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Márcia Polese-Bonatto

ANÁLISE DE ALTERAÇÕES DE SEQUÊNCIA NOS GENES ASSOCIADOS À DOENÇA DE NIEMANN-PICK TIPO C E AVALIAÇÃO DE GENES CANDIDATOS A MODIFICADORES DE FENÓTIPO

Porto Alegre
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“A confiança não vem do ato de estar sempre certo, mas de não ter medo de estar errado”

Peter T. McIntire

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PARTE I

RESUMO

A doença de Niemann-Pick tipo C (NP-C) é uma doença autossômica recessiva causada por mutações nos genes *NPC1* ou *NPC2* e se caracteriza principalmente pelo acúmulo do colesterol não esterificado no lisossomo/endossomo tardio. Aproximadamente 95% dos pacientes apresentam mutação no gene *NPC1* enquanto 5% em *NPC2*. O gene *NPC1* codifica uma glicoproteína transmembrânica de 1278 aminoácidos, com 13 domínios que reside na membrana do lisossomo/endossomo tardio. O produto do gene *NPC2* é uma glicoproteína de 132 aminoácidos expressa em todos os tecidos, sendo uma proteína solúvel que se liga ao colesterol. A incidência de NP-C é estimada em 0,95 casos para cada 100.000 nascidos vivos. O objetivo geral deste trabalho foi identificar o genótipo de pacientes com NP-C, determinando a frequência de mutações comuns e caracterizando mutações novas nos genes associados à doença, e analisar variantes de sequência em genes candidatos a modificadores de fenótipo dessa doença. As mutações mais frequentemente encontradas foram p.Ala1035Val (27,3%), p.Pro1007Ala (17,0%) e p.Phe1221Serfs*20 (14,8%). Na identificação das variantes de sequência, foram identificadas 5 novas variantes potencialmente patogênicas, sendo 1 deleção pequena (p.Lys38_Tyr40del), 1 mutação do (p.Asn195Lysfs*2) e 3 mutações de ponto (p.Cys238Arg, p.Ser365Pro e p.Val694Met). Um outro subgrupo de amostras (n=34), proveniente de indivíduos com um único alelo mutante, foram submetidos a uma análise molecular mais abrangente visando a identificação de possíveis rearranjos gênicos. Os resultados obtidos confirmam os relatos que indicam uma frequência muito baixa de rearranjos gênicos associados à NP-C, pois não foi identificado nenhum paciente com perfil anormal através desse protocolo abrangente. Considerando que chaperonas diferentes foram associadas à expressão do gene *NPC1,* polimorfismos de nucleotídeos únicos (SNPs) nos genes que codificam a chaperona DNAJB6 e a cochaperona CHIP, ambas proteínas do sistema de controle de qualidade proteica, foram selecionados e analisados para investigar uma possível associação com variações fenotípicas de pacientes com NP-C. Essa avaliação permitiu estabelecer as frequências alélicas e genotípicas dos SNPs rs9647660, rs12668448, rs4716707 e rs6459770 (no gene *DNAJB6*) e o rs6597 (no gene *CHIP*) nos pacientes e em amostras de indivíduos normais. Os resultados obtidos permitiram comparar os grupos (pacientes e controles) e, dentro do grupo de pacientes, comparar os diferentes fenótipos, mas não foram encontradas diferenças estatisticamente significativas. Além disso, foi avaliado o gene que codifica a apolipoproteína E (ApoE), uma lipoproteína envolvida no metabolismo do colesterol, que se caracteriza por apresentar 3 alelos distintos a partir de dois polimorfismos específicos (rs429358 e rs7412) no gene *ApoE*. As frequências alélicas de *ApoE ε2*, *ApoE ε3* e *ApoE ε4* no grupo de pacientes foi estabelecida em 6,3%, 82,9% e 10,8%, respectivamente. O genótipo mais frequente foi o *ApoE ε3/ε3* (63,8%). Apesar de não terem sido encontradas diferenças estatisticamente significativas, não podemos descartar completamente o efeito da ApoE no fenótipo dos pacientes, considerando que esta proteína está presente no metabolismo do colesterol. Por fim, os dados produzidos através desse trabalho contribuem para o melhor conhecimento das bases moleculares da NP-C.

ABSTRACT

Niemann-Pick type C (NP-C) disease is an autosomal recessive disease caused by mutations in the *NPC1* or *NPC1* genes and is mainly characterized by the storage of non-esterified cholesterol in lysosome/late endosome. Approximately 95% of patients carry mutations in the *NPC1* gene while 5% in *NPC2*. The *NPC1* gene encodes a 1278 amino acid transmembrane glycoprotein with 13 domains placed in the membrane of the lysosome/late endosome membrane. The product of *NPC2* gene is a 132 amino acid glycoprotein expressed in all tissues, being a soluble protein that binds to cholesterol. The incidence of NP-C is estimated to be 0.95 cases per 100,000 live births. The general aim of this work was to identify the genotype of patients with NP-C, determining the frequency of common mutations and characterizing novel mutations in the genes associated with the disease, and analyzing sequence variants in candidate genes of phenotypic modifiers of the disease. More frequently found mutations were p.Ala1035Val (27.3%), pPro1007Ala (17.0%) and p.Phe1221Serfs*20 (14.8%). In the identification of sequence variants, 5 new potentially pathogenic variants were identified, 1 small deletion (p.Lys38_Tyr40del), 1 mutation (p.Asn195Lysfs*2) and 3 point mutations (p.Cys238Arg, p.Ser365Pro and p.Val694Met). A further subgroup of samples, from individuals with a single mutant allele, were subjected to a more comprehensive molecular analysis to identify possible gene rearrangements. Results of this work confirms previous reports that indicate a very low frequency of gene rearrangements associated with NP-C, since no patient with an abnormal profile was identified through this protocol. Considering that different chaperones were associated with NPC1 gene expression, single nucleotide polymorphisms (SNPs) in the genes encoding the chaperone DNAJB6 and cochaperone CHIP, both proteins of the protein quality control system, were selected and analyzed to investigate a possible association with phenotypic variations of NP-C patients. This evaluation allowed to establish allelic and genotype frequencies of SNPs rs9647660, rs12668448, rs4716707 and rs6459770 (in the DNAJB6 gene) and rs6597 (in the CHIP gene) in samples of NP-C patients and in samples from normal individuals. Results allowed to compare both groups (patients and controls) and, within the group of patients, to compare different clinical phenotypes, but no statistically significant differences were found. In addition, the gene encoding apolipoprotein E (ApoE), a lipoprotein involved in cholesterol metabolism, was evaluated, which is characterized by 3 distinct alleles from two specific polymorphisms (rs429358 and rs7412) in the ApoE gene. The allelic frequencies of *ApoE ε2*, *ApoE ε3* and *ApoE ε4* in patient group were established as 6.3%, 82.9%, and 10.8%, respectively. The most frequent genotype was *ApoE ε3/ε3* (63.8%). No statistically significant difference was found in the analysis performed. However, we cannot rule out the effect of ApoE on phenotype of patients, considering that this protein is related to cholesterol metabolism. Finally, the data produced through this work contribute to a better understanding of the molecular basis of NP-C.

LISTA DE ABREVIATURAS

ApoE – apolipoproteína E

Aβ – beta-amiloide

CHIP – *Carboxy-terminus of Hsc70-interaction protein*

C-triol – *cholestane-3β,5α,6β-triol*

DA – doença de Alzheimer

DLD – doenças de depósito lisossomal

ERAD – *endoplasmatic-reticulum associated-protein degradation*

HGMD – *The Human Gene Mutation Database*

Hsc70 – *Heat Shock Cognate 70*

HSP 40 – *heat shock protein 40*

Hsp70 – *heat shock protein 70*

Hsp90 – *heat shock protein 90*

kDa – quilodalton

LDL – doenças lisossômicas de depósito

MLPA – *Multiplex Ligation-dependent Probe Amplification*

NGS – *Next Generation Sequencing*

NP-A – Niemann-Pick tipo A

NP-B – Niemann-Pick tipo B

NP-C – Niemann-Pick tipo C

PAS – pré-autofagossomo

PCR – *Polymerase chain reaction*

SNP – *single nucleotide polymorphisms*

7-KC - *7-ketocholesterol*

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1 INTRODUÇÃO

1.1 Doenças lisossômicas de depósito e a doença de Niemann-Pick

As doenças lisossômicas de depósito (DLD) são um grupo heterogêneo, com mais de 40 desordens genéticas. Mutações nos genes que codificam enzimas lisossomais, proteínas transmembranas, proteínas de transporte entre outras proteínas encontradas nos lisossomos são responsáveis pelas patologias. A doença de Niemann-Pick é uma doença autossômica recessiva e classifica-se como uma DLD, caracterizando-se pela alteração no metabolismo lipídico (Marsden & Levi, 2010).

Até o momento, foram descritas três formas da doença: a doença de Niemann-Pick tipo A (NP-A), a doença de Niemann-Pick tipo B (NP-B) e a doença de Niemann-Pick tipo C (NP-C). NP-A e NP-B são causadas pela mesma deficiência enzimática: a deficiência da esfingomielinase ácida. Entretanto, elas apresentam manifestações clínicas distintas devido a diferentes mutações encontradas no gene *ASM*. A NP-A é a forma mais grave, pois apresenta degeneração neurológica que leva a óbito em torno dos 3 anos de idade. A NP-B não apresenta manifestações neurológicas e, consequentemente, é a forma mais branda da doença (Wasserstein & Schuchman, 2006).

1.2 Doença de Niemann-Pick tipo C

A doença de Niemann-Pick tipo C (NP-C) é uma doença autossômica recessiva causada por mutações em um dos dois genes: no gene *NPC1* ou no gene *NPC2*. A patologia se caracteriza pelo acúmulo de colesterol não esterificado nos lisossomos e no endossomo tardio causado pela falha no tráfego intracelular dos lipídeos (Vanier, 2015). Também é observado níveis

aumentados de glicoesfingolipídeos, que ocorre pela inibição da enzima esfingomielinase ácida, devido ao excesso de colesterol no interior do lisossomo (Devlin *et al.*, 2010).

A idade de início da doença é variável, podendo ter início na infância até a idade adulta. As manifestações clínicas são heterogêneas, diferenciando-se entre estes grupos (Vanier, 2010; Patterson *et al.*, 2012; Jahnova *et al.*, 2014; Schultz *et al.*, 2016). O tempo de vida dos pacientes pode variar desde alguns dias até mais de 60 anos, mas na maioria dos casos, o óbito ocorre entre 10 a 25 anos de idade (Vanier, 2010).

Dois genes foram identificados como os responsáveis pela doença de NP-C, *NPC1* (OMIM ID: 607623) e *NPC2* (OMIM ID: 601015) (Vanier *et al.*, 1996). Aproximadamente 95% dos pacientes apresentam mutação no gene *NPC1* enquanto de 4% a 5% em *NPC2*, até o momento mais 500 mutações já foram descritas (478 no gene *NPC1* e 27 no gene *NPC2*) (www.hgmd.cf.ac.uk). As mutações podem ser do tipo *missense*, *nonsense*, *frame shift* (deleções e inserções) e alterações em introns, este último podendo levar a criação de sítios alternativos de *splicing* (Bounford and Gissen, 2014).

1.2.1 Manifestações Clínicas

As manifestações viscerais (fígado, baço e em alguns casos, pulmão) em conjunto com sintomas neurológicos ou psiquiátricos auxiliam no diagnóstico diferencial de NP-C, mas casos atípicos são relativamente frequentes (Vanier, 2016). O diagnóstico na infância acaba sendo problemático, devido ao fato que os mesmos sintomas podem ser encontrados em outras doenças neurológicas (Vance & Karten, 2014) e, em outros erros inatos do metabolismo, como exemplo, podemos citar Doença de Gauche tipo 3, Doença de Tay-Sachs,

Síndrome de MEGDEL e doença de Krabbe (Sobrido *et al.*, 2019). Mais recentemente, escores clínicos estão sendo desenvolvidos para auxiliar o diagnóstico (NP-C *Suspicion index* – SI) (Jahnova *et al.*, 2014).

Os sintomas viscerais e os neurológicos e/ou psiquiátricos apresentam diferenças temporais, ocorrendo em idades precoces até idade tardia, respectivamente (Vanier, 2010).

Em um estudo multicêntrico, que envolveu 4 países (República Tcheca, Iran, Espanha e dois centros na Eslováquia) 63 pacientes com diagnóstico de NP-C foram divididos em infantil (< 4 anos), juvenil (≥ 4 anos - <16 anos) e adulto (≥ 16 anos) com idade média de diagnóstico de 2,15 anos, 10,97 anos e 25,98 anos respectivamente. No grupo infantil, manifestações viscerais apareceram em mais de 50% dos casos, no grupo juvenil foram observadas manifestações viscerais e neurológicas, já no grupo adulto, foram frequentes os sintomas psicóticos (Pineda *et al.*, 2019).

As manifestações perinatais são comumente relacionadas a alterações no fígado, incluindo ainda hidropisia fetal, ascite e icterícia prolongada associada com a progressão da hepatoesplenomegalia (Yerushalmi *et al.*, 2002). No período neonatal não são observadas manifestações neurológicas, as quais são importantes para o diagnóstico diferencial de NP-C (Vanier & Millat, 2003).

No diagnóstico infantil precoce, o qual ocorre entre 2 meses até 2 anos, observa-se hepatoesplenomegalia, já o atraso no desenvolvimento motor e a hipotonia são os primeiros sintomas neurológicos a serem observados entre os 8 e 9 meses, tornando-se evidente entre 1 a 2 anos. Nestes casos, dificilmente os pacientes ultrapassam 5 anos de vida (Vanier, 2010).

No diagnóstico infantil tardio, de 2 até 6 anos, os pacientes podem apresentar hepatomegalia e/ou esplenomegalia e manifestações neurológicas, como atraso na fala, ataxia progressiva, atraso no movimento ocular, cataplexia, atraso nos movimentos motores, atraso mental, disfagia, disartria e crises convulsivas. O óbito geralmente ocorre entre 7 e 12 anos de vida (Vanier, 2010).

No período juvenil, de 6 até 15 anos, classificado como a forma “clássica”, os pacientes apresentam esplenomegalia, raramente hepatoesplenomegalia. Problemas na escrita e dificuldade de atenção também são observadas nestes casos. Ocorre o aparecimento de ataxia, disartria, disfagia, problemas motores e variáveis graus de crises convulsivas. A sobrevida nestes casos é de 30 anos ou mais (Vanier, 2010).

