-Telangiectasia

ATXN2 — Gene *ataxin-2*

ATXN3 — Gene *ataxin-3*

CAGexp — Expansão do trinucleotídeo CAG

DRPLA — Atrofia Dentato-Rubro-Palido-Luisiana | *Dentatorubral-Pallidoluysian Atrophy*

FAN1 — Gene *Fanconi anemia FANC1/FANCD2-associated [endo] nuclease 1*

FRDA — Ataxia de Friedreich | *Friedreich Ataxia*

HCPA — Hospital de Clínicas de Porto Alegre

MJD — Doença de Machado-Joseph | *Machado-Joseph disease*

PCR — Reação em Cadeia da Polimerase | *Polymerase Chain Reaction*

polyQ — Poliglutamina

RS — Estado do Rio Grande do Sul

SCA1 — Ataxia Espinocerebelar tipo 1 | *Spinocerebellar Ataxia type 1*

SCA2 — Ataxia Espinocerebelar tipo 2 | *Spinocerebellar Ataxia type 2*

SCA3/MJD — Ataxia Espinocerebelar tipo 3 | *Spinocerebellar Ataxia type 3*

SCA6 — Ataxia Espinocerebelar tipo 6 | *Spinocerebellar Ataxia type 6*

SCA7 — Ataxia Espinocerebelar tipo 7 | *Spinocerebellar Ataxia type 7*

SCA17 — Ataxia Espinocerebelar tipo 17 | *Spinocerebellar Ataxia type 17*

SGM-HCPA — Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre

SNV — Variação de nucleotídeo único | *single nucleotide variation*

SUMÁRIO

1. INTRODUÇÃO

1.1. Ataxias Espinocerebelares

As ataxias espinocerebelares (SCAs — do inglês "*spinocerebellar ataxias*") são caracterizadas como um grupo diverso, composto por mais de 30 doenças neurodegenerativas e hereditárias, com heranças variadas entre autossômicas dominantes e recessivas, além de algumas ligadas ao X. De forma geral, possuem idade de início de sintomas tardio (na vida adulta) e caracterizam-se por disfunção e degeneração cerebelar de forma lenta e progressiva, levando os indivíduos acometidos à morte cerca de 10 a 20 anos após o início dos primeiros sintomas. Os sintomas que convergem entre quase todas são: perda e/ou falta de coordenação de movimentos voluntários dos membros (a ataxia), dificuldades na articulação de palavras (disartria), além de tremores. Outros sintomas comuns ao grupo, mas não presentes em todas as doenças que o compõem: dificuldades de deglutição (disfagia), movimentos involuntários dos olhos (nistagmo), sinais piramidais e extrapiramidais e comprometimento cognitivo. Além desses sintomas comuns, algumas SCAs podem apresentar características relativas àquela SCA específica (Bird, 1998; Orr e Zoghbi, 2007).

Dentre as SCAs, podemos destacar a ataxia espinocerebelar tipo 1 (SCA1 — do inglês "*Spinocerebellar Ataxia type 1*"; OMIM #164400), a ataxia espinocerebelar tipo 2 (SCA2 — do inglês "*Spinocerebellar Ataxia type 2*"; OMIM #183090), a ataxia espinocerebelar tipo 3 ou doença de Machado-Joseph (SCA3/MJD — do inglês "*Spinocerebellar Ataxia type 3*"; OMIM #109150), a ataxia espinocerebelar tipo 6 (SCA6 — do inglês "*Spinocerebellar Ataxia type 6*"; OMIM #183086), e a ataxia espinocerebelar tipo 7 (SCA7 — do inglês "*Spinocerebellar Ataxia type 7*"; OMIM #164500) como as mais prevalentes entre as autossômicas dominantes, enquanto a ataxia de Friedreich (FRDA — do inglês "*Friedreich Ataxia*"; OMIM #229300), ataxia telangiectasia (A-T — do inglês "*Ataxia Telangiectasia*"; OMIM #208900), ataxia com apraxia Oculomotora tipo 1 (AOA1 — do inglês "*Ataxia Oculomotor Apraxia type 1*"; OMIM #208920) e ataxia com apraxia oculomotora tipo 2 (AOA2 — do inglês "*Ataxia Oculomotor Apraxia type 2*"; OMIM #606002) são as mais prevalentes entre as autossômicas recessivas (Bird, 1998; Orr e Zoghbi, 2007). A atrofia dentato-rubro-palido-luisiana (DRPLA — do inglês "*Dentatorubral-Pallidoluysian Atrophy*"; OMIM #125370) também compartilha dos sintomas e características patofisiológicas das SCAs.

As SCAs podem ser divididas em três grupos, dependendo da sua etiologia: (1) expansão de repetições do trinucleotídeo CAG (CAGexp) em regiões codificantes; (2) CAGexp em

regiões não codificantes; por fim, (3) as causadas por outros tipos de mutação, como deleções, mutações de ponto de sentido trocado, mutações de ponto sem sentido e mutações em sítios de *splicing* gênico (Soong e Paulson, 2007). As seis SCAs mais comuns — a saber: SCA1, SCA2, SCA3/MJD, SCA6, SCA7 e ataxia espinocerebelar tipo 17 (SCA17 — do inglês "*Spinocerebellar Ataxia type 17*"; OMIM #607136) — representam mais de 50% dos casos de SCAs a nível global. Todas são causadas por uma causa comum: um gene com a presença de CAGexp em alguma de suas regiões codificantes (Soong & Paulson, 2007).

1.2. Poliglutaminopatias

As poliglutaminopatias são um grupo de dez doenças de origem genética onde a expansão de um trato de poliglutamina (polyQ — do inglês "*polyglutamine*") — uma sequência de repetições do aminoácido glutamina com mais repetições do que o normal — causa um ganho de função tóxico do produto gênico, visto especialmente em células neuronais. As ataxias espinocerebelares SCA1, SCA2, SCA3/MJD, SCA6, SCA7 e SCA17 fazem parte desse grupo de doenças (Bunting, Hamilton & Tabrizi, 2022).

O grupo também é composto pela DRPLA, a atrofia muscular bulbar e espinhal (SBMA — do inglês "*Spinal And Bulbar Muscular Atrophy*"; OMIM #313200) e a doença de Huntington (HD — do inglês "*Huntington Disease*"; OMIM #143100), sendo que dentre as doenças causadas por polyQ, a HD é a mais estudada (Lieberman, Shakkottai & Albin, 2019).

1.3. Doença de Machado-Joseph/Ataxia Espinocerebelar tipo 3 (SCA3/MJD)

A ataxia espinocerebelar tipo 3 ou doença de Machado-Joseph (SCA3/MJD — do inglês "*Machado-Joseph Disease*"; OMIM #109150) é uma doença neurodegenerativa de origem genética, apresentando herança autossômica dominante. É a forma de ataxia dominante mais prevalente no mundo, assim como no Brasil, representando 78,4% dos diagnósticos no Sul do Brasil (Schöls et al, 2004; de Castilhos et al, 2014; Saute e Jardim, 2015). A doença é caracterizada pela presença de uma expansão da repetição do trinucleotídeo CAG (códon que codifica para o aminoácido glutamina) numa região de repetições em tandem no éxon 10 do gene *ATXN3*. Este gene está localizado no braço longo do cromossomo 14.

O gene, caracterizado em 1994 por Kawaguchi e colaboradores como o *locus* gênico da

mutação que causa a SCA3/MJD, foi então nomeado como *MJD1*, mas hoje é melhor conhecido por *ATXN3* (Kawaguchi et al, 1994). Ele codifica a ataxina-3, uma proteína com função deubiquitinadora envolvida na manutenção da homeostase proteica, transcrição, regulação de citoesqueleto e degradação de substratos de chaperona enovelados de maneira incorreta (Li et al, 2002; Mao et al, 2005; Tzvetkov et al, 2007; Seki et al, 2013; Ashkenazi et al, 2017).