O período adulto se caracteriza pela presença de sintomas como a ataxia cerebelar, atraso no movimento ocular, distonia, disartria, problemas cognitivos, epilepsia, desordem nos movimentos, disfagia, esplenomegalia e transtornos psiquiátricos, entre eles, crises paranoicas, depressão, problemas comportamentais agressivos e isolamento social, não sendo necessário o aparecimento concomitante destes sintomas para o diagnóstico de NP-C (Vanier, 2010).

1.2.2 Genes *NPC1* e *NPC2* e interação de seus produtos de expressão

O gene *NPC1* está localizado no *locus* 18q11-q12 e abrange 57 kb de DNA genômico, sendo dividido em 25 éxons (Carstea *et al.*, 1997; Patterson, 2000). Esse gene codifica uma glicoproteína transmembrânica de 1278 aminoácidos, com 13 domínios que reside na membrana dos lisossomos e endossomos tardio (Carstea *et al.*, 1997). A proteína apresenta um domínio

sensível ao esterol (SSD), o qual medeia o tráfico intracelular de colesterol através de sua ligação ao domínio N-terminal (Ioannou, 2000).

O gene *NPC2* está localizado no *locus* 14q24.3, abrange 13,5 kb de DNA genômico e sua região codificante apresenta 5 éxons. O produto desse gene é uma glicoproteína de 132 aminoácidos, expressa em todos os tecidos com alta concentração nos testículos, rins e fígado. É uma proteína solúvel, que se liga ao colesterol e é capaz de reverter parcialmente o acúmulo de lipídios celular (Naureckiene *et al.*, 2000). A proteína NPC2 apresenta isoformas com glicosilação variável, pesos moleculares variando entre 19 a 23 kDa e, distribuição em diferentes tecidos (Vanier & Millat 2004).

A localização e a estrutura dos genes *NPC1* e *NPC2* encontram-se representadas, de forma esquemática, na figura 1.

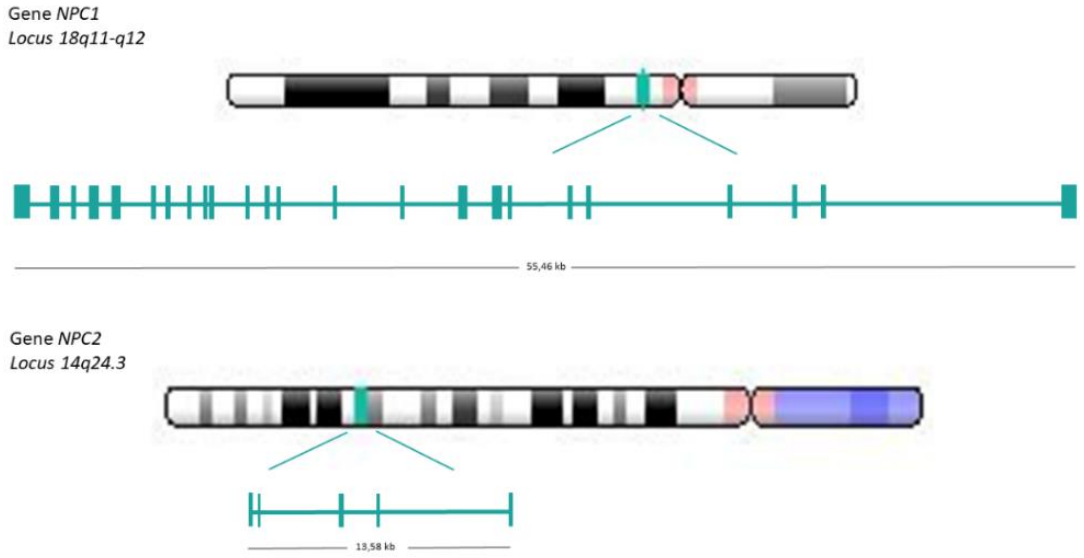

Figura 1: Representação esquemática dos genes *NPC1* e *NPC2*. O gene *NPC1* é composto de 25 éxons, com tamanho total de 55,46 kb; gene *NPC2* é composto por 5 éxons com tamanho total de 13,58 kb (Fonte: Elaborada pelo autor).

O mecanismo de transporte intracelular do colesterol vem sendo chamado de “time NPC” por alguns autores. Entretanto, o mecanismo completo ainda não

está completamente elucidado (Vance *et al.*, 2010). Relação direta entre as proteínas NPC1 e NPC2 já foi evidenciada (Figura 2). Após a endocitose do colesterol para o interior do lisossomo, a proteína NPC2 liga-se a região hidrofóbica do colesterol, proporcionando o movimento deste no meio aquoso. O colesterol ligado a NPC2 é então transferido para a proteína transmembrânica NPC1, a qual é responsável pela exportação do colesterol para fora do lisossomo/endossomo tardio (Wang *et al.*, 2010).

O transporte do colesterol do lisossomo para o citoplasma via proteína NPC1 ainda não foi elucidado, sendo questionado se esse transporte ocorre via inserção da molécula na membrana do lisossomo. Questões como: na presença de colesterol, a proteína NPC1 pode sofrer mudanças conformacionais importantes para a sua função de transporte? O colesterol, ligando-se ao domínio SSD vai regular as mudanças conformacionais ou vai realmente realizar o transporte desse composto? Estes questionamentos estão por serem esclarecidos (Pfeffer, 2019).

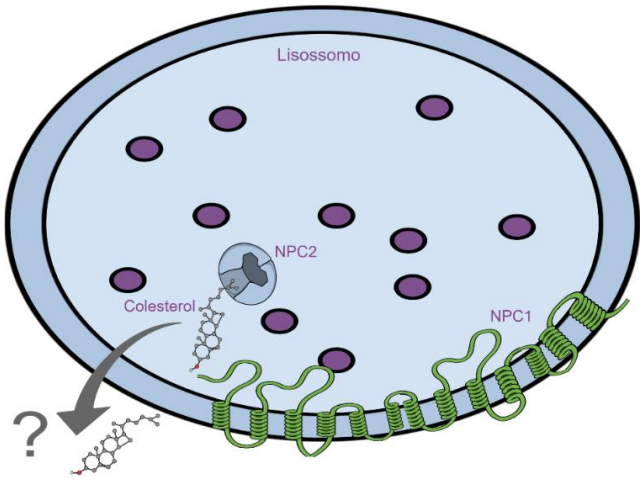

Figura 2: Mecanismos proposto de exportação do colesterol a partir do lisossomo/endossomo tardio mediado pelas proteínas NPC1/NPC2. O colesterol não esterificado entra nos lisossomo/endossomo tardio via endocitose mediada por receptores de LDL (*low-density lipoproteins*); em seguida ocorre ligação à proteína NPC2, a qual encontra-se no lúmen da organela, sendo transportado para a proteína transmembrânica NPC1, a qual será responsável pela exportação da molécula para o citoplasma (Fonte: Elaborada pelo autor).

1.2.3 Dados Epidemiológicos

NP-C é uma doença pan-étnica com prevalência no oeste europeu estimada em 1 para cada 120.000 a 150.000 nascidos vivos (Vanier & Millat, 2003). Atualmente, estima-se que a incidência seja de 0,95 casos em cada 100.000 nascidos vivos (Vanier, 2015). Na República Tcheca, a prevalência estimada entre os anos de 1975-2012 foi de 0,93 casos a cada 100.000 nascidos vivos (Jahnova *et al.*, 2014). No Brasil, ainda não há dados disponíveis referentes à prevalência e incidência de NP-C.

Acredita-se que estes valores sejam subestimados devido à dificuldade de diagnóstico desta patologia (Mengel *et al.*, 2013). Em uma análise mais recente, dados gerados por sequenciamento do exoma, onde as variações identificadas foram classificadas como patogênicas ou não-patogênicas baseadas numa combinação de revisão da literatura e análise de bioinformática, sugeriu uma incidência de 1,12 a cada 100.000 nascidos vivos (Wassif *et al.*, 2016).

1.2.4 Diagnóstico de Niemann-Pick tipo C

O diagnóstico de NP-C é realizado através de uma combinação de análises laboratoriais, com análises bioquímicas e moleculares (Vanier, 2016). O teste do Filipin foi desenvolvido há mais de 30 anos e é utilizado para o diagnóstico de NP-C. Esse teste se caracteriza pela observação de uma intensa fluorescência ao redor do núcleo, pelo acúmulo de colesterol não esterificado (Wraith *et al.*, 2009). A utilização apenas deste teste para confirmar casos de NP-C pode ser dificultada pelo fato de que formas “variantes” da doença resultarem em análises inconclusivas. Esses casos podem estar relacionados a

mutações específicas na sequência gênica, como é o caso da mutação p.Pro1007Ala e em alguns casos de heterozigotos compostos (Vanier, 2016).

A busca de novos biomarcadores que possam ser identificados em amostras de sangue e que ofereçam especificidade e baixo custo estão sendo colocados na rotina de triagem para diagnóstico de NP-C (Vanier, 2016). A atividade da quitotriosidase é utilizada para diagnóstico da doença de Gaucher, mas também se encontra elevada em outras doenças lisossômicas, podendo estar aumentada em casos de NP-C (Ries *et al.*, 2006).

A dosagem de oxiesteróis está sendo utilizada para auxiliar no diagnóstico de NP-C. Oxiesteróis correspondem a uma classe de moléculas as quais são geradas a partir da oxidação do colesterol. Elevadas concentrações destas moléculas foram observadas em plasma de camundongos NPC1-/- e, concentrações levemente aumentadas foram observadas em pacientes heterozigotos para NPC1. Dois metabólitos em especial chamam atenção: *cholestane-3β,5α,6β-triol* (C-triol) e *7-ketocholesterol* (7-KC). A dosagem de oxiesteróis é uma análise que utiliza amostra de sangue como material biológico e, as técnicas utilizadas podem ser a cromatografia gasosa acoplada a espectrometria de massa e cromatografia líquida acoplada à espectrometria de massa em tandem (Porter *et al.*, 2010; Jiang *et al.*, 2011).

O C-triol e 7-KC apresentam uma alta correlação entre si, porém estes biomarcadores não estão correlacionados com a idade de início dos sintomas e nem com a gravidade da doença (Pajares *et al.*, 2015). Mais de 30 laboratórios ao redor do mundo já disponibilizam a dosagem de oxiesterol no plasma para auxiliar no diagnóstico de NP-C (Sobrido *et al.*, 2019). Muitos estudos

encontram-se em andamento para validar este biomarcador como teste auxiliar no diagnóstico desta patologia.

No laboratório de análises bioquímicas do Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, 76 amostras de indivíduos com suspeita para NP-C foram dosadas os níveis de oxiesteróis, especificamente o C-triol e 7-KC, a atividade da quitotriosidase, além da realização do teste de Filipin e da análise molecular (sequenciamento de Sanger). Os resultados obtidos neste trabalho apresentaram 88,0% de sensibilidade e 96,1% de especificidade na dosagem de C-triol, reafirmando, desta forma, que este metabolito pode ser um bom biomarcador para diagnóstico de NP-C (Hammerschmidt *et al.*, 2018).

Outros candidatos a biomarcadores também vem sendo observados, como o aumento dos lisoesfingolipídeos e presença de ácidos biliares na urina. A detecção destes marcadores apresenta baixo custo, são de fácil implementação em laboratório e permitem a primeira triagem da amostra do paciente. Porém, eles ocorrem em outras doenças e seus níveis normais não excluem o diagnóstico. Desta forma, ainda não há dados suficientes para a validação no diagnóstico complementar de NP-C (Vanier, 2015).

A análise molecular é necessária para confirmar o diagnóstico após a identificação de biomarcadores alterados e em resultados inconclusivos no teste de Filipin (Patterson *et al.*, 2012). Esta análise pode ser realizada através da técnica de sequenciamento das regiões codificantes e flanqueadoras dos genes *NPC1* e *NPC2* utilizando a metodologia de Sanger.

Além das análises bioquímicas e moleculares, o diagnóstico de NP-C pode contar com biomarcadores de imagem. A ressonância magnética (*Magnetic Resonance Imaging* – MRI), DaTSCAN™ SPECT (*Single-photon Emission*

Computed Tomography), tensor de difusão (*diffusion tensor imaging* – DTI) e tomografia por emissão de pósitrons (*positron emission tomography* – PET) são alguns exemplos que, apesar de não serem específicos para a patologia, podem ser utilizados para verificar a gravidade da doença bem como para o acompanhamento de tratamentos (Benussi *et al*., 2018).

O sequenciamento de nova geração (*Next Generation Sequencing* – NGS) está começando a ser validado para ser utilizado como mais uma ferramenta de diagnóstico. O NGS apresenta maior cobertura dos genes, permitindo uma análise que inclui íntrons e regiões regulatórias, mas algumas alterações como duplicações podem passar despercebidas por essa metodologia (Macias-Vidal *et al.*, 2011).

Dentre os fatores que podemos considerar como sendo possíveis causas da doença estão as alterações de maior extensão, como grandes deleções e inserções de DNA. Para tal, a técnica de amplificação de múltiplas sondas dependente de ligação (*Multiplex Ligation-dependent Probe Amplification* - MLPA), descrita por Schouten e colaboradores (2002), e posteriormente comercializada pela empresa holandesa MRC-Holland, pode ser aplicada. A técnica se baseia no princípio que a amostra de DNA genômico é hibridizada a uma mistura de sondas, com amplificação posterior dos produtos de ligação por PCR (*Polymerase chain reaction*), utilizando um par de primers universal. Os fragmentos finais são resolvidos por eletroforese capilar, sendo possível a quantificação relativa de cópias gênicas. A técnica de MLPA permite avaliar alterações do tipo grandes deleções e duplicações de éxons e até mesmo uma duplicação do gene inteiro (Macias-Vidal *et al.*, 2011).

Além do auxílio laboratorial, pode ser utilizado o *Score NP-C SI* em combinação com análise de biomarcadores e análise genética garantindo uma melhor eficiência e uma diminuição do custo-efetividade para o diagnóstico preciso de NP-C (Sobrido *et al.*, 2019).

1.2.5 Tratamentos disponíveis

O Miglustat (Zavesca®), vem sendo utilizado como tratamento paliativo de NP-C, o qual tem apresentado redução de progressão da doença. Este fármaco foi aprovado pela Agência Nacional de Vigilância Sanitária (ANVISA), pela União Europeia e ainda por países como Canadá e Japão. Esse medicamento também é utilizado para tratamento da doença de Gaucher. O Miglustat leva a redução da glicosilceramida através da inibição de glicosilceramida sintase (Patterson *et al.*, 2007). Estudos recentes apresentam resultados que indicam que o há uma redução dos scores de incapacidade em pacientes que fazem uso deste medicamento (Pineda *et al.*, 2019).

Exaustivos ensaios clínicos com β-ciclodextrina vem sendo realizados. Resultados preliminares apresentaram atenuação do fenótipo de NP-C. A internalização da molécula para o lisossomo ocorre via pinocitose, a qual possivelmente realiza a função de NPC1 e de NPC2. Como ocorre esse efeito ainda não está determinado, mas uma das hipóteses é de que ela estabiliza o colesterol e o efluxo ocorre via transporte vesicular (Rosenbaum & Maxfield, 2010). Outra hipótese é que a β-ciclodextrina esteja associada à proteína LAMP1, uma outra proteína relacionada com o tráfego do colesterol para o endossomo tardio bem como para o lisossomo, especialmente quando NPC1 está ausente ou limitada (Singhal *et al.*, 2018)

Atualmente, os pacientes são assistidos com tratamentos paliativos, com o objetivo de amenizar os sintomas. Eles seguem acompanhados por fisioterapeutas, fonoaudiólogos e nutricionistas, além da administração de algumas drogas que amenizam a progressão da doença, mas a recuperação total do fenótipo ainda não é possível.

1.3 Genes modificadores

Os genes *NPC1* e *NPC2* estão associados à doença NP-C. Porém, ainda existem muitas lacunas que necessitam ser preenchidas, pois o fenótipo dos pacientes pode ser variável ainda que na presença da mesma alteração gênica. Genes modificadores, são aqueles capazes de modular a gravidade da doença, juntamente com fatores ambientais e o gene responsável pela doença, podendo estar relacionados às diferenças de fenótipos observados nessa patologia.

A busca para encontrar uma possível associação entre genes, pode ser visto como encontrar uma agulha no palheiro, mas a seleção daqueles nos quais seus produtos estão relacionados à proteína NPC1 pode ser uma boa ferramenta para elencar os possíveis candidatos a modificadores. Como alternativa metodológica, a análise de polimorfismo de nucleotídeo único (*single nucleotide polymorphisms* – SNP) torna-se uma técnica factível e que pode auxiliar na tentativa de achar as respostas.

1.4 Homeostase proteica

A procura de novos candidatos que possam estar envolvidos na fisiopatologia de NP-C é constante. Atualmente, estudos visando o controle de qualidade tem se mostrado promissores. O controle de qualidade garante a homeostase proteica necessária para o correto funcionamento celular, as

proteínas sintetizadas devem ser enoveladas ao seu estado nativo para que consigam exercer suas funções. Porém, condições ambientais e estresse fisiológico podem levar a interações não-nativas que guiam para o enovelamento incorreto bem como para a agregação.

A proteína NPC1 é sintetizada no reticulo endoplasmático (RE), transportada para o complexo de Golgi onde é glicosilada para posterior envio ao lisossomo/endossomo tardio (Ioannou, 2001). Em um trabalho recente, os resultados obtidos indicaram como sendo 9 horas o tempo de meia vida da proteína selvagem, enquanto a meia vida da proteína mutada (NPC1 p.I1061T) foi de 6,5 horas. Neste trabalho, a degradação NPC1 p.I1061T foi observada em duas vias distintas, a proteossomal e a de degradação associada ao RE, mas ambas com a função de alcançar a homeostasia proteica (Schultz *et al.*,2018).

A autofagia seletiva do RE (*selective ER-phagy*) está associada ao RE e se refere a uma rota autofágica descrita recentemente, a qual contempla alvos específicos e apresenta a missão de manter a forma e a função deste compartimento celular (Dikic, 2018). No trabalho de Schultz e colaboradores (2018), foi evidenciada a associação da proteína NPC1 mutada (p.I1061T) como um substrato endógeno pelo receptor FAM134B, o qual é um dos quatro receptores específicos identificados até o momento como integrantes da autofagia seletiva do RE (Schultz *et al.*,2018). Essa ligação entre a proteína NPC1 e o receptor FAM134B, sugere que, possivelmente, a autofagia seletiva do RE contempla alvos específicos (Dikic, 2018).

Outro trabalho utilizou modelos celulares de NP-C para testar a administração de indutores de autofagia. Como resultado, foi observado a

diminuição no armazenamento do colesterol, indicando o possível comprometimento desta via (Sarkar *et al.*, 2013).

Ainda para o controle proteico, há outras rotas autofágicas que envolvem a participação de várias proteínas, entre elas as chaperonas (Smith & Cheetham, 2015). As chaperonas são moléculas que interagem com outras proteínas participando da sua estabilização e ganho de função sem estarem presentes no produto final (Ellis, 1987).

O acúmulo de proteínas mal enoveladas é citotóxico e pode implicar em patologias. Entre elas, podemos citar doenças neurodegenerativas e também doenças lisossômicas de depósito (Osellame & Duchen, 2013; Smith & Cheetham, 2015). Além da função de enovelamento correto, as chaperonas participam do processo de degradação proteica através do envio das proteínas para o proteossomo e para a via autofágica (Kim *et al.*, 2013).

As chaperonas Hsp70 (*heat shock protein 70*), Hsp90 (*heat shock protein 90*) e a cochaperona CHIP (*Carboxy-terminus of Hsc70-interaction protein*) aparecem no controle de qualidade da proteína NPC1 selvagem e também na mutante (p.Ile1061Thr) estando a Hsc70 (*heat shock cognate 70*) envolvida na degradação via proteossoma, enquanto Hsp70 estaria envolvida no enovelamento e na estabilização (Nakasone *et al.*, 2014).

Além da Hsp70 (família HSPA) e Hsp90 (família HSPC), as chaperonas Hsp40 (*heat shock protein 40*) (DNJAB6) e a cochaperona CHIP vêm sendo associadas a doenças neurodegererativas.

1.4.1 Chaperona DNAJB6

As moléculas J, também chamadas de Hsp40, estão envolvidas na regulação da atividade da Hsp70 através da indução da hidrólise de ATP,

estimulando a atividade ATPase, dessa forma estabilizando a interação desta com seu substrato e garantindo a especificidade do mesmo (Kampinga & Craig, 2010; Mittra *et al.*, 2009).

Até o momento, 49 diferentes DNAJ são conhecidas, sendo divididas em três classes de acordo com os seus domínios. Essa diversidade de domínios promove a interação específica entre a Hsp70 e sua proteína alvo (Figura 3). A DNAJB6 é uma integrante da classe II das Hsp40, a qual está envolvida no bloqueio da agregação beta-amiloide (Aβ), agregados de poliglutaminas e toxicidade celular (Smith & Cheetham, 2015).

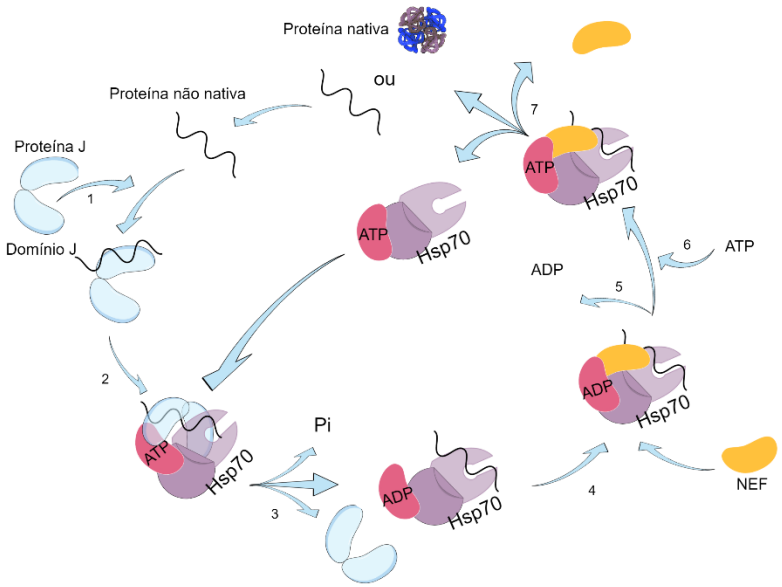

Figura 3: Modelo de associação da maquinaria de Hsp70 no enovelamento de proteínas. 1) Proteína J (Hsp40) liga-se a proteína cliente; 2) Interação Hsp70 via domínio J; 3) Hidrólise de ATP e liberação do domínio J; 4) Ligação dos NEF (*Nucleotide Exchange Factor*); 5) Associação de ADP ao complexo; 6) Ligação de um ATP; 7) Proteína "cliente" é liberada devido a baixa afinidade pela Hsp70 (ATP) (Fonte: Elaborada pelo autor).

1.4.2 Cochaperona CHIP

A CHIP pertencente à família das E3 ligases, sendo uma cochaperona (Ballinger *et al.*, 1999) que se liga ao domínio C-terminal das Hsp/c70 e Hsp90,

através do seu domínio N-terminal (Matsumura *et al.*, 2013). Ela está envolvida na ubiquitinação das chaperonas para o envio das mesmas para a via ubiquitina-proteossomo (Narayan *et al.*, 2015).

Em modelos animais e celulares da doença de Machado-Joseph, a expressão baixa desta cochaperona foi associada ao aumento da gravidade da doença (Miller *et al.*, 2005; Williams *et al.*, 2009). A mesma também foi identificada em imunoprecipitado juntamente com a proteína NPC1 mutada, indicando a participação no processo de ubiquitinação da proteína mutada para posterior envio ao proteossomo (Nakasone *et al.*, 2014). Após o enovelamento, a proteína pode ser enviada para 3 destinos distintos: para o lisossomo/endossomo tardio, para a via proteossomal ou para a via autofágica. Essas duas últimas vias não são utilizadas somente para proteínas mutadas, mas também para proteínas selvagens quando mal enoveladas, o que acontece com relativa frequência (figura 4).

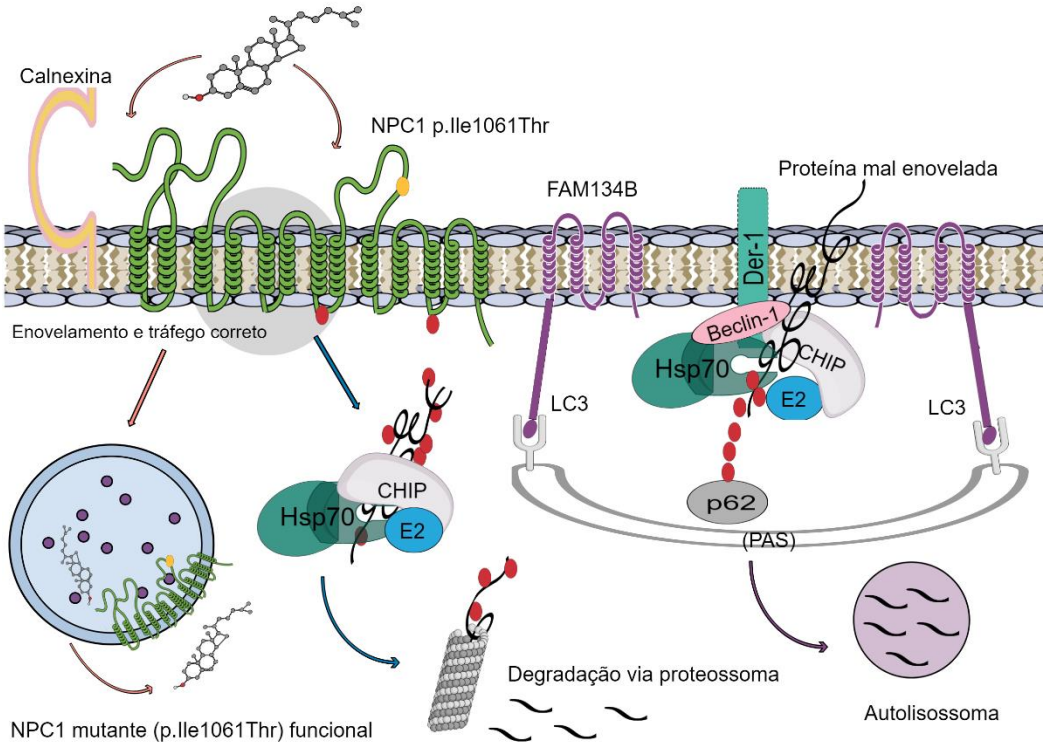

Figura 4: Via de degradação de NPC1 selvagem e mutada (p.Ile1061Thr). NPC1 é uma proteína transmembrânica cujo enovelamento é facilitado pela maquinaria calnexina/calreticulina. A marcação cinza indica a região SSD, importante para a ligação do colesterol. As proteínas, selvagem ou mutada, podem ser degradadas no proteossomo se não tiverem um enovelamento correto. Não há uma definição de todos os componentes que participam da via de degradação ERAD (*endoplasmatic-reticulum associated-protein degradation*), mas a maquinaria contendo Hsp70 e a CHIP já vêm sendo descritas neste processo. A ubiquitinação da NPC1 ocorre em três resíduos de lisina (318, 792 e 1180), voltados para o citoplasma. O outro processo que também está envolvido na degradação das proteínas mal enoveladas é a autofagia, onde as proteínas são engolfadas pelo pré-autofagossomo (PAS), o qual subsequentemente formará o autofagossomo que se fusionará com o lisossomo, formando o autolisossomo onde ocorrerá a degradação do substrato. Ainda não se conhece completamente a via de degradação de NPC1 (Fonte: Elaborada pelo autor).

A utilização de chaperonas como ferramenta para o desenvolvimento de fármacos para casos de NP-C vêm se mostrando promissora. Além das chaperonas biológicas, as chaperonas químicas também têm funcionado para o correto enovelamento das proteínas mutadas. Há trabalhos indicando que na superexpressão da proteína mutada de NPC1 e o uso de chaperonas químicas

é possível recuperar o fenótipo e, a explicação desse efeito é que o excesso de proteína sobrecarrega a via de degradação. Dessa forma, proteínas mutadas escapam do controle de qualidade e, com o auxílio de chaperonas químicas, conseguem ser enoveladas corretamente e tornarem-se funcionais (Gelsthorpe *et al.*, 2008).

1.5 Apolipoproteína E

A apolipoproteína E (ApoE), não faz parte das rotas autofágica e proteossomal discutidas até o momento. Porém, ela tem sido apontada como um possível modificador, uma vez que se encontra diretamente relacionada ao metabolismo do colesterol. Trata-se de uma proteína de transporte do colesterol e de outros lipídios no plasma e no sistema nervoso central, ligando-se aos receptores ApoE de superfície celular (Mahley & Rall, 1988; Zhong *et al.*, 2016). A proteína contém 299 aminoácidos, a região N-terminal (resíduos 136-150) interage com os receptores de ApoE e a ligação dos lipídios ocorre na região C-terminal (resíduos 244-272) (Hatters *et al.*, 2006; Bu, 2009).