Já se sabe que a idade de início (AO — do inglês "*age of onset (of symptoms)*") do fenótipo é variável e, em geral, relaciona-se diretamente com o tamanho do trato CAGexp no gene *ATXN3*, com 55,2% sendo explicados pela quantidade de repetições dessa região polimórfica (de Mattos et al, 2019a). Com a adição de alguns fatores modificadores à comparação, como o tamanho da região polimórfica do gene *ATXN2*, polimorfismos nos genes *FAN1* (que codifica proteína de uma via de reparo de DNA) e *CAST* (envolvido em via de clivagem proteica ligada à SCA3/MJD) , além de fatores familiares, pode-se elucidar até 73,5% da AO de indivíduos afetados (de Mattos et al, 2019a; Mergener et al, 2020; Martins et al, 2021).

1.3.1. Etiologia da SCA3/MJD

Como mencionado, a etiologia da SCA3/MJD é a expansão de uma repetição em tandem no gene *ATXN3*, que possui 62,1 kb e é localizado na fita antissenso do cromossomo 14, na banda 14q32.12. (Figura 1). No éxon 10 deste gene, encontra-se a região de repetição em tandem do trinucleotídeo CAG (GRCh38:CM000676.2; Saute e Jardim, 2015).

Em uma população normal, o número de repetições do trinucleotídeo CAG nessa região varia entre 12 e 44. O fenótipo da SCA3/MJD é visto em indivíduos com 56 ou mais repetições, podendo chegar a 86 e até 91 (Saute e Jardim, 2015; Ashizawa et al, 2018). Já no intervalo compreendendo 45 a 55 repetições, foi visto que há penetrância incompleta dos sintomas da doença (Ashizawa et al., 2018).
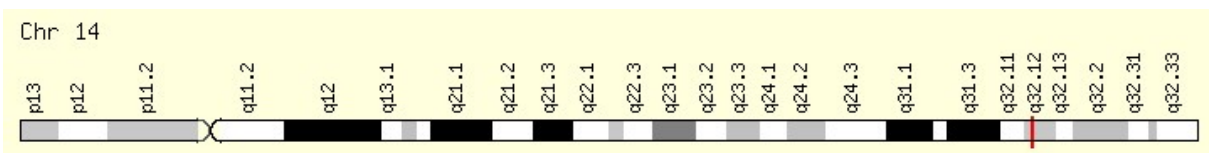

Figura 1 Localização genômica do gene *ATXN3* no cromossomo 14. Adaptado de genecards (id: GC14M099702).

A AO da SCA3/MJD é variável, com média entre 34 e 40 anos de idade, mas já foram encontrados casos cuja AO variava desde 4 até 78 (Saute e Jardim, 2015). Existe uma correlação

inversamente proporcional entre as CAGexp e a AO. Isto é, quanto mais repetições, mais precoce é a manifestação dos primeiros sintomas (de Mattos et al, 2019b)

A doença é caracterizada principalmente pelo aparecimento de ataxia e sua progressão, causados pela degeneração progressiva do cerebelo e tronco encefálico dos indivíduos afetados pela mutação. A degeneração dessas duas estruturas encefálicas desencadeia diversas outras manifestações clínicas, dentre disfunção dos sistemas oculomotor, piramidal e extrapiramidal, além de disfunção e perda dos neurônios motores e sensoriais (principalmente associados ao sistema nervoso periférico). Outras manifestações podem ser observadas a nível visual e oculomotor, como movimentos involuntários e repetitivos dos olhos (nistagmo), redução da velocidade de sacadas oculares e perda de associação dos movimentos dos dois olhos. Com a progressão da SCA3/MJD, os pacientes tendem a perder diversas habilidades motoras, apresentando disfagia, disartria, distonia, amiotrofia, atrofia facial e temporal. A perda de capacidades motoras leva o paciente a necessitar de auxílio para realização de tarefas, inicialmente podendo ser bengalas, cadeiras de roda, até etapas mais avançadas, onde podem requerer alimentação por sonda ou via parenteral (Paulson, 2012).

Diferente do que acontece na doença de Huntington, as pessoas afetadas pela SCA3/MJD não costumam desenvolver demências. Seu viés psiquiátrico costuma aparecer mais fortemente associado à depressão. Foram observadas questões relacionadas à redução de capacidade em atividades de atenção e capacidade de construir fonemas (Paulson, 2012; Zawacki et al, 2002).

Atualmente, não existe tratamento para a doença em si; os principais tratamentos são voltados à atenção aos sintomas que a SCA3/MJD desenvolve nos indivíduos afetados. Os principais são farmacológicos, mas alguns outros, como fisioterapêuticos, fonoaudiológicos e voltados à terapia ocupacional também podem auxiliar na melhora das condições de vida e independência de pacientes (Duarte-Silva e Maciel, 2018; Saute et al, 2015).

1.3.2. Epidemiologia da SCA3/MJD

A SCA3/MJD é a forma mais comum de ataxia hereditária de herança autossômica dominante no mundo. As taxas de prevalência da doença no estado do Rio Grande do Sul (RS) foram estimadas em 7:100.000 em 2020, variando de 17 a 166:100.000 em algumas cidades (Rodríguez-Labrada et al, 2020). Em estudo anterior, foi estimada em 1,8:100.000 a prevalência da SCA3/MJD no estado, enquanto de outras SCAs seria de 0,2:100.000 (Jardim et al, 2001).

Esse estudo indica que a prevalência de SCA3/MJD no RS era ao menos 9 vezes maior que a prevalência de outras SCAs.

Martins e colaboradores descreveram, em 2007, três SNVs relacionadas com as famílias cujos sobrenomes nomeiam a doença (família Joseph, da ilha de Flores, em Açores/Portugal; e a família Machado, da ilha de São Miguel, também em Açores). Os haplótipos relacionados às famílias Machado e Joseph configuram 94% das famílias estudadas (Martins et al, 2007).

A história da colonização do RS pode explicar a aglomeração de casos nessa região: a Coroa Portuguesa enviou entre dois e cinco mil de seus cidadãos da Europa para o RS no intuito de popular a região, até então habitada de forma esparsa apenas por ameríndios. Esse povoamento acabou por perpetuar uma grande ancestralidade portuguesa no estado. Foi visto por Rodríguez-Labrada e colaboradores que de 178 famílias estudadas no RS, 170 (92%) carregavam o haplótipo da família Joseph, sugerindo que suas mutações possuem a mesma origem ancestral (Rodríguez-Labrada et al, 2020).

1.3.3. Modificadores de fenótipo

A SCA3/MJD é causada pela CAGexp no éxon 10 do gene *ATXN3* e o seu tamanho pode explicar, em média, apenas 55,2% das AO dos pacientes com a doença, apesar de existir uma correlação inversamente proporcional já estabelecida entre AO e CAGexp. Alguns modificadores já conhecidos do fenótipo, quando adicionados à equação, auxiliam a elucidar melhor as AO. Os mais conhecidos são genótipo da apoliproteína E, número de repetições do trinucleotídeo CAG no gene *ATXN2*, além de fatores ambientais e outros fatores genéticos ainda não elucidados (Saute e Jardim, 2015; de Mattos, 2019b). Foi visto em 2020 que a variante rs3512 no gene *FAN1* pode explicar uma redução de 2,44 anos na AO de pacientes com SCA3/MJD na população gaúcha (Mergener et al, 2020). Em outro estudo envolvendo também a população do RS, foi encontrado um pequeno efeito neuroprotetor na presença da variante rs1559089 no gene *CAST* (Martins et al, 2021).

Essas variações genéticas indicam uma possibilidade de que outros genes podem ter efeitos interessantes tanto de forma neuroprotetora como promotora da doença. O estudo de outras variações e mutações em genes candidatos a modificadores pode levar a um melhor entendimento da fisiopatologia da SCA3/MJD.

1.4. Estudos prévios

Em estudo colaborativo entre vários grupos de pesquisadores, incluindo o grupo de Neurogenética do Serviço de Genética Médica (SGM) do Hospital de Clínicas de Porto Alegre (HCPA) e liderado por um grupo português, variações de nucleotídeo único (SNVs — do inglês "*single nucleotide variants*") foram identificadas em um pequeno número de pacientes com SCA3/MJD através de sequenciamento completo do exoma (WES — do inglês "*whole exome sequencing*") (Raposo et al, 2021). Dentre as variantes encontradas, podemos citar a rs2293909, localizada no gene *DLGAP2*. Essa variante está localizada em uma região intrônica que, quando observado no grupo estudado (n=78), estaria associado à antecipação da idade de início, explicando 10% da variância da AO. Essa foi a primeira vez em que este gene foi associado como modificador de fenótipo da SCA3/MJD, o que suscitaria novos estudos acerca dessa variante e seus possíveis impactos na idade de início em indivíduos afetados.

1.4.1. *DLGAP2*

O gene *DLGAP2* (do inglês "*discs large homolog associated protein 1*"; genecar) codifica a proteína DAP-2 (do inglês, "*Disks large-associated protein 2*"), a qual foi identificada em 1997 (Satoh et al, 1997). Esse gene está localizado na banda 23.3 do braço curto do cromossomo 8 (8p23.3; coordenadas genômicas: GRCh38: 8:737,628-1,708,476), compreendendo cerca de 970,8 kb.

A proteína codificada pelo gene *DLGAP2* atua principalmente em sinapses e está relacionada com regulação da região dos terminais pós-sinápticos e sinalização celular neuronal, sendo observada interação com as proteínas *human homologue of the Drosophila discs large tumour suppressor protein* (hDLG) e *postsynaptic density protein 95 kDa* (PSD-95). Ambas são associadas a receptores pós-sinápticos, canais de íons e a proteína *adenomatous polyposis coli protein* (APC), uma proteína relacionada com a adesão celular (Satoh et al, 1997).
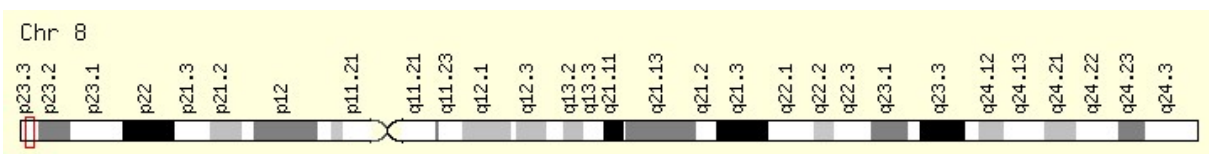
Figura 2 Localização cromossômica do gene *DLGAP2*. Adaptado de genecards (id: GC08P000739)

1.5.   JUSTIFICATIVA

Tendo em vista a alta prevalência da SCA3/MJD no Rio Grande do Sul e a falta de informações que justifiquem as peculiaridades da fisiopatologia da doença entre pacientes com uma mesma CAGexp no gene *ATXN3*, é possível que outros fatores, sejam ambientais ou genéticos, influenciam nessa diferença. A análise de outras regiões do DNA além desse gene pode auxiliar na compreensão da diferença da idade de início entre eles. Quando novos alvos são identificados, podem dar um melhor entendimento de como esses modificadores de fenótipo influenciam no desenrolar clínico e no entendimento da fisiopatologia da SCA3/MJD.

1.6.   OBJETIVOS

1.6.1.   Objetivo geral:

O presente estudo tem como objetivo principal a investigação do papel da variante rs2293909 no gene *DLGAP2* como modificadora da idade de início da doença em pacientes com SCA3/MJD.

1.6.2.   Objetivos específicos:

- Determinar as frequências alélicas e genotípicas da variante rs2293909 em pacientes com SCA3/MJD;
- Comparar dados obtidos no grupo de pacientes com o grupo de controles local e com dados de bancos de dados internacional;
- Verificar a associação de dados das variantes com a idade de início (AO) dos pacientes.

2. ARTIGO CIENTÍFICO

O artigo intitulado "Spinocerebellar ataxia type 3/Machado-Joseph disease: variant in the *DLGAP2* gene as an additional modifier of age of onset of the disease" foi formatado conforme normas para publicação de acordo com o periódico NeuroMolecular Medicine.

1 Spinocerebellar ataxia type 3/Machado-Joseph disease: variant in the *DLGAP2* gene as an

2 additional modifier of age of onset of the disease

3

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33 **Abstract**

34

35 Spinocerebellar ataxia type 3, or Machado-Joseph disease (SCA3/MJD), is a neurodegenerative

36 disorder caused by an expansion of CAG repeats. This expansion is inversely correlated to age

37 of onset (AO) of symptoms. However, on average, just up to 55.2% of variation in AO can be

38 explained by CAG length. Then, additional modulators, either genetic or environmental, can

39 play a role in modulating disease onset. Recent data demonstrated that an intronic variant at

40 *DLGAP2* gene (rs2293909) was associated with an anticipation of AO in a Brazilian group of

41 SCA3/MJD patients. In the present study, genotype frequency of rs2293909 was demonstrated

42 in a group of SCA3/MJD patients from South Brazil. Allele frequency distribution was 0.207

43 for C allele and 0.793 for T allele in SCA3/MJD patients, and 0.300 for C allele and 0.700 for

44 T allele in local controls. There was no statistically significant difference in allele (p=0.048)

45 nor genotype (p=0.149) frequencies between patients and controls, although a tendency was

46 seen in the allele frequency. Therefore, we established allele and genotype frequencies of

47 rs2293909 in a group of Brazilian SCA3/MJD patients as well as controls. In this study, we

48 have an indication that the C allele of rs2293909 is associated with early onset of the disease.

49 Therefore, this variant can be an additional factor to modulate AO in SCA3/MJD. As previously

50 stated, combined effects are very likely to be involved in disease modulation.

51

52 Keywords: Spinocerebellar ataxia type 3; Machado-Joseph disease; PolyQ; *DLGAP2* gene;

53 rs2293909.

Introduction

Spinocerebellar ataxia type 3 or Machado-Joseph disease (SCA3/MJD; OMIM #109150) is an inherited neurodegenerative disorder of autosomal dominant trait. SCA3/MJD is by far the most prevalent form of dominant ataxia worldwide, representing up to 78.4% of cases in Southern Brazil (de Castilhos et al, 2014). The disease is characterized by the presence of an expansion of the CAG trinucleotide repeat (codon that codes for the amino acid glutamine) in a region of tandem repeats in exon 10 of the *ATXN3* gene, which is located on the long arm of chromosome 14. The *ATXN3* gene encodes the ataxin-3, a protein with deubiquitinating function involved in the maintenance of protein homeostasis, transcription, cytoskeletal regulation, and degradation of misfolded chaperone substrates (Li et al, 2002; Mao et al, 2005; Tzvetkov et al., 2007; Seki et al, 2013; Ashkenazi et al, 2017).

The disease is mainly characterized by the appearance of ataxia and its progression, caused by the progressive degeneration of the cerebellum and brainstem of individuals affected by the mutation. The degeneration of these two brain structures triggers several other clinical manifestations, including dysfunction of the oculomotor, pyramidal and extrapyramidal systems, in addition to dysfunction and loss of motor and sensory neurons (mainly associated with the peripheral nervous system). Other manifestations can be observed at the visual and oculomotor level, such as involuntary and repetitive movements of the eyes (nystagmus), reduction in the speed of ocular saccades and loss of association of the movements of the two eyes. With the progression of SCA3/MJD, patients tend to lose several motor skills, presenting dysphagia, dysarthria, dystonia, amyotrophy, facial and temporal atrophy. The loss of motor skills leads the patient to need help to carry out tasks, initially being canes, wheelchairs, to more advanced stages, where they may require feeding by tube or parenteral route (Paulson, 2012).