Em humanos, o gene *ApoE* apresenta dois polimorfismos (rs429358 e rs7412) que resultam em três alelos (ε2, ε3 e ε4), os quais possibilitam a ocorrência de seis diferentes genótipos (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4). O alelo ε3 é o mais frequente (77,9%) e o alelo ε2, o mais raro (8,4%) (Mahley, 1988; Bu, 2009; Zhong *et al.*, 2016). A presença do alelo ε4 ocorre em aproximadamente 15% da população em geral, mas em pacientes com a doença de Alzheimer (DA) pode chegar a 40% (Bu, 2009).

As três isoformas, ApoE2, ApoE3 e ApoE4, se diferenciam apenas nas posições 112 e 158 de sua proteína, onde a cisteína (Cys) e arginina (Arg) estão presentes: ε2 (112 Cys, 158 Cys), ε3 (112 Cys, 158 Arg) e ε4 (112 Arg, 158 Arg).

A diferença de um aminoácido entre as isoformas interfere na estrutura e influencia a associação de lipídios e a ligação com receptores (Hatters *et al.*, 2006).

A ApoE2 é menos eficiente no transporte de lipídios, estando associada a hiperlipoproteinemia do tipo III e, em algumas populações, também tem demonstrado efeito protetivo contra DA (Bu, 2009; Tyrrel *et al.*, 1998). A ApoE4 liga-se a lipoproteínas de maior tamanho, estando associada ao risco aumentado de doenças cardiovasculares e a DA (Bu, 2009). Já a ApoE3 aparece como tendo um efeito “neutro” (Fu *et al.*, 2012).

Em uma coorte de 15 pacientes com NPC1, o alelo ApoE ε4 foi associado ao início precoce da doença, enquanto o alelo ApoE ε2 ao início tardio (Fu *et al.*, 2012). A presença do alelo ε4 tem sido associado como um fator de risco para a DA, e parece estar associada a outras doenças. No primeiro trabalho, onde os autores buscaram uma associação deste alelo com a gravidade da doença de NP-C, os resultados apontaram como um possível modificador, mesmo com um número reduzido de pacientes (n=15).

Para a presente tese foram escolhidos três genes candidatos a modificadores, DNAJB6, CHIP e ApoE, por apresentarem dados na literatura que indicam possível relevâncias destes na fisiopatologia da doença de NP-C.

2 OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste trabalho foi identificar o genótipo de pacientes com doença de Niemman-Pick tipo C (NP-C), determinando a frequência de mutações comuns e caracterizando mutações novas nos genes associados à doença, e analisar variantes de sequência em genes candidatos a modificadores de fenótipo dessa doença.

2.2 Objetivos Específicos

- Descrever o espectro de mutações em uma coorte de pacientes com NP-C através de ampla análise genética;
- Determinar a frequência de mutações comuns em pacientes brasileiros com NP-C;
- Aplicar um protocolo mais abrangente para investigar a possível ocorrência de rearranjos gênicos nos genes *NPC1* e *NPC2* em indivíduos com suspeita clínica de NP-C;
- Investigar polimorfismos de nucleotídeo único em genes que codificam chaperonas e cochaperonas como potenciais modificadores de fenótipo em pacientes com NP-C;
- Determinar as diferentes isoformas da apolipoproteína E na coorte de pacientes com NP-C e verificar seu potencial efeito sobre o fenótipo dos pacientes.

PARTE II

CAPÍTULO I

Manuscrito I - “Niemann-Pick disease type C: mutation spectrum and novel sequence variations in the human NPC1 gene”

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Niemann-Pick Disease Type C: Mutation Spectrum and Novel Sequence Variations in the Human *NPC1* Gene

Márcia Polese-Bonatto[1,2] · Hugo Bock[1,2] · Ana Carolina S. Farias[1] · Rafaella Mergener[1] · Maria Cristina Matte[1] · Mirela S. Gil[1] · Felipe Nepomuceno[3] · Fernanda T. S. Souza[4,5] · Rejane Gus[4] · Roberto Giugliani[1,4,5,6] · Maria Luiza Saraiva-Pereira[1,2,4,6,7]

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Abstract

Niemann-Pick type C (NP-C) is a rare autosomal recessive disorder characterized by storage of unesterified glycolipids and cholesterol in lysosome and/or late endosome due to mutations in either *NPC1* or *NPC2* gene. This study aims to identify the spectrum of sequence alterations associated to NP-C in individuals with clinical suspicion of this disease. The entire coding region and flanking sequences of both genes associated to NP-C were evaluated in a total of 265 individuals that were referred to our laboratory. Clinical and/or biochemical suspicion of NP-C was confirmed by molecular analysis in 54 subjects. In this cohort, 33 different sequence alterations were identified in *NPC1* and one in *NPC2*. Among those, 5 novel alterations in *NPC1* gene were identified as follows: one deletion (p.Lys38_Tyr40del), one frameshift (p.Asn195Lysfs*2), and three missense mutations (p.Cys238Arg, p.Ser365Pro and, p.Val694Met) that are likely to be pathogenic through different approaches, including in silico tools as well as multiple sequence alignment throughout different species. We have also reported main clinical symptoms of patients with novel alterations and distribution of frequent symptoms in the cohort. Findings reported here contribute to the knowledge of mutation spectrum of NP-C, defining frequent mutations as well as novel sequence alterations associated to the disease.

Keywords Niemann-Pick type C disease · *NPC1* gene · *NPC2* gene · Mutation spectrum · Novel variation

Introduction

Niemann-Pick type C disease (NP-C disease; OMIM #257220) is a rare autosomal recessive neurodegenerative disorder characterized by storage of unesterified glycolipids and cholesterol in lysosome and/or late endosome (LE/L) due to mutations in either *NPC1* or *NPC2* genes [1]. This disorder causes premature death, and subjects from different ethnic groups can be affected [2–4]. NP-C prevalence is approximately 1/100,000 live births, but incidence can vary among different countries [4]. Hepatosplenomegaly, vertical supranuclear ophthalmoplegia, progressive ataxia, dystonia, and dementia are among symptoms characterized as the "classic" phenotype [5–7]. Mutations in genes coding for the large transmembrane endosomal NPC1 and a small soluble lysosomal NPC2 proteins result in intracellular sterol

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✉ Maria Luiza Saraiva-Pereira
mlpereira@hcpa.edu.br

[1] Laboratório de Identificação Genética, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

[2] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[3] Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[4] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

[5] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[6] INAGEMP—Instituto Nacional de Genética Médica Populacional, Porto Alegre, RS, Brazil

[7] Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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cycling alterations [8]. Great majority of NP-C cases is due to mutations in *NPC1* gene (95%) whereas the remaining are caused by mutations in *NPC2* gene [4, 9]. The human *NPC1* gene is located at *locus* 18q11, spans more than 47 kb, and is organized into 25 exons. The transcript is 4.9 kb long encoding a protein with 1278 amino acids [10]. NPC1 protein has 13 transmembrane domains, 3 large and 4 small luminal loops, 6 small cytoplasmic loops, and a cytoplasmic tail [11]. High homology was observed among NPC1 protein and other NP-C orthologs, such as mouse, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans* [12]. High sequence homology between NPC1 and other proteins that are involved in cholesterol metabolism was also observed [13, 14]. The human *NPC2* gene is located at *locus* 14q24.3, spans more than 13 kb, and is organized into 5 exons. The transcript of 0.9 kb produces a small soluble glycoprotein that contains 131 amino acid residues [5, 15]. *NPC1* and *NPC*2 genes have many mammalian orthologs with highly conserved primary sequences [16].

Diagnosis of NP-C requires biochemical evaluation, such as Filipin staining test in fibroblasts or plasma oxysterols evaluation, and/or molecular analysis of *NPC1* and *NPC2* genes [5, 9]. To date, more than 460 different sequence alterations have been reported to be associated to NP-C.

This study describes the mutation spectrum of a broad genetic analysis in a cohort of patients with NP-C, including five novel sequence variants and rare mutations.

Material and Methods

Patients

In this study, we have included biological samples from 265 individuals that were sent to our laboratory from different regions of Brazil, from 2011 to 2017, through the NPC Network. Inclusion criteria were positive or inconclusive result in the Filipin staining test or a strong clinical suspicion of NP-C, regardless the biochemical evaluation outcome. This study was approved by our local Institutional Review Board (project number 05168).

DNA Isolation and Amplification of *NPC1* and *NPC2* Genes

Genomic DNA was isolated from peripheral white blood cells using standard protocols and stored at − 20 °C. Polymerase chain reaction (PCR) was used to selectively amplify specific fragments of *NPC1* (NG_012795.1) and *NPC2* (NG_007117.1) genes. Primer sequences can be found in Supplementary Table S1. Coding sequences and flanking regions (exons 1 to 25 of *NPC1* gene and exons 1 to 5 of *NPC2* gene) were amplified by PCR using genomic DNA as template. The whole coding region of *NPC1* was divided into 24 different amplicons (exons 15 and 16 were amplified as one fragment). *NPC2* coding region was divided into 5 different amplicons. Amplification reaction was performed in final volumes of 25 μL containing 25 ng genomic DNA, 200 mM of each dNTP, 2.5 μM of each primer (forward and reverse), 2.5 mM of $MgCl_2$, 200 mM of Tris-HCl (pH 8.4), 50 mM of KCl, and 1.25 U of *Taq* DNA Polymerase (Invitrogen™, Carlsbad, CA, USA). Cycling conditions were initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Each PCR product was verified by electrophoresis on a 1.5% (*w*/*v*) agarose gel and visualization under UV light.

DNA Sequencing

Amplicons were purified using 2.5 U of Exonuclease I (USB, Cleveland, OH, USA) and 0.25 U of Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA). DNA sequencing was performed using BigDye® Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) from universal M13 (− 20) forward and reverse primers, following the manufacturer's instructions. Sequences were analyzed with DNA Sequencing Analysis software v. 5.2 (Applied Biosystems) in an ABI PRISM® 3130*xl* Genetic Analyzer. All identified sequence variations were confirmed by sequencing an independent sample from both forward and reverse primers. Sequence variations were compared to data available in the NP-C database in the Human Gene Mutation Database (HGMD® - http://www.hgmd.cf.ac.uk), the Exome Aggregation Consortium (ExAC) browser (http://exac.broadinstitute.org/), the Genome Aggregation Database (gnomAD) browser (http://gnomad.broadinstitute.org/), and 1000genomes (http://www.internationalgenome.org/home).

Evaluation of Novel Mutations

Amino acid sequences of *NPC1* from 10 different species were compared by multiple alignment in order to determine whether changes identified in their amino acid sequences were associated to conserved residues. Sequences were searched for using the protein database from the National Center for Biotechnology Information (NCBI - https://www.ncbi.nlm.nih.gov/). Amino acid sequences were aligned with Clustal Omega using FASTA format (https://www.ebi.ac.uk/Tools/msa/clustalo/). In order to assess their potential pathogenicity, novel sequence variations in the *NPC1* coding region were analyzed using eight web-based tools. Those tools were PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) [17], SNPs3D (http://www.snps3d.org/) [18], Align GVGD (http://agvgd.iarc.fr/) [19], Mutation Taster (http://www.mutationtaster.org/) [20], Mendelian Clinically Applicable Pathogenicity (M-CAP) Score (http://bejerano.

Table 1 Alleles defined by this study. Novel sequence variants are shown in italics

Mutation	cDNA nucleotide substitution	Exon	# of alleles	Allelic frequence
NPC1 gene				
p.Lys38_40Tyrdel	*c.114-122del9*	*2*	*1*	*0.011*
p.Gln117*	c.349C > T	4	1	0.011
p.Cys177Tyr	c.530G > A	5	2	0.023
p.Ala183Thr + *p.Ser365Pro*	c.547G > A + *c.1093 T> C*	5 and 8	1	0.011
p.Ser151Phefs*70	c.451_452delAG	4	1	0.011
*p.Asn195Lysfs*2*	*c.584dupA*	*5*	*1*	*0.011*
p.Cys238Arg	*c.712 T> C*	*6*	*1*	*0.011*
p.Cys247Tyr	c.740G > A	6	1	0.011
p.Arg372Trp	c.1114C > T	8	1	0.011
p.Arg615His	c.1844G > A	12	1	0.011
p.Val664Met	c.1990G > A	13	2	0.023
p.Ser667Leu	c.2000C > T	13	1	0.011
p.Val694Met	*c.2080G> A*	*13*	*1*	*0.011*
p.Gly710Alafs*19	c.2129delA	13	2	0.023
p.Pro733Serfs*10	c.2196_2197insT	14	1	0.011
p.Ala764Val	c.2291C > T	15	1	0.011
p.Ser865Leu	c.2594C > T	17	1	0.011
p.Ala926Thr	c.2776G > A	18	1	0.011
p.Trp942Cys	c.2826G > T	19	1	0.011
p.Asp945Asn	c.2833G > A	19	1	0.011
p.Cys957Tyr	c.2870G > A	19	1	0.011
p.Gly992Arg	c.2974G > C	20	1	0.011
p.Pro1007Ala	c.3019C > G	20	15	0.170
p.Ala1035Val	c.3104C > T	21	24	0.273
p.Ile1061Thr	c.3182 T > C	21	4	0.045
p.Gly1140Val	c.3419G > T	22	1	0.011
p.Asn1156Ile	c.3467A > T	22	1	0.011
p.Leu1157Pro	c.3470 T > C	22	1	0.011
p.Val1165Met	c.3493G > A	23	1	0.011
p.Glu1166Lys	c.3496G > A	23	1	0.011
p.Arg1186His	c.3557G > A	23	2	0.023
p.Phe1221Serfs*20	c.3662delT	24	13	0.148
Total			88	
NPC2 gene				
p.Glu20*	c.58G > T	2	2	1.000
Total			2	

stanford.edu/mcap/) [21], Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/snv) [22], Rare Exome Variant Ensemble level (REVEL) (https://sites.google.com/site/revelgenomics/) [23], and Variant Effect Scoring Tool (VEST3) (http://hg19.cravat.us/CRAVAT/) [24]. Mutalyzer 2.0 was used as a reference for naming novel sequence variations (https://mutalyzer.nl/) [25]. Model structure of *NPC1* was generated by PyMOL 2.0 software (https://pymol.org/2/), and mutant models by Modeler 9.1 software [26], using PDB ID code 3JD8.