Age of onset (AO) of the disease onset is variable and directly related to the length of the CAG tract, with 55.2% being explained by the number of repeats of this polymorphic region (de Mattos et al, 2019a). Therefore, additional factors, such as genetic or environmental, can contribute to the AO variation observed in SCA3/MJD. Our group has been working on some genetic modifying factors, such as the length of the CAG tract in the *ATXN2* gene, variants in *FAN1* (which encodes a protein of a DNA repair pathway) and *CAST* (involved in a protein cleavage pathway linked to SCA3/MJD) genes. Together with family factors, these variants can explain up more than just those 55.2% of the OA of affected individuals (de Mattos et al, 2019a; Mergener et al, 2020; Martins et al, 2021).

87   A very recent whole-exome sequencing (WES) study proposed novel MJD-modifying
88 genes and pathways to be further investigated as new disease-modifying targets. In a subset of
89 patients included in this study of Brazilian origin, an intronic variant at *DLGAP2* gene
90 (rs2293909) was associated with an anticipation of AO, explaining 10% of the variance in the
91 group (Raposo et al, 2022).

92   The product of *DLGPA2* gene, which is expressed in the brain, encodes the disks large-
93 associated protein 2 (DAP-2). This protein plays a role in synapse organization and neuronal
94 cell signaling. Variants in *DLGAP2* have been observed in individuals with autosomal dominant
95 complex neurodevelopmental disorders, including autism spectrum disorder, among others
96 (Pouquet et al, 2017).

97   In order to contribute to the understanding of factors that modulate AO in SCA3/MJD
98 patients, we have investigated the role of variant rs2293909 as a disease modifier of AO in a
99 wider Brazilian group of SCA3/MJD patients.

100   **Methods**

101

102   **Samples**

103   Subjects included in this study were evaluated in the Neurogenetics outpatients clinic of

104   the Medical Genetics Service and samples analyzed in the Translational Neurogenetics

105   laboratory, both at Hospital de Clínicas de Porto Alegre, Rio Grande do Sul, Brazil. Informed

106   consent was obtained from all individual participants included in this study. A total of 184

107   SCA3/MJD patients were included. **Table 1** shows specific information about the studied

108   population. The outcome was AO, a quantitative variable, which was defined as the age at the

109   first symptom. A control group composed of 50 unrelated healthy individuals was also

110   evaluated in order to determine allelic and genotypic frequencies in our population.

111

112   **Genotyping analysis**

113   Most, if not all, samples were available in the laboratory's biorepository. In all samples

114   analyzed, DNA was isolated from peripheral blood leukocytes using standard methods. The

115   CAG repeat length analysis was performed by the polymerase chain reaction (PCR) using

116   fluorescent labeled primers flanking the CAG repeat tract at the *ATXN3* gene, followed by

117   capillary electrophoresis into the genetic ABI3130*xl* (Applied Biosystems, Foster City, CA,

118   USA). Results were analyzed through GeneMapper® ID v 3.2 software (Applied Biosystems,

119   Foster City, CA, USA), as described by França et al. (2012).

120   rs2293909 genotyping was performed using TaqMan SNP Genotyping Assay

121   (C__16185513_10) in a final volume of 8 μL containing 2 ng of DNA, according to assay

122   protocol (Applied Biosystems, Foster City, CA, USA). Amplification was performed in the ABI

123   7500 Real-Time PCR System® equipment (Applied Biosystems, Foster City, CA, USA) as

124   follows: one cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95 °C for 15

125   s and 60 °C for 1 min.

126

127   **Statistical Analyses**

128   Pearson's correlation was used to determine the association between AO and CAGexp.

129   Chi-square test was used to check for Hardy-Weinberg equilibrium (HWE). Data on allele and

130   genotype frequencies were searched for into two databases, 1000 Genomes Project (Zerbino et

131   al. 2018) and gnomAD (Lek et al. 2016), in order to compare with both our local control and

132   SCA3/MJD groups. Data were analyzed by Student t-test for allele association, and by one-way

133   analysis of variance (ANOVA) for genotype. As shown by the linear R^2, the observed degree

134   of explanation of the variability in AO by CAGexp was reported. Predicted AO was calculated

135   based on the CAGexp length (de Mattos et al, 2019b). All statistical analyses were made using

136   Predictive Analytics SoftWare - PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). The

137   significance level was set as 5%. All graphics were created using GraphPad Prism version 5 for

138   Windows (GraphPad Software, San Diego, CA, USA).

139   **Results**

140

141   **Table 1** summarizes mean and range of AO and allele length of patients. Allele

142   frequency distribution was 0.207 for C allele and 0.793 for T allele in SCA3/MJD patients, and

143   0.300 for C allele and 0.700 for T allele in local controls. Frequencies of both patients and

144   control groups were in Hardy-Weinberg equilibrium. There was no statistically significant

145   difference in allele (p=0.048) nor genotype (p=0.149) frequencies between patients and

146   controls, although a tendency was seen in the allele frequency. Distribution of allele and

147   genotype frequencies in SCA3/MJD patients and in controls are shown in **table 2**. Allele

148   frequencies determined in local controls were similar to those global frequencies found in two

149   different international databases (1000 Genomes Project and GnomAD). However, SCA3/MJD

150   group frequencies were slightly different.

151   We have also compared AO and length of the CAGexp of *ATXN3* with patient′s

152   genotype. As expected, a strong inverse correlation between AO and CAGexp repeat length at

153   *ATXN3* was observed. Genotype distribution can be observed in **figure 1. Figure 1A** shows

154   correlation of AO and CAG repeat length in each genotype group, while, in **Figure 1B**, subjects

155   were placed into two groups: one with patients that carry at least one C allele (C/C genotype

156   and C/T genotype) and the other, subject with T/T genotype at rs2293909.

157   Genotype distribution is also shown in **figure 2** as two different groups, and no clear

158   difference can be observed in this case. However, when patients were divided into early,

159   intermediate or late groups, according to expected AO, we can see a slight difference among

160   them (**Figure 3**), with a tendency of C allele being more frequent in earlier onset patients.

161 **Discussion**

162

163   Considering allele and genotype frequencies distribution, there were no clear differences

164 found between patients and controls, although a tendency was observed when considered allele

165 frequency in the SCA3/MJD patients' group. Databases as 1000 genome (Zerbino et al. 2018)

166 and gnomAD (Lek et al. 2016) show a lower frequency of the minor allele (C allele) in Europe

167 (24-26%) and, in this current work, frequency of minor allele in SCA3/MJD patients' group

168 was estimated in 20,5%, while frequency of this same allele was estimated in 30,0% in local

169 controls. This data might be related to an estimated high rate (more than 80%) of European

170 ancestry in the South region of Brazil (Ruiz-Linares et al. 2014). This outcome can be also due

171 to a founder effect of Portuguese (from the Azorean islands) in our SCA3/MJD group, as

172 previously reported (Saute & Jardim, 2015).

173   The data presented here indicate that the C allele at rs2293909 seems to be more frequent

174 in SCA3/MJD subjects with earlier AO. This impact on AO was reported recently and

175 associated with an earlier onset, explaining 10% of AO variance in Brazilian SCA3/MJD

176 patients (Raposo et al, 2022). It is relevant to mention that, in the Portuguese group included in

177 the same study, the effect of this variant was observed in the opposite direction. Therefore,

178 further studies are needed to a better understanding of a possible interaction between those

179 proteins.

180   To date, there is no report that DAP-2 interacts with ataxin-3 (products of *ATXN3* gene).

181 However, this interaction cannot be ruled out when considering that both are associated with

182 neurodegenerative processes. In SCA3/MJD as well as in other late onset neurodegenerative

183 disorders, neurodegeneration is expected to start much earlier than onset of first symptom.

184   In summary, we established allele and genotype frequencies of rs2293909 in a group of

185 Brazilian SCA3/MJD patients as well as controls. In this study, we have an indication that the

186 C allele of rs2293909 is associated with early onset of the disease. Therefore, this variant can

187 be an additional factor to modulate AO in SCA3/MJD. As previously stated, combined effects

188 are very likely to be involved in disease modulation.

196 **Compliance with Ethical Standards Conflict of interest**

197 The authors declare that they have no conflict of interest.

198

199 **Ethical Approval**

200 The study was performed as per the revised Helsinki declaration following approval of the

201 ethics committee of the hospital from where samples were collected.

202

203 **Informed Consent**

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

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282 **Figure Legends**

283

284 **Fig. 1** Correlation between age of onset (AO), CAGexp repeat length at *ATXN3* and genotypes

285 from SCA3/MJD subjects (n=184). **A)** Different genotypes are represented by squares (C/C),

286 triangles (C/T) or circles (T/T). Lines represent the linear regression model of AO, expanded

287 CAG and genotype. **B)** Different genotypes are represented by squares (C/C and C/T) or circles

288 (T/T). Lines represent the linear regression model of AO, expanded CAG and genotype.

289

290 **Fig. 2** Distribution of genotypes of rs2293909 in SCA3/MJD subjects, according to AO.

291

292 **Fig. 3** Distribution of genotypes of rs2293909 in SCA3/MJD subjects, according to AO and

293 subdivided into early, intermediate and late onset.

294 **Table 1**: Sample characterization
295

Sample	SCA3/MJD (*n*=184)
Female	106 (57.6%)
AO (years)	34.05 (9 to 56)
Normal Allele (CAG length)	22.36 (13 to 37)
Expanded Allele (CAG length)	75.38 (68 to 84)

296 AO = age of onset. Data are given as *n* (%) and mean (range).
297

298

299 **Table 2:** Allele and genotype frequencies of rs2293909.
300

	Allele			Genotype				Total
	C	T	*p*	C/C	C/T	T/T	*p*	
SCA3/MJD	76 (20.7)	292 (79.3)	0.048	10 (5.4)	56 (30.4)	118 (64.1)	0.149	184
Local controls	30 (30.0)	70 (70.0)		6 (12.0)	18 (36.0)	26 (52.0)		50

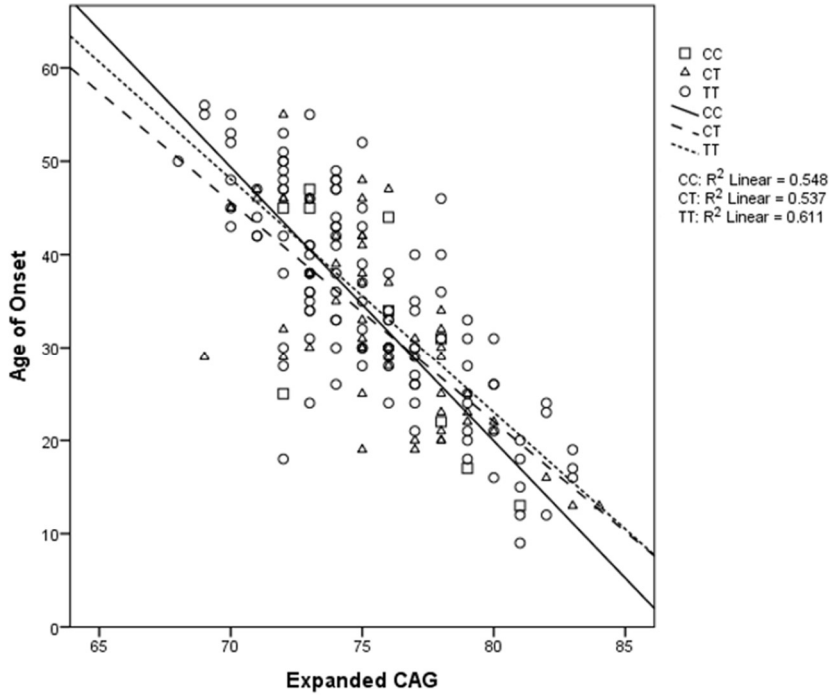Data are given as *n* (%); Percentage is group related. Pearson chi square
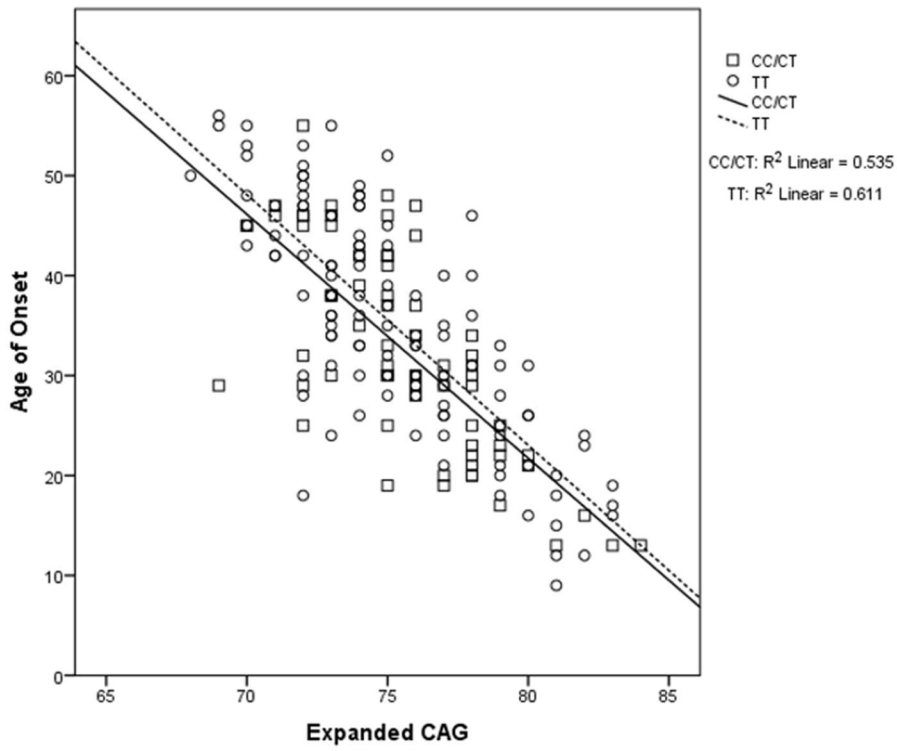
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302 **Figure 1**
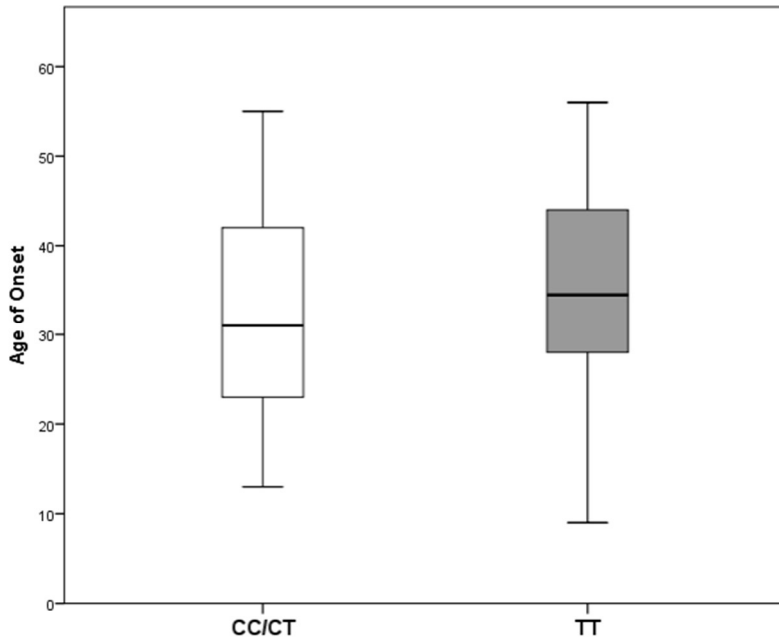
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304 **A**