Results

Genotype of NP-C patients was defined through identification of novel and/or rare sequence alterations in *NPC1* or *NPC2* genes. Mutant alleles were confirmed in 54 out of 265 individuals, being 29 (53.7%) females and 25 (46.3%) males. Within NP-C confirmed patients, 18 (33.3%) were from consanguineous marriage. Among NP-C cases identified in this work, 52 (96.3%) patients have mutations in *NPC1* gene while the remaining 2 (3.7%) patients carry mutations in *NPC2*. In

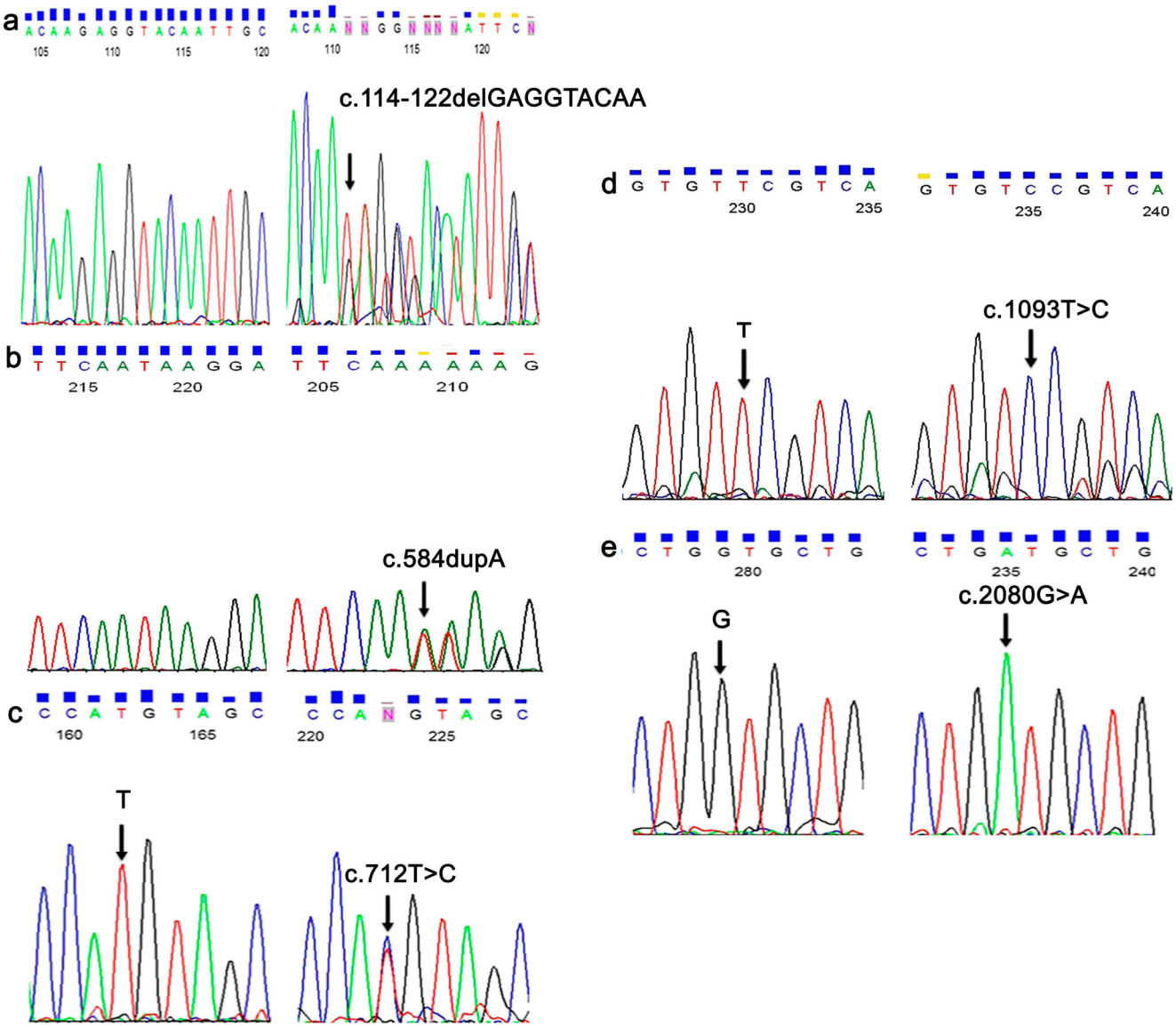

Fig. 1 DNA sequencing of novel sequence variations identified in *NPC1* gene. (a) Direct sequencing of exon 2 from the forward primer. Arrow indicates the beggiging of the deletion in the p.Lys38_Tyr40del (c.114_122delGAGGTACAA) variation. Patient is heterozygous for this variation; therefore, after sequence variation, two different profiles can be seen in the figure: one from wild-type allele and the other from mutant allele. (b) Direct sequencing of exon 5 from the forward primer. The arrow shows nucleotide duplication that characterizes the p.Asn195Lysfs*2 (c.584dupA) variation. Patient is heterozygous for this variation; therefore, after sequence variation, two different profiles can be seen in the figure: one from wild-type allele and the other from mutant allele. (c) Direct sequencing of exon 6 from the forward primer. The arrow indicates T to C substitution in the p.Cys238Arg (c.712 T > C) variation. Patient is heterozygous for this variation. (d) Direct sequencing of part of exon 8 from the forward primer. The arrow indicates T to C substitution in the p.Ser365Pro (c.1093 T > C) variation. Patient is homozygous for this variation. (e) Direct sequencing of part of exon 13 from the forward primer. The arrow indicates G to A substitution in the p.Val694Met (c.2080G > A) variation. Patient is homozygous for this variation

total, 34 different sequence alterations were identified, including 5 novel variations in *NPC1*, and a detailed distribution of mutations is shown in Table 1. Frequency of variants was estimated using unrelated chromosomes only; i.e., we have just considered one allele from homozygous patients of consanguineous marriages, giving a total of 90 alleles. The most frequent mutation was p.Ala1035Val (27.0%), followed by p.Pro1007Ala (16.9%), and p.Phe1221Serfs*20 (14.6%).

All novel variants described here are located in *NPC1* gene, and distributed as follows: one small deletion (p.Lys38_Tyr40del), one frameshift (p.Asn195Lysfs*2), and 3 missense mutations (p.Cys238Arg, p.Ser365Pro, and p.Val694Met). Sequencing profile of novel mutations is in Fig. 1. These novel changes were not found among 400 alleles from normal individuals. All 5 patients carrying novel mutations were previously evaluated by Filipin staining test and results were positive. A brief clinical description of patients

with novel sequence variations can be found in Table 2. Variations cited in this work as novel were not found in the Human Gene Mutation Database (HGMD®), ExaC, gnomAD, and 1000genomes. Findings regarding these novel changes are described below.

The p.Lys38_Tyr40del (c.114-122del9) mutation is a deletion of nine nucleotides within exon 2 (Fig. 1(a)) that leads to a protein with three missing amino acids (Lys, Arg, and Tyr). This mutation was found in a compound heterozygote carrying p.Phe1221Serfs*20 in the other allele. This male patient was diagnosed when he was 1 year old and clinical symptoms include dysphagia, cognitive decline, and developmental delay (Table 2).

Variation p.Asn195Lysfs*2 is due to a duplication of one nucleotide (adenine) in exon 5 of *NPC1* gene (Fig. 1(b)). This frameshift was identified in a female patient *in trans* with p.Phe1221Serfs*20. She was diagnosed at 6 months of age, and main symptoms were hepatosplenomegaly, hypotonia, and developmental delay. This mutation produces a truncated protein that is expected to have a defective function.

Regarding missense mutations, p.Cys238Arg is located at exon 6, due to T to C change (Fig. 1(c)), which was found in a female patient diagnosed at 2 years of age. Her clinical symptoms included cerebellar ataxia, developmental delay, and cataplexy. The p.Cys238Arg was found *in trans* with p.F1221Sfs*20 (patient was a compound heterozygote), and variants were confirmed in her parents.

Novel variation p.Ser365Pro is due to T to C change in exon 8 of *NPC1* (Fig. 1(d)) and it was found *in cis* with p.Ala183Thr. This complex allele was found in a homozygous male patient from a consanguineous marriage, and both mutations were confirmed in his parents.

The other novel missense mutation, p.Val694Met, is due to G to A change in exon 13 (Fig. 1(e)), and this alteration was detected in a female patient from consanguineous marriage. She was 14 years old at diagnosis and clinical symptoms include cerebellar ataxia, and vertical supranuclear gaze palsy. Patient was homozygous for this alteration, and variant was also confirmed in her parents.

All novel sequence variations were evaluated through alignment of amino acid sequences from 10 different organisms, and alterations are located within conserved residues, suggesting an effect on protein function or structure (Fig. 2). Pathogenicity of novel missense variations was evaluated using different web-based tools (supplementary Table S2), following the guidelines by the American College of Medical Genetics and Genomics (ACMG) to the interpretation of sequence variants [27]. Two missense mutations (p.Cys238Arg and p.Val694Met) were defined as being pathogenic by all different tools. p.Ser365Pro was also classified as pathogenic by great majority of tools except by one (CADD software). However, considering that this serine residue is conserved among species (Fig. 2), and that this substitution introduces a novel imino group in the protein, a pathogenic effect might be also expected in this case.

Finally, distribution of age at diagnosis ranged from 2 months to 46 years with an average of 11.3 years, and 32 cases (59.3%) were diagnosed in patients of up to 10 years of age. Patients included in the analysis were from different regions of Brazil, and more detailed description of symptoms was available from 33 confirmed cases. Therefore, more frequent symptoms, based on cases with complete clinical description, were splenomegaly, hepatomegaly, cerebellar ataxia, and vertical supranuclear gaze palsy, and distribution of symptoms according to age group is shown in supplementary Fig. S1.

Discussion

We identified mutations in 54 NP-C patients, being 96.3% in *NPC1* gene and 3.7% in *NPC2* gene. Distribution of mutations shown here is similar to the described in the literature from studies worldwide, where mutations in *NPC1* gene occur in 95% of the NP-C patients [2, 12, 15, 28].

The most frequent mutation in our sample population was p.Ala1035Val (27.0%) that is different from cohorts reported in North hemisphere, where p.Ile1061Thr is described as the most frequent one [29–38]. As previously reported, high frequency of p.Ile1061Thr in Hispanic patients suggest a founder effect originated in Western Europe [32, 39]. A prevalence of a different mutation in this studied cohort suggests a different

Table 2 Brief clinical description of patients that carry novel sequence variations identified by this study

Mutation	Genotype	Age at diagnosis	Gender	Clinical symptoms
p.Lys38_Tyr40del	p.Lys38_Tyr40del/p.Phe1221Serfs*20	1 year	Male	Dysphagia, cognitive decline, developmental delay
p.Ser365Pro	[p.Ser365Pro + p.Ala183Thr]/ [p.Ser365Pro + p.Ala183Thr]	27 years	Male	Neurological regression
p.Asn195Lysfs*2	p.N195Kfs*2/p.F1221Sfs*20	6 months	Female	Hepatomegaly, splenomegaly, hypotonia, developmental delay
p.Cys238Arg	p.Cys238Arg/p.Phe1221Serfs*20	2 years	Female	Cerebellar ataxia, developmental delay, cataplexy
p.Val694Met	p.Val694Met/p.Val694Met	14 years	Female	Cerebellar ataxia, vertical supranuclear gaze palsy

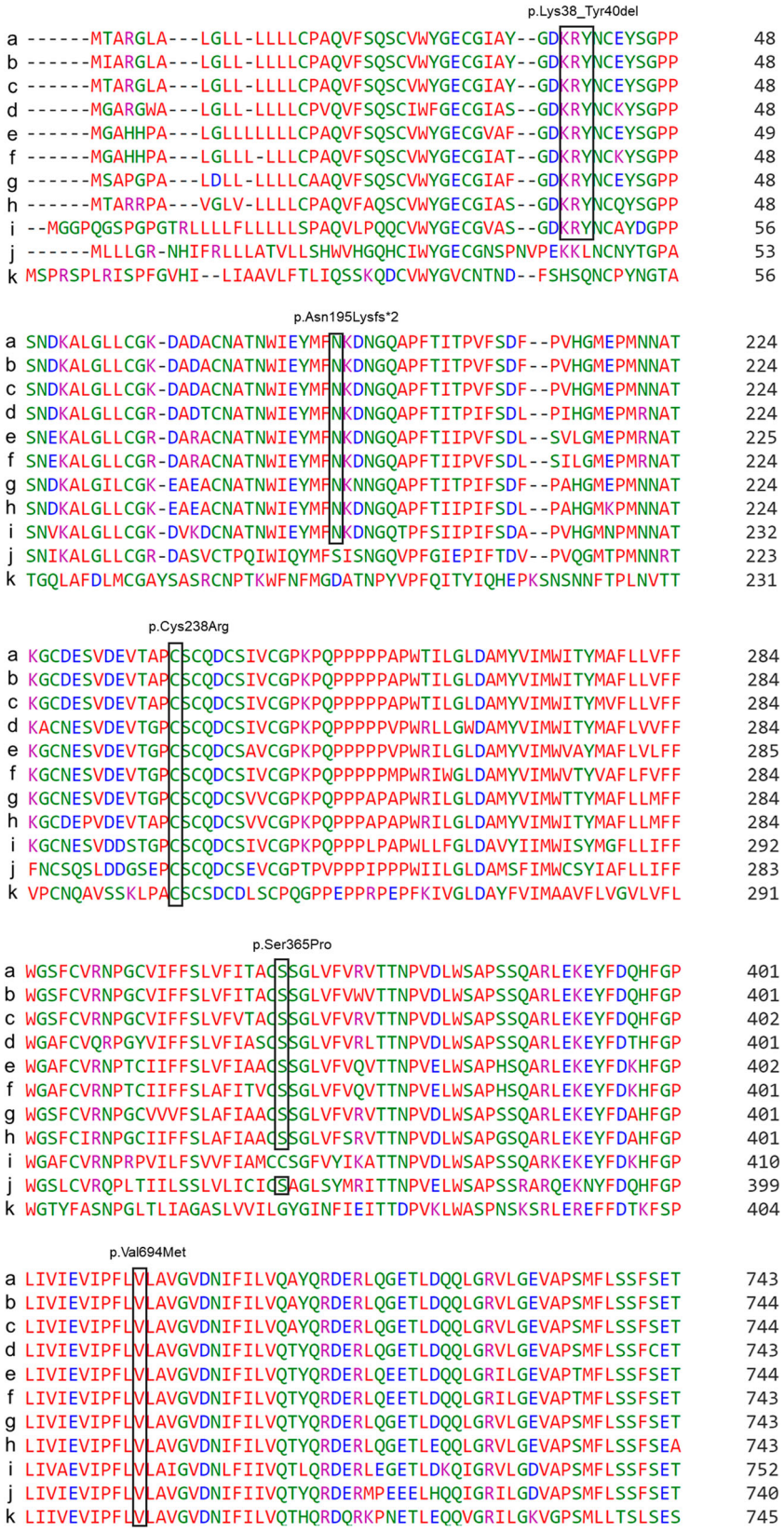

Fig. 2 Alignment of amino acid sequences of several organisms. Specific regions flanking mutations are represented. (a) Human (NP_000262.2); (b) Chimpanzee (XP_016788941.1); (c) Orangutan (XP_009250570.1); (d) Domestic guinea pig (XP_003474080.2); (e) Rat (NP_705888.2); (f) Mouse (NP_032746.2); (g) Horse (XP_005612821.1); (h) Dog (NP_001003107.1); (i) Chicken (XP_419162.3); (j) Zebrafish (NP_001230804.1); (k) Fruit fly (NP_001188769.1). Boxes indicate conserved residues associated to novel mutations. Designation of each mutation is given above the appropriate box

ethnic background of NP-C patients in Brazil. The second most common mutation in our study was p.Pro1007Ala (16.9%), and this alteration was also reported as being frequent in different European countries [5]. Frequency data of