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306
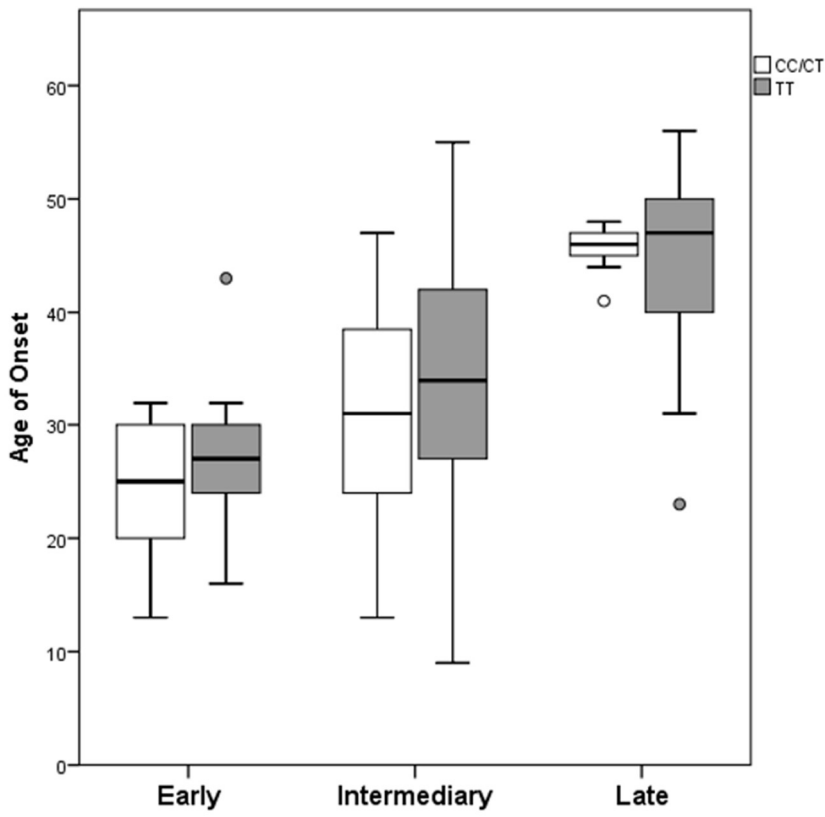307

308 **B**

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312 **Figure 2**

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315

316 **Figure 3**

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318

3.   CONCLUSÃO

O trabalho aqui apresentado propõe investigar o papel da variante rs2293909 no gene *DLGAP2* como possível modificadora da idade de início da doença em pacientes com SCA3/MJD. Em outro estudo recente, foi visto que a variante estava associada com até 10% da variância da idade de início entre os pacientes investigados. Quando a mesma investigação foi feita nos pacientes e indivíduos controles locais, não foi vista diferença entre a frequência dos alelos ou genótipos entre os dois grupos, entretanto, uma tendência pode ser vista na frequência alélica. Quando subdividimos os pacientes entre início precoce, intermediário ou tardio da doença, aqueles com ao menos um alelo C tendem a ter início mais precoce em todos os grupos, corroborando o estudo anterior e abrindo mais portas para o estudo da *DLGAP2* como um modificador de fenótipo em SCA3/MJD e até em outras doenças do grupo das poliglutaminopatias e ataxias espinocerebelares.

Este estudo soma-se a vários outros que visam entender melhor a natureza complexa das manifestações fisiopatológicas da SCA3/MJD. Como seguimento, pretendemos ampliar o número de indivíduos investigados para fortalecer a evidência. Em seguida, vamos testar o efeito da variante rs2298141, localizada no gene *ITGB1*, que foi identificada no mesmo estudo de whole-exome sequencing que detectou o potencial da *DLGAP2* como possível modificador de fenótipo da SCA3/MJD.

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ANEXO A - NORMAS PARA SUBMISSÃO DE ARTIGO NA REVISTA NEUROMOLECULAR MEDICINE

Instructions for Authors

Article Types

Original Articles: Full-length reports of current research. Abstract: 250 words maximum. Introduction: 500 words max; Discussion: 1,500 words max. Article: 6,000 words including abstract and acknowledgement but excluding author contributions statement, disclosure, references, figure legends tables and figures (Up to 6 in total figures + tables). Each figure should have a maximum of 6 panels. A maximum of 40 references are permitted.

Review Articles: Reviews are comprehensive analyses of specific topics relevant to mechanistic understanding or therapeutic development of a CNS condition. Abstract: 250 words maximum Article: 10,000 words including abstract, figure legends and acknowledgements. A maximum of 150 references are permitted.

Mini-reviews: Should be on an interesting and cutting-edge topic pertinent to mechanistic understanding or therapeutic development of a CNS condition. Usually by invitation only. However, authors can submit a 1-page pre-submission inquiry to Profs Raghu Vemuganti (vemuganti@neurosurgery.wisc.edu) or Thiruma Arumugam (g.arumugam@latrobe.edu.au) highlighting the importance of the topic of their review. Maximum length of 2,000 words excluding Title page, References and acknowledgments. Can have 2 cartoons or figures. Abstract maximum length of 150 words. A maximum of 40 references are permitted.

Nano-reviews: Should be on a novel, emerging and hot topic pertinent to mechanistic understanding or therapeutic development of a CNS condition. Usually by invitation only. However, authors can submit a 1-page pre-submission inquiry to Profs Raghu Vemuganti (vemuganti@neurosurgery.wisc.edu) or Thiruma Arumugam (g.arumugam@latrobe.edu.au) highlighting the importance of the topic of their review. Maximum length of 1,000 words excluding Title page, References and acknowledgments. Can have 1 cartoon or figure. Abstract

maximum length of 100 words. A maximum of 15 references are permitted.

Rapid Communications: Rapid communications are aimed at disseminating new data in an extremely short process . This can include negative results, and limited-scope findings. Rapid communications are prepared as 1,500 words (including abstract and acknowledgements but excluding author contributions statement, disclosure, references, figure legends, tables and figures). Up to 2 in total figures + tables are permitted. Each figure should have a maximum of 6 panels. A maximum of 15 references are permitted. Response regarding acceptance revision or rejection is usually given within 1 week. Rapid communications can only be submitted in the following fields:

Alzheimer's Disease
Parkinson's Disease
Vascular Dementia
Adult Neurogenesis
Exercise-related Metabolism
Learning and Memory
Neuroinflammation
Brain Tumors
Stroke

Commentary Articles: Commentary articles are short, narrowly focused articles that are commissioned by the journal. Commentary articles seek to provide a critical viewpoint on a key subject or provide an insight into an important development in neuroscience. These articles are generally not peer-reviewed.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly

– at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink "Submit manuscript" and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Title Page

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by "retrospectively registered", for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

- **Competing Interests:** Authors are required to disclose financial or non-financial

interests that are directly or indirectly related to the work submitted for publication. Please refer to "Competing Interests and Funding" below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

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Please note all authors must provide an institutional email as a prerequisite to submitting a manuscript.

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Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Terminology

- Please always use internationally accepted signs and symbols for units (SI units).

Scientific style

- Nomenclature: Insofar as possible, authors should use systematic names similar to

those used by Chemical Abstract Service or IUPAC.

- Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson, 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott, 1991; Barakat et al., 1995; Kelso & Smith, 1998; Medvec et al., 1999).

Authors are encouraged to follow official APA version 7 guidelines on the number of authors included in reference list entries (i.e., include all authors up to 20; for larger groups, give the first 19 names followed by an ellipsis and the final author's name). However, if authors shorten the author group by using et al., this will be retained.

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

Reference list entries should be alphabetized by the last names of the first author of each work.

Journal names and book titles should be *italicized*.

If available, please always include DOIs as full DOI links in your reference list (e.g. "https://doi.org/abc").