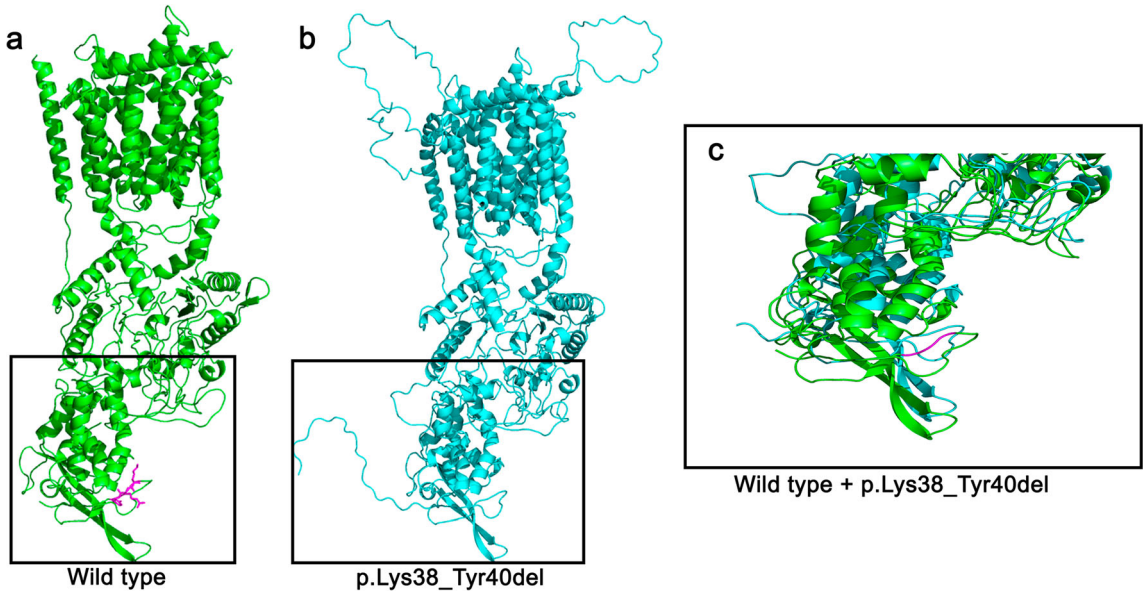

Fig. 3 Location of p.Lys38_40Tyr in the NPC1 protein. (a) Image represents the wild-type NPC1 protein; region of three amino acids (lysine, arginine, and tyrosine) involved in the deletion is represented in pink. (b) Image represents mutant NPC1 protein p.Lys38_40Tyr. (c) Close-up and superposed view of wild-type (in green) and mutant (in blue) NPC1 proteins. Figures were generated by PyMOL 2.0

this mutation reported in Portuguese, British, and German patients ranged from 15 to 20% [37].

We have observed higher frequency of some mutations in specific regions as follows: p.Ala1035Val was present in 32.0% of mutant alleles identified in São Paulo state, p.Pro1007Ala was found in 53.3% of mutant alleles from Paraná state, and p.Phe1221Serfs*20 was identified in 75.0% of alleles from Pernambuco state. Although preliminary, these data indicate that regional variation of ethnic background in a huge country as Brazil might determine higher frequencies of mutations in specific places. Several studies have reported specific disease-causing mutations among different populations and ethnic groups associated to NP-C [30, 31, 38–40]. Additional analyses are required to further investigate this issue.

Distribution of confirmed cases among geographical regions of Brazil was as follows: 46.3% of cases in Southeast, followed by 25.9% of cases from Northeast, 24.1% of cases from South, and 3.7% from West Central. This higher frequency of cases in Southeast might be a combination of highly populated region as well as a more facilitated access to health system.

Clinical presentation of NP-C can be heterogeneous and nonspecific, which makes more difficult to reach a correct diagnosis [9]. Symptoms of pediatric patients (≤4 years) described here are in agreement to a previous report that more discriminatory signs for NP-C in pediatric patients are splenomegaly, hepatomegaly, dysphagia, cognitive decline, delayed neuro-psychomotor, ataxia, and cataplexy [41]. Clinical findings reported here in adult patients, such as seizures, neurological regression, splenomegaly, cognitive decline, cerebellar ataxia, and vertical

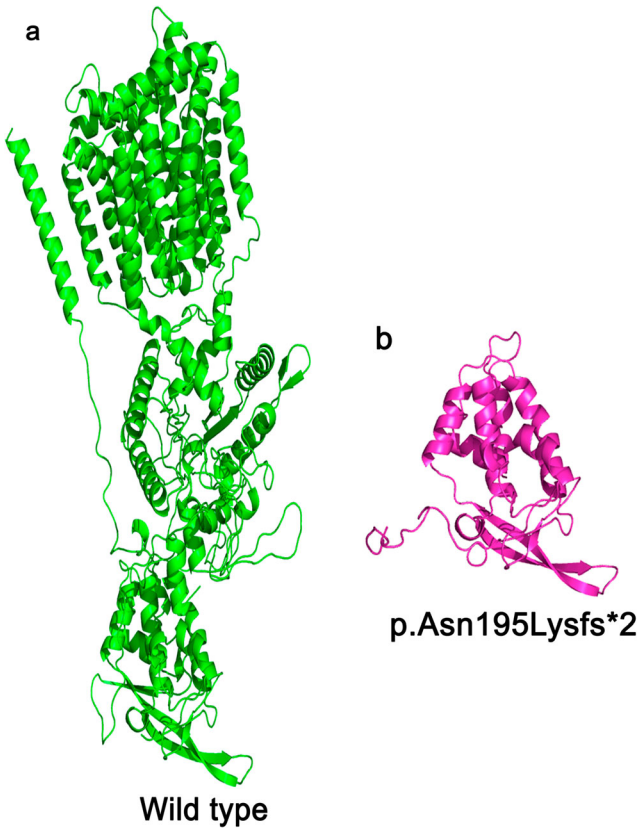

Fig. 4 Images representing wild-type NPC1 protein and NPC1 protein produced in the presence of p.Asn195Lysfs*2. (a) Wild-type NPC1 protein (1278 amino acids). (b) Mutant NPC1 protein produced by p.Asn195Lysfs*2 mutation (197 amino acids). Figures were generated by PyMOL 2.0

supranuclear gaze palsy, were described as more commonly found in older patients (supplementary Fig. S1).

Novel sequence variants appear to be widespread along different regions in the protein: p.Lys38_Tyr40del, p.Asn195Lysfs*2, and p.Cys238Arg are located within lumen A domain, p.Ser365Pro in the transmembrane II (TMII) domain, and p.Val694Met in the transmembrane V (TMV). Position of these variations can be visualized in NPC1 protein topology generated by Protter software [42] (supplementary Fig. S2).

Mutation p.Lys38_Tyr40del is an in-frame deletion that leads to a protein lacking three amino acids (Lys, Arg, and Tyr). This deletion is located within the N-terminal domain (NTD) (supplementary Fig. S2), which is the first luminal domain composed by 240 amino acids [43]. This type of mutation generates a mutant protein with a different tertiary structure (Fig. 3) that will likely affect protein function.

The frameshift variation p.Asn195Lysfs*2 produces a truncated small protein that is expected to have a defective function. The wild-type NPC1 protein has 1278 amino acids, and mutant protein produced by this variation would be expected to have 197 amino acids only (Fig. 4). Therefore, essential domains of NPC1 protein will be missing, and normal function highly impaired.

Missense mutation p.Cys238Arg is also located in a very relevant domain of the protein. The amino acid cysteine at position 238 establishes one of two disulfide bonds (C97–C238 and C227–C243) from Ψ loop. This particular loop has been reported before as being an important interface between N-terminal domain (NTD) and middle luminal domain (MLD) [44]. Therefore, the replacement of this cysteine residue by an arginine prevents the establishment of a disulfide bond, which changes protein conformation as can be observed in Fig. 5(b, c).

p.Ser365Pro variation introduces a novel imino group in the protein (Fig. 5(d, e)). This imino group will be part of a transmembrane domain (supplementary Fig. S2) and can cause a very relevant change in the protein structure by itself. In this particular case, as this mutation is found in a complex allele *in cis* with another mutation, an even stronger impact in protein function is expected due to a combination of effects.

The remaining missense mutation p.Val694Met is also located within a transmembrane region, which is important for NPC1 (Fig. 5(f) and Fig. 5(g)). This region shows high homology to the sterol-sensing domains (SSD) of HMG-Co A

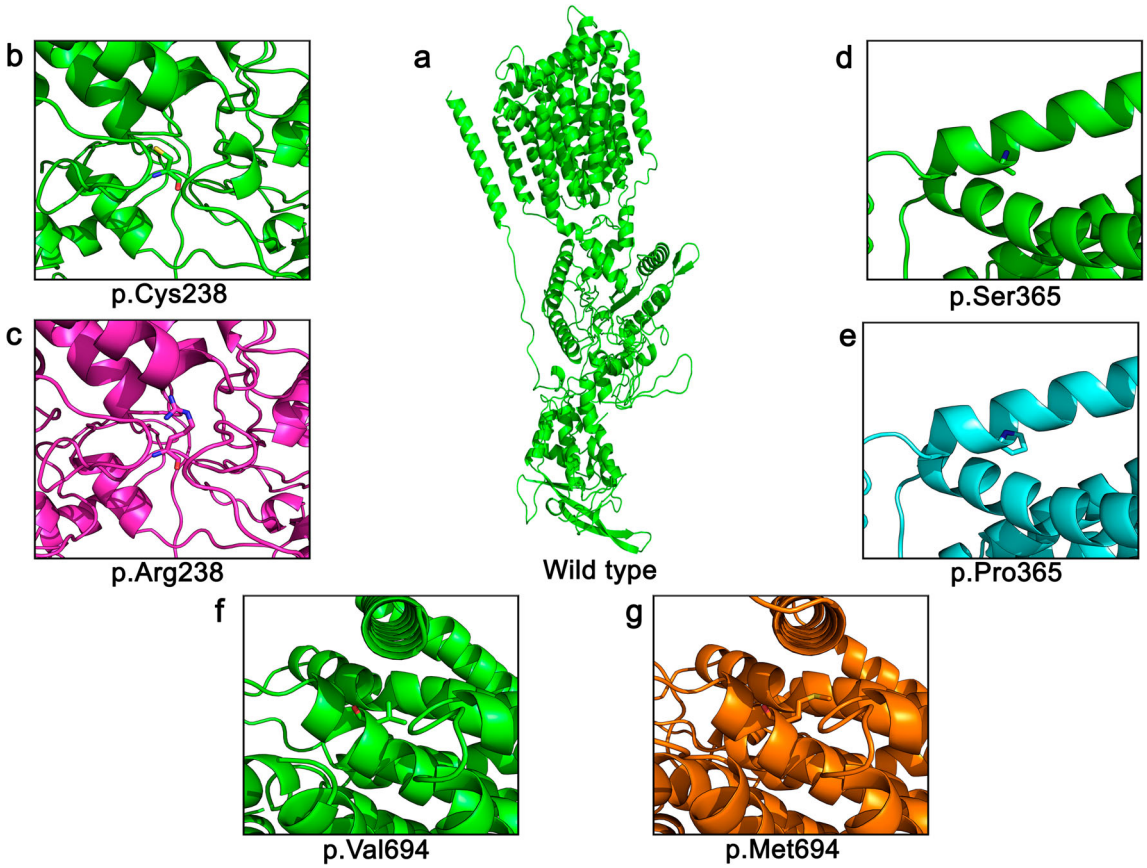

Fig. 5 Missense mutations in NPC1 when compared to the wild-type amino acido residue. Arrows indicate location of wild-type and mutant amino acid residue in each variant. (a) Wild-type NPC1 protein. (b) Wild-type residue Cys (cysteine) compared to (c) mutant residue Arg (arginine). (d) Wild-type residue Ser (serine) compared to (e) mutant residue Pro (proline). (f) Wild-type residue Val (valine) compared to (g) mutant residue Met (methionine). Figures were generated by PyMOL 2.0

reductase that is involved in cholesterol synthesis, and to the sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP), which is an activator of a transcription factor in cholesterol biosynthesis [11, 29, 30]. The insertion of cholesterol into the lysosomal membrane involves NPC1 transmembrane domains, including the sterol-sensing motif that has been identified in other proteins as involved in cholesterol homeostasis [45]. The majority of mutations in the SSD region is associated to a severe phenotype [30, 46].

All novel variants were located within conserved regions when multiple alignment analysis was performed with sequences of 10 different species (Fig. 2). These conserved regions imply a requirement of those amino acids for normal protein structure and/or activity throughout species. In addition, all novel variations were tested for hydrophobicity prediction and variations of hydrophobicity levels are expected in each one. This is further evidence related to protein topology susceptibility associated to novel sequences alterations reported here.

In summary, data provided here contribute to the knowledge of worldwide mutation spectrum associated to NP-C. Combination of molecular analyses with in silico tools, as well as molecular modeling, can generate a more comprehensive insight into NP-C associated proteins, with a potential to identify additional targets to the development of novel therapeutics for Niemann-Pick type C.

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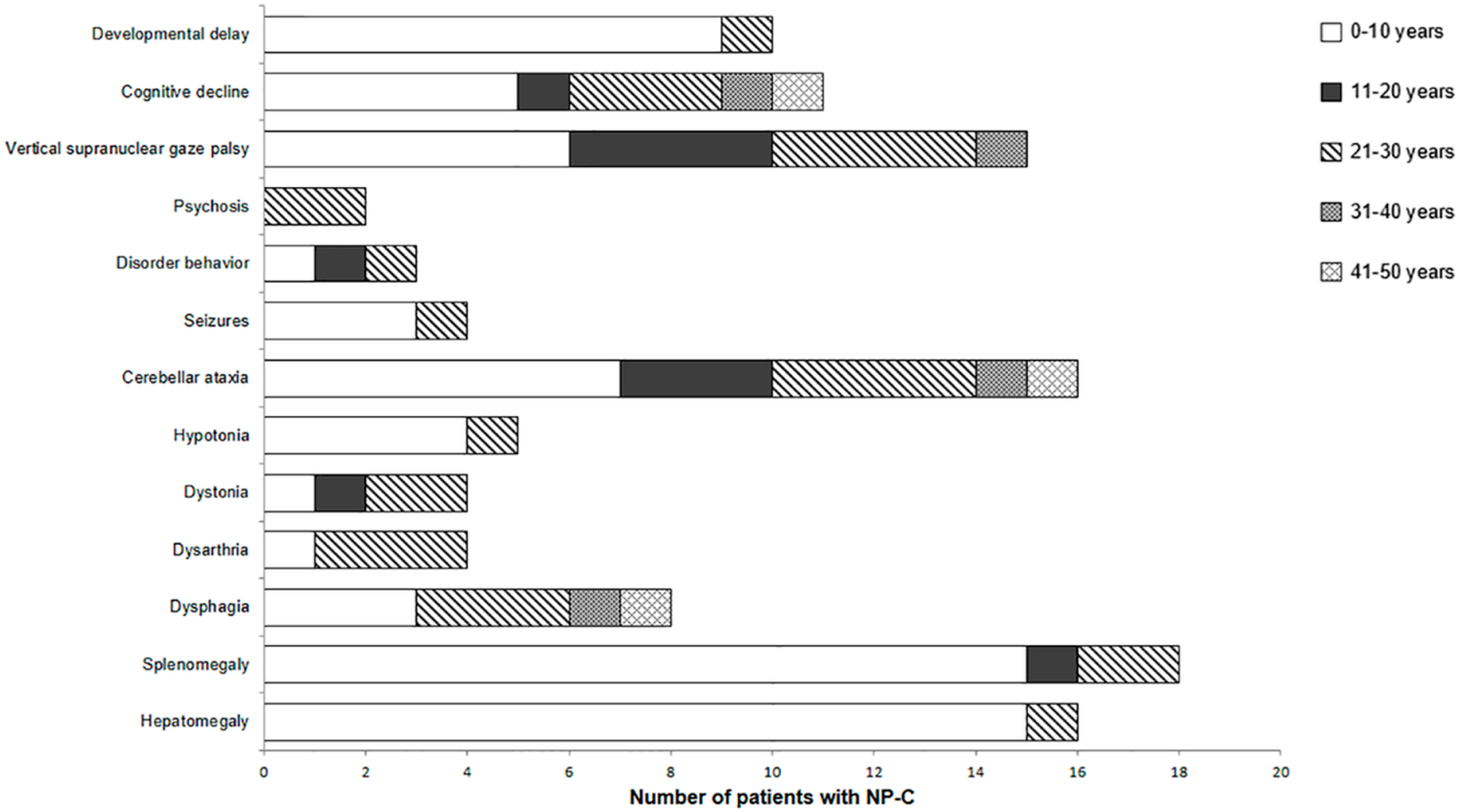
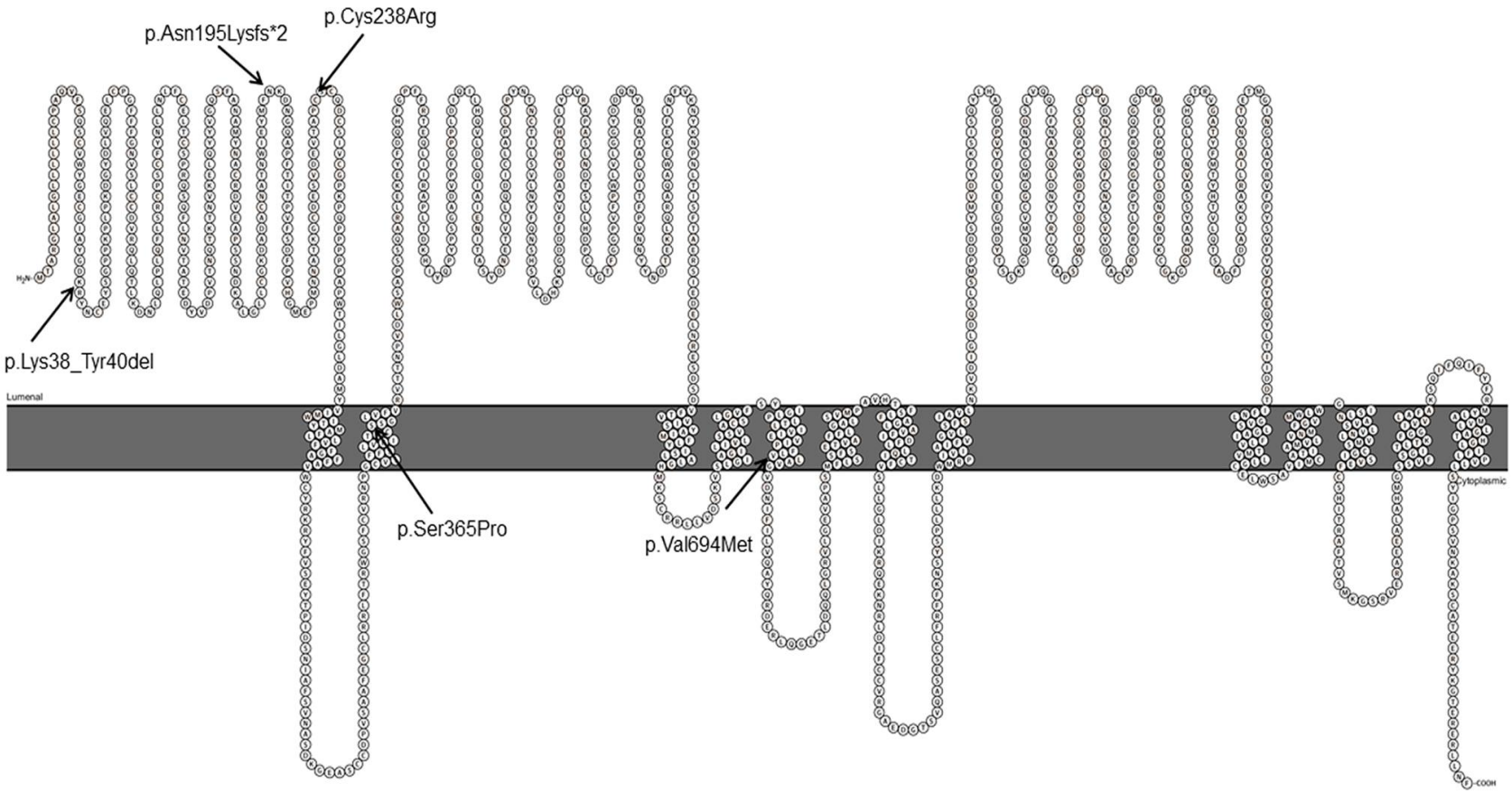
Supplementary figures



Supplementary Fig. S1 Distribution of symptoms according to age group of NP-C patients. Symptoms were available in 33 patients.



Supplementary Fig. S2 NPC1 protein topology and distribution the novel mutations. The 13 transmembrane protein domains are represented in the middle; cytosolic domains are shown at the bottom part, and luminal domains are shown at the upper part of the figure. Novel sequence alterations are indicated at their positions.

Table S1. Location and sequence of primers used to analyze *NPC1* and *NPC2* genes.

Exons and flanking regions	Primer sequence (5´ > 3´)		Length (bp)
	Forward	Reverse	
NPC1 gene			
1	GTAAAACGACGGCCAGTAGAAACCGTTGGCACAACTC	AACAGCTATGACCATGCAGACCAACTTCCCCAGGAC	471
2	GTAAAACGACGGCCAGTTTTGCAGGTAGCATTGGAAG	AACAGCTATGACCATGCACCTCCACCCTGCAATAAC	325
3	GTAAAACGACGGCCAGTTTGTCAGGAGAGGAGAGAAAGC	AACAGCTATGACCATGCAATCACTGGAGCCTAGGAAG	674
4	GTAAAACGACGGCCAGTTGATGCTCCAAAGTCCCTTTAC	AACAGCTATGACCATGTGGATGCAAACTTGCACATAC	753
5	GTAAAACGACGGCCAGTCATGGTGCATATGGAGTTCG	AACAGCTATGACCATGCTCAGTCTCCCCAAGCACTG	623
6	GTAAAACGACGGCCAGTTTTCAGTGGGCTTTTCTTTG	AACAGCTATGACCATGTGGAGGTATTTGTTTCTTGTCC	451
7	GTAAAACGACGGCCAGTGCCAGGAGGAGGAAGAAAG	AACAGCTATGACCATGCACACCACCTCACCCACTG	279
8	GTAAAACGACGGCCAGTTCCTGCCATGAGATAGCAAC	AACAGCTATGACCATGATACCATGACATTCAGCCCC	576
9	GTAAAACGACGGCCAGTTGACCCTCAGGGCAATG	AACAGCTATGACCATGTGTTGTTTGCTCACCTCTGG	419
10	GTAAAACGACGGCCAGTCAGCCTCATCAAATGTTCACTG	AACAGCTATGACCATGGGTAAGAAATTAACAAAACTGCCC	310
11	GTAAAACGACGGCCAGTGAGCCCAGAGATACAGTCCATAG	AACAGCTATGACCATGCGTAACTCAGATCTGCCATTG	329
12	GTAAAACGACGGCCAGTAAAACGTGGCCTTTGTATCG	AACAGCTATGACCATGTGAAGAAAATAGATGTAGGCAACAG	451
13	GTAAAACGACGGCCAGTTTCAACTCTAGGTTTAATACAGCCC	AACAGCTATGACCATGCCTCACAGGTCACACTCACG	391
14	GTAAAACGACGGCCAGTGCTTAGAAGACACTGCTAATCGTC	AACAGCTATGACCATGAAAGGAAGCAACACAAAGGG	667
15 and 16	GTAAAACGACGGCCAGTCTGTGCTGGCTCCTTGTATC	AACAGCTATGACCATGAATCTCCTTCCCAGGCTGTC	598

17	GTAAAACGACGGCCAGTCCCTGTACTCCCTATTAGCCTG	AACAGCTATGACCATGGGAAACCCTGTCACCATTTG	356
18	GTAAAACGACGGCCAGTCTCCTGGCACCCTCTTATTC	AACAGCTATGACCATGAAAGCCAGATTTAACATACATTTTG	417
19	GTAAAACGACGGCCAGTCTTTGAAAGGACTAATTTACCACC	AACAGCTATGACCATGATGATGACACAGGGAGACCC	430
20	GTAAAACGACGGCCAGTAAGAAAGTAATGCCCCTCACTG	AACAGCTATGACCATGGAGAAGAGACGTTCCCATGC	332
21	GTAAAACGACGGCCAGTTGGGTCTGACCTCTGAGTCC	AACAGCTATGACCATGCCTGAAAATCAGCATCTTGC	412
22	GTAAAACGACGGCCAGTTTTCTGCAAGGGATGTTTCC	AACAGCTATGACCATGTCCATCTTTAGGGTTTACATGG	439
23	GTAAAACGACGGCCAGTTGGGTAATTAGCACCCATCC	AACAGCTATGACCATGGCTTGCAATCCTTAGAAGCTG	316
24	GTAAAACGACGGCCAGTCTTGAACCTGGGAGAAATCC	AACAGCTATGACCATGTTATCAAATGACCATTAGTATGAGTTC	367
25	GTAAAACGACGGCCAGTCCATCTCCAAAAGAGAGGGAG	AACAGCTATGACCATGGAACTTGTGGGATGGCTTACTC	625
NPC2 gene			
1	GTAAAACGACGGCCAGTGAGACTGCAGGCTTCTGGG	AACAGCTATGACCATGCAGTTAGGTAGGGTCCAAGGC	413
2	GTAAAACGACGGCCAGTGAGAGCAGAGCACCTTCCC	AACAGCTATGACCATGATTCATGACTGCCAATTCCC	306
3	GTAAAACGACGGCCAGTGGAATGCTGTTGCTTGGG	AACAGCTATGACCATGTCTTCATCATAGAGATAAGGGGC	382
4	GTAAAACGACGGCCAGTAGCTGTGCCCACATGCTAAG	AACAGCTATGACCATGCAGGAAATAGGGTCTCAGATGC	382
5	GTAAAACGACGGCCAGTGGAATGTCTGATAACTTGCCC	AACAGCTATGACCATGTGTCTTCAGTGCACTCTGGG	540

Table S2. Data from *in silico* analyses of novel missense variations.

***NPC1* mutations**	**# of alleles**	**SNPs3D**	**Align GVGD**	**PolyPhen2**	**Mutation Taster**	**M-CAP**	**CADD**	**REVEL**	**VEST3**	**Classification**
p.Cys238Arg	1	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Likely Pathogenic
p.Ser365Pro	1	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Non-Pathogenic	Pathogenic	Pathogenic	Likely Pathogenic
p.Val694Met	1	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Likely Pathogenic

CAPÍTULO II

Manuscrito II - “Preliminary experience of a Brazilian reference center with a comprehensive genetic test for Niemann-Pick type C”

Artigo a ser submetido para a revista *Genetics & Molecular Research*

Preliminary experience of a Brazilian reference center with a comprehensive genetic test for Niemann-Pick type C

Running Title: Experience of a Brazilian center with genetic test for Niemann-Pick type C

M. Polese-Bonatto[1,2], H. Bock[1,2], M.C. Matte[1], F.T.S. Souza[4], R.G. Kessler[4], R. Giugliani[4,5] and M.L. Saraiva-Pereira[1,2,3,4,6]

1Laboratório de Identificação Genética, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brasil

2Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil

3Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil

4Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brasil

5Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

6Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil.