- Journal article Grady, J. S., Her, M., Moreno, G., Perez, C., & Yelinek, J. (2019). Emotions in storybooks: A comparison of storybooks that represent ethnic and racial groups in the United States. *Psychology of Popular Media Culture, 8*(3), 207–217. https://doi.org/10.1037/ppm0000185

- Article by DOI Hong, I., Knox, S., Pryor, L., Mroz, T. M., Graham, J., Shields, M. F., & Reistetter, T. A. (2020). Is referral to home health rehabilitation following inpatient rehabilitation facility associated with 90-day hospital readmission for adult patients with stroke? *American Journal of Physical Medicine & Rehabilitation*. Advance online publication. https://doi.org/10.1097/PHM.0000000000001435

- Book Sapolsky, R. M. (2017). *Behave: The biology of humans at our best and worst*. Penguin Books.

- Book chapter Dillard, J. P. (2020). Currents in the study of persuasion. In M. B. Oliver, A. A. Raney, & J. Bryant (Eds.), *Media effects: Advances in theory and research* (4th ed., pp. 115–129). Routledge.

- Online document Fagan, J. (2019, March 25). *Nursing clinical brain*. OER Commons. Retrieved January 7, 2020, from https://www.oercommons.org/authoring/53029-nursing-clinical-brain/view

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art
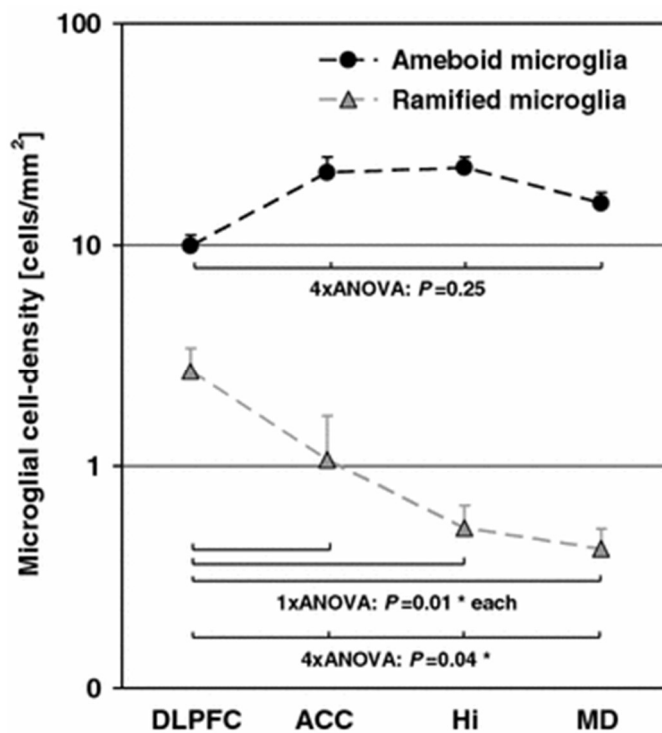
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art
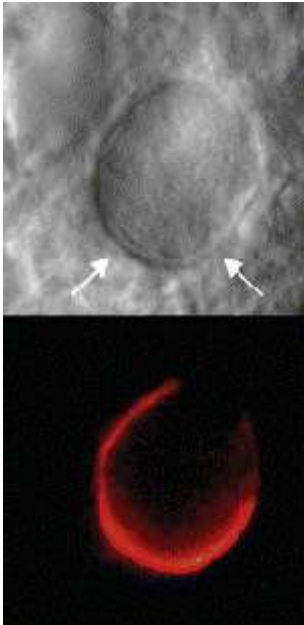
- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art
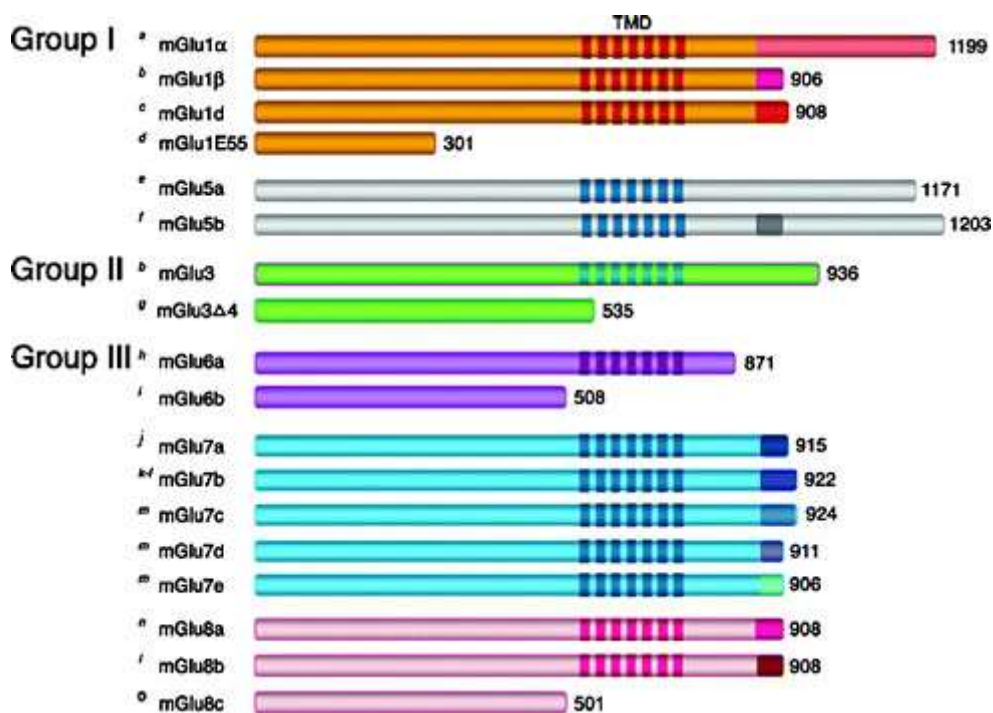
- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
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Figure Numbering

- All figures are to be numbered using Arabic numerals.
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Research involving human participants, their data or biological material

Ethics approval

When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

Retrospective ethics approval

If a study has not been granted ethics committee approval prior to commencing, retrospective ethics approval usually cannot be obtained and it may not be possible to consider the manuscript for peer review. The decision on whether to proceed to peer review in such cases is at the Editor's discretion.

Ethics approval for retrospective studies

Although retrospective studies are conducted on already available data or biological material (for which formal consent may not be needed or is difficult to obtain) ethics approval may be required dependent on the law and the national ethical guidelines of a country. Authors should check with their institution to make sure they are complying with the specific requirements of

their country.

Ethics approval for case studies

Case reports require ethics approval. Most institutions will have specific policies on this subject. Authors should check with their institution to make sure they are complying with the specific requirements of their institution and seek ethics approval where needed. Authors should be aware to secure informed consent from the individual (or parent or guardian if the participant is a minor or incapable) See also section on **Informed Consent**.

Cell lines

If human cells are used, authors must declare in the manuscript: what cell lines were used by describing the source of the cell line, including when and from where it was obtained, whether the cell line has recently been authenticated and by what method. If cells were bought from a life science company the following need to be given in the manuscript: name of company (that provided the cells), cell type, number of cell line, and batch of cells.

It is recommended that authors check the [NCBI database](#) for misidentification and contamination of human cell lines. This step will alert authors to possible problems with the cell line and may save considerable time and effort.

Further information is available from the [International Cell Line Authentication Committee](#) (ICLAC).

Authors should include a statement that confirms that an institutional or independent ethics committee (including the name of the ethics committee) approved the study and that informed consent was obtained from the donor or next of kin.

Research Resource Identifiers (RRID)

Research Resource Identifiers (RRID) are persistent unique identifiers (effectively similar to a DOI) for research resources. This journal encourages authors to adopt RRIDs when reporting key biological resources (antibodies, cell lines, model organisms and tools) in their manuscripts.