Corresponding author:

Maria Luiza Saraiva-Pereira, PhD

Laboratório de Identificação Genética - Centro de Pesquisa Experimental

Hospital de Clínicas de Porto Alegre

Ramiro Barcelos 2350

CEP 90035-903, Porto Alegre, Brasil

e-mail: mlpereira@hcpa.edu.br

ABSTRACT

Niemann-Pick type C (NP-C) is a neurodegenerative disorder characterized by storage of unesterified cholesterol in liver, spleen, and central nervous system. To date, two genes are associated to this disease, *NPC1* and *NPC2*, being 95% of disease-associated mutations found in the former, and just 4% in the latter. The aim of the current study was to apply a more comprehensive genetic analysis protocol, including Sanger sequencing and the multiplex ligation-dependent probe amplification (MLPA), to search for sequence variants in *NPC1* or in *NPC2* genes. A total of 12 distinct sequences variants were found in 18 different samples, including one frameshift novel variant (p.Val231Glyfs*2), detected in just one allele. In addition, an update in our diagnosis strategy was implemented using a different biomarker, due to high incidence of false positive results in the filipin staining test. We can then conclude that a combination of a screening test using a more specific biochemical biomarker with a more comprehensive genetic test is essential for achieving an accurate diagnosis of NP-C.

Keywords: Niemann-Pick type C disease; *NPC1* gene; *NPC2* gene; Multiplex ligation-dependent probe amplification (MLPA); NP-C diagnosis

INTRODUCTION

Niemann-Pick disease type C (NP-C; OMIM: NP-C1 #257220, NP-C2 #607625) is a rare, autosomal recessive neurovisceral disease with a broad clinical spectrum. NP-C is caused by mutations in one of two genes, *NPC1* and *NPC2* (Vanier, 2015). *NPC1* is located on chromosome 18q11-q12, spans 57 kb divided into 25 exons (Carstea, 1997; Patterson et al., 2000). This gene encodes for a large glycoprotein of 1278 amino acid located in late endosomes, characterized by 13 transmembrane domains (Carstea, 1997). *NPC2* maps to 14q24.3 and it was found to be identical to *HE1*, a previously known gene encoding a major secretory protein in the human epididymis (Naureckiene et al., 2000). The *HE1/NPC2* gene, renamed *NPC2* gene, spans 13.5 kb of genomic DNA divided into 5 exons, and encodes for a small soluble glycoprotein of 132 amino acid that is ubiquitously expressed (Vanier and Millat, 2004).

Great majority of mutations (95%) associated to NP-C is found in the *NPC1* gene, with just approximately 4% in *NPC2*. There are few cases with a positive outcome in the biochemical evaluation and no pathogenic alteration detected in those genes (Patterson et al., 2012; Papandreou and Gissen, 2016).

The estimated prevalence of NP-C is approximately 1 in 100,000 live births, although recent studies suggest that this figure might be underestimated, considering that many patients fails to reach a diagnosis (Wassif et al., 2016; Hendriksz et al., 2017). Nonspecific clinical presentation can be responsible for the delay in the establishment of a NP-C diagnosis. Therefore, an efficient laboratorial evaluation is crucial in order to allow that the proper diagnosis is

achieved at an early stage of disease, enabling prompt start of appropriate clinical management (Papandreou and Gissen, 2016).

Filipin staining test used to be the ‘gold standard’ diagnostic test for NP-C, where a positive result would be a remark of impaired intracellular cholesterol transport (Vanier, 2010). However, both false negative as well as false positive results have been reported; those false negatives are represented by a small group of patients with a “variant phenotype” staining (Papandreou and Gissen, 2016; Vanier et al., 2016). Therefore, genetic analysis is recommended to confirm a diagnosis of NP-C (Patterson et al., 2012; Vanier and Latour, 2015).

More than 460 different sequence alterations have been reported in those genes so far [Human Gene Mutation Database (HGMD) – Professional version - http://www.hgmd.cf.ac.uk]. DNA sequencing is required for the analysis of the whole coding regions of *NPC1* and *NPC2*, and Sanger sequencing can be used as a diagnostic tool for point mutations or small genomic rearrangements. When gross genomic rearrangements, such as deletions and/or insertions, have to be investigated, additional tools, such as such as multiplex ligation-dependent probe amplification (MLPA), are required to confirm or rule out those types of variant, as indicated by a recently revised diagnostic algorithm (Petterson et al., 2017). More recently, next generation sequencing (NGS) is beginning to be employed for investigation of more complex clinical phenotypes (Sudrié-Arnaud et al., 2018). However, NGS is not successful in all cases, and depending upon type of genomic rearrangement, this alteration might escape detection.

Therefore, the aim of the current study was to apply a more comprehensive genetic analysis protocol, based on the revised algorithm for use of biomarkers and genetic testing, including Sanger sequencing and the multiplex

ligation-dependent probe amplification (MLPA) of *NPC1* or in *NPC2* genes, in a cohort of individuals with clinical suspicion of NP-C.

MATERIALS AND METHODS

Subjects

Clinical information and biological samples from 265 unrelated individuals from different regions of Brazil were obtained through Rede NPC Brasil, a Brazilian network for diagnosis of NP-C. From this large cohort, a subset composed by 34 individuals was carefully selected according to the following inclusion criteria: (i) a strong clinical suspicion of NP-C and/or (ii) a positive or inconclusive/variant result in the filipin staining test. Strong clinical suspicion was defined based on leading manifestations, such as ataxia, cognitive decline, psychosis, and splenomegaly. A positive result was defined by high fluorescence in the test while inconclusive or “variant” would be those tests showing moderate fluorescence. This data was collected retrospectively, not being performed during this study. The other individuals were excluded due to negative results at both biochemical biomarker and genetic testing, excluding a NP-C diagnosis, or due to the presence of two pathogenic mutations into two different alleles that confirms a NP-C diagnosis. The study was approved by our local institutional review board (IRB), Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (CEP-HCPA), under registration #05-168.

DNA Isolation

Blood samples (5 mL) were collected into EDTA containing tubes, genomic DNA was isolated from peripheral blood leukocytes according to standard procedures and kept at –20˚C until further analyses. DNA was quantified using a

NanoDrop ND-1000 Spectrophotometer, and samples were diluted to 50 ng/uL before molecular analysis.

Genetic testing

Polymerase chain reaction (PCR) was used to selectively amplify the whole coding sequences of *NPC1* and *NPC2* genes as specific fragments, using DNA genomic as template. The whole coding region of *NPC1* was divided into 24 different amplicons (exons 15 and 16 were amplified within the same fragment). *NPC2* coding region was divided into 5 different amplicons. Sequences of primers used for PCR amplification, as previously reported (Polese-Bonatto et al, 2019). Amplicons were purified using 2.5 U of Exonuclease I (USB, Cleveland, OH, USA) and 0.25 U of Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA), according to standard procedures. Sanger sequencing was performed using BigDye® Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) using universal M13 primers, following manufacturer's instructions.

Multiplex Ligation-Dependent Probe Amplification (MLPA) was carried out using MRC-Holland commercial kits for *NPC1* and *NPC2* (SALSA P193-A2), according to the manufacturer's instructions. Following ligation and amplification reaction, products were subjected to capillary electrophoresis on an ABI3130xl Genetic Analyzer (Applied Biosystems – Foster City, CA, USA). Data from electrophoresis were analyzed using GeneMapper® v 3.2 software (Applied Biosystems – Foster City, CA, USA) and Coffalyser v.9 software (MRC-Holland, http://www.mrc-holland.com/).

In silico analyses

The Human Gene Mutation Database (HGMD® – Professional version - http://www.hgmd.cf.ac.uk) was the primary reference database for verifying sequence variants. The Exome Aggregation Consortium (ExAC) browser (http://exac.broadinstitute.org/) and The Genome Aggregation Database (gnomAD) browser (http://gnomad.broadinstitute.org/) (Lek et al., 2016) were also used to search for variants in this study, and the Human Splicing Finder (HSF) software (http://www.umd.be/HSF3/) was used to additionally check the intron variant. Different predictor tools were applied to verify the impact of variants on protein structure and/or function, including Mendelian Clinically Applicable Pathogenicity (M-CAP) Score (http://bejerano.stanford.edu/mcap/), Mutation Taster (http://www.mutationtaster.org/), and Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/). Variants are named according to Human Genome Variation Society (HGVS) nomenclature guidelines (http://www.hgvs.org/content/guidelines), using NM_000271.4 reference sequence for NPC1 transcript and NM_006432.4 for NPC2 transcript.

RESULTS

Twelve different variants (all present in just one allele) in 18 (53%) individuals were identified by a more comprehensive genetic testing applied in this study, from the total of 34 individuals included in this cohort. Table 1 shows a full list of sequence variants.

Table 1. Sequence variants detected by genetic testing and result of filipin staining test, when available.

Sample ID	Sequence variation in one allele		Result of Filipin staining test	Allele frequency*
	Nomenclature at protein level	Nomenclature at DNA level		
	NPC1 gene			
1	p.Ser151Phefs*18	c.451_452del	inconclusive	4.062e-6
2				
3	p.Val231Glyfs*2	c.692_693del	----	----
4	p.Pro237Ser	c.709C>T	inconclusive	0.01039
5				
6				
7	p.Pro434Ser	c.1300C>T	---	0.004110
8	p.Gly911Ser	c.2731G>A	---	0.001082
9	p.Ser954Leu	c.2861C>T	“variant” profile	7.223e-5
10	p.Asn961Ser	c.2882A>G	----	0.001098
11			inconclusive	
12	p.Ala1035Val	c.3104C>T	inconclusive	8.122e-6
13			positive	
14	p.Val1115Phe	c.3343G>T	----	0.0008911
15	p.Arg1183His	c.3548G>A	inconclusive	0.0001589
16			----	
	NPC2 gene			
17	p.Val30Met	c.88G>A	inconclusive	0.002009
18	c.363+7g>a	c.363+7G>A	positive	0.001252

* Allele frequencies data from the genome aggregation database

Eleven out of those 12 variants were already found at HGMD and/or in databases that combines both exome and genome sequencing data from a wide variety of large-scale sequencing projects (defined in the Material and Methods section). Some of these variants are reported at a very low allelic frequencies

(table 1). In this group, we have observed cases with either positive or inconclusive (or “variant”) results in the filipin staining test.

Different predictors tools were employed to evaluate pathogenicity of variants within coding sequences due to single nucleotide change, according to publish guidelines (Richards et al., 2015; Ghosh et al., 2017). This group includes nine variants (missense), and those with low allele frequencies were classified as probably pathogenic mutations, according to *in silico* analyses using tools indicated by Ghosh and colleagues (2017).

One variant (c.363+7G>A in *NPC2* gene) was found in one sample (Table 1, #18), which was included in our subset due to a positive result in the filipin test. This nucleotide change was detected by both Sanger sequencing (Figure 1) and MLPA analysis (Figure 2). As this variant was located within a non-coding sequence, HSF software was applied to confirm or ruled out a possible splicing defect, and no significant splicing motif alteration was detected by this tool in the presence of this variant.

Variant p.Val231Glyfs*2 (Figure 3) detected in one allele was not found among variants listed in the databases, being an indication that this variant is a novel finding and possibly very rare. No further sequence alterations were identified in the remaining 16 samples after the complete genetic testing protocol.

DISCUSSION

A highly variable clinical phenotype makes the diagnosis of NP-C a challenge task. Therefore, the combination of a well-defined clinical suspicion and an extensive laboratory evaluation, including biomarkers as well as genetic confirmation, is desirable for confirming or exclude a diagnosis.

The strategy applied in this cohort was able to detect 12 different sequences variants in total. Each of them was present in just one allele, i.e., individuals were heterozygous for the variant. Great majority (10 out of 12) was located in *NPC1* gene, and few were detected in more than one sample (Table 1). The most frequent variant was p.Pro237Ser that was present in samples from 3 unrelated individuals. Allele frequency of this variant is just over 1% according to gnomAD being categorized as a polymorphism. Other variants in *NPC1* gene with low allelic frequencies were defined as rare variants. However, p.Ser151Phefs*18, p.Asn961Ser, p.Ala1035Val, and p.Arg1183His were identified in 2 unrelated individuals each. Variants located in *NPC2* gene, p.Val30Met and c.363+7G>A, were found in one sample each.

Variant c.363+7G>A (*NPC2*) was detected by Sanger sequencing (Figure 1), but an abnormal profile was also observed by MLPA (Figure 2). No additional alteration was found using MLPA. Data presented here further suggest that copy number variation is a rare event in *NPC1* and *NPC2* genes.

To our current knowledge, the frameshift variation p.Val231Glyfs*2 (Figure 3) is likely to be a novel variant due to the fact that this change was not found in variant databases. In addition, produces a truncated small protein that is expected to have a defective function. The wild-type NPC1 protein has 1278

amino acids, and mutant protein produced by this variation would be expected to have just 233 amino acids. Therefore, essential domains of NPC1 protein will be missing, and normal function highly impaired.

In the present cohort, we have seen a number of cases reported as having a strong clinical suspicion or a positive result in the filipin staining test that no pathogenic sequence alteration in either NP-C associated genes were found. Outcomes like that are in line with previously published reports (Vanier et al., 2016; Patterson et al., 2017), and confirm that more specific biomarkers have to be included in a laboratory diagnosis protocol for NP-C. Hence, we are currently using oxysterols measurements as an additional biomarker in the NP-C diagnosis protocol, as recently reported (Hammerschmidt et al., 2017).

We are aware that no new case of NP-C was reported here. Nevertheless, several individuals considered as doubtful for the diagnosis were excluded from this clinical suspicion and are currently being reassessed for other clinical conditions with confounder symptoms that is very relevant for those patients.

In summary, we reported here our experience on applying a revised genetic testing in selected individuals with a strong clinical suspicion of NP-C or a positive or inconclusive result in the filipin staining test. We have reported that sequence variants were found in 53% of samples, including one polymorphism and 11 potential pathogenic sequences variants. High incidence of false positive results in the filipin staining test was also observed, even among individuals with no variants in both NP-C associated genes that led to a recent update in NP-C diagnostic strategy in our center. Hence, data reported here greatly support that a combination of a screening test using a more specific biochemical biomarker with the genetic testing is crucial to achieve an accurate diagnosis in NP-C.

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Legends of figures

Figure 1. Location and identification of c.363+7G>A sequence alteration in NPC2. A) Partial sequence of the gene, including whole of exon 3 (white letters) and introns 2 and 3. MLPA probe sequence is underline. Location of sequence alteration (g>a) is boxed. Sequence obtained by Sanger sequencing is shown in B) from the forward primer, where g>a alteration can be visualized, and in C) from the reverse primer, where c>t nucleotide change represents the reported sequence variant.

Figure 2. MLPA fragment profile. Results from sample 18 (Table 1) are shown in A) as ratio of tested sample