Examples:

Organism: *Filip1^{tm1a(KOMP)Wtsi}* **RRID:MMRRC_055641-UCD**

Cell Line: RST307 cell line **RRID:CVCL_C321**

Antibody: Luciferase antibody DSHB Cat# LUC-3, **RRID:AB_2722109**

Plasmid: mRuby3 plasmid **RRID:Addgene_104005**

Software: ImageJ Version 1.2.4 **RRID:SCR_003070**

RRIDs are provided by the Resource Identification Portal. Many commonly used research resources already have designated RRIDs. The portal also provides authors links so that they can quickly register a new resource and obtain an RRID.

Clinical Trial Registration

The World Health Organization (WHO) definition of a clinical trial is "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes". The WHO defines health interventions as "A health intervention is an act performed for, with or on behalf of a person or population whose purpose is to assess, improve, maintain, promote or modify health, functioning or health conditions" and a health-related outcome is generally defined as a change in the health of a person or population as a result of an intervention.

To ensure the integrity of the reporting of patient-centered trials, authors must register prospective clinical trials (phase II to IV trials) in suitable publicly available repositories. For example www.clinicaltrials.gov or any of the primary registries that participate in the WHO International Clinical Trials Registry Platform.

The trial registration number (TRN) and date of registration should be included as the last line of the manuscript abstract.

For clinical trials that have not been registered prospectively, authors are encouraged to register retrospectively to ensure the complete publication of all results. The trial registration number (TRN), date of registration and the words 'retrospectively registered' should be included as the

last line of the manuscript abstract.

Standards of reporting

Springer Nature advocates complete and transparent reporting of biomedical and biological research and research with biological applications. Authors are recommended to adhere to the minimum reporting guidelines hosted by the EQUATOR Network when preparing their manuscript.

Exact requirements may vary depending on the journal; please refer to the journal's Instructions for Authors.

Checklists are available for a number of study designs, including:

Randomised trials (CONSORT) and Study protocols (SPIRIT)

Observational studies (STROBE)

Systematic reviews and meta-analyses (PRISMA) and protocols (Prisma-P)

Diagnostic/prognostic studies (STARD) and (TRIPOD)

Case reports (CARE)

Clinical practice guidelines (AGREE) and (RIGHT)

Qualitative research (SRQR) and (COREQ)

Animal pre-clinical studies (ARRIVE)

Quality improvement studies (SQUIRE)

Economic evaluations (CHEERS)

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

Examples of statements to be used when ethics approval has been obtained:

- All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and

with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Medical University of A (No. ...).

- This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

- Approval was obtained from the ethics committee of University C. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

- The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

- Ethical approval was waived by the local Ethics Committee of University A in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

- This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.

- This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

Examples of statements to be used when no ethical approval is required/exemption granted:

- This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.

- The data reproduced from Article X utilized human tissue that was procured via our Biobank AB, which provides de-identified samples. This study was reviewed and deemed exempt by our XYZ Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Informed consent

All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. This is especially true concerning images of vulnerable people (e.g. minors, patients, refugees, etc) or the use of images in sensitive contexts. In many instances authors will need to secure written consent before including images.

Identifying details (names, dates of birth, identity numbers, biometrical characteristics (such as facial features, fingerprint, writing style, voice pattern, DNA or other distinguishing characteristic) and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scholarly purposes and the participant (or parent/guardian if the participant is a minor or incapable or legal representative) gave written informed consent for publication. Complete anonymity is difficult to achieve in some cases. Detailed descriptions of individual participants, whether of their whole bodies or of body sections, may lead to disclosure of their identity. Under certain circumstances consent is not required as long as information is anonymized and the submission does not include images that may identify the person.

Informed consent for publication should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort meaning.

Exceptions where it is not necessary to obtain consent:

- Images such as x rays, laparoscopic images, ultrasound images, brain scans, pathology

slides unless there is a concern about identifying information in which case, authors should ensure that consent is obtained.

- Reuse of images: If images are being reused from prior publications, the Publisher will assume that the prior publication obtained the relevant information regarding consent. Authors should provide the appropriate attribution for republished images.

Consent and already available data and/or biologic material

Regardless of whether material is collected from living or dead patients, they (family or guardian if the deceased has not made a pre-mortem decision) must have given prior written consent. The aspect of confidentiality as well as any wishes from the deceased should be respected.

Data protection, confidentiality and privacy

When biological material is donated for or data is generated as part of a research project authors should ensure, as part of the informed consent procedure, that the participants are made aware what kind of (personal) data will be processed, how it will be used and for what purpose. In case of data acquired via a biobank/biorepository, it is possible they apply a broad consent which allows research participants to consent to a broad range of uses of their data and samples which is regarded by research ethics committees as specific enough to be considered "informed". However, authors should always check the specific biobank/biorepository policies or any other type of data provider policies (in case of non-bio research) to be sure that this is the case.

Consent to Participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript. In the case of articles describing human transplantation studies, authors must include a statement declaring that no organs/tissues were obtained from prisoners and must also name the institution(s)/clinic(s)/department(s) via which organs/tissues were obtained. For manuscripts reporting studies involving vulnerable groups where there is the potential for coercion or where

consent may not have been fully informed, extra care will be taken by the editor and may be referred to the Springer Nature Research Integrity Group.

Consent to Publish

Individuals may consent to participate in a study, but object to having their data published in a journal article. Authors should make sure to also seek consent from individuals to publish their data prior to submitting their paper to a journal. This is in particular applicable to case studies. A consent to publish form can be found
[here. (Download docx, 36 kB)](here.)

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Consent to participate' and/or 'Consent to publish'. Other declarations include Funding, Competing interests, Ethics approval, Consent, Data and/or Code availability and Authors' contribution statements.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Sample statements for **"Consent to participate"**:

Informed consent was obtained from all individual participants included in the study.
Informed consent was obtained from legal guardians.
Written informed consent was obtained from the parents.
Verbal informed consent was obtained prior to the interview.

Sample statements for **"Consent to publish"**:

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.
The participant has consented to the submission of the case report to the journal.
Patients signed informed consent regarding publishing their data and photographs.

Sample statements if identifying information about participants is available in the article: Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Images will be removed from publication if authors have not obtained informed consent or the paper may be removed and replaced with a notice explaining the reason for removal.

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This journal operates a type 3 research data policy. A submission to the journal implies that materials described in the manuscript, including all relevant raw data, will be freely available to any researcher wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Data availability

All original research must include a data availability statement. Data availability statements should include information on where data supporting the results reported in the article can be found, if applicable. Statements should include, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. For the purposes of the data availability statement, "data" is defined as the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. When it is not possible to share research data publicly, for instance when individual privacy could be compromised, data

availability should still be stated in the manuscript along with any conditions for access. Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

1. The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]

2. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

3. All data generated or analysed during this study are included in this published article [and its supplementary information files].

4. The datasets generated during and/or analysed during the current study are not publicly available due [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.].

5. Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

6. The data that support the findings of this study are available from [THIRD PARTY NAME] but restrictions apply to the availability of these data, which were used under licence for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [THIRD PARTY NAME].

More templates for data availability statements, including examples of openly available and restricted access datasets, are available here:

Data availability statements

Data repositories

This journal strongly encourages that all datasets on which the conclusions of the paper rely are available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Please see Springer Nature's information on recommended repositories.

List of Repositories

General repositories - for all types of research data - such as figshare and Dryad may be used where appropriate.

Data citation

The journal also requires that authors cite any publicly available data on which the conclusions of the paper rely. Data citations should include a persistent identifier (such as a DOI), should be included in the reference list using the minimum information recommended by DataCite, and follow journal style. Dataset identifiers including DOIs should be expressed as full URLs.

Research data and peer review

Peer reviewers are encouraged to check the manuscript's Data availability statement, where applicable. They should consider if the authors have complied with the journal's policy on the availability of research data, and whether reasonable effort has been made to make the data that support the findings of the study available for replication or reuse by other researchers. Peer reviewers are entitled to request access to underlying data (and code) when needed for them to perform their evaluation of a manuscript.

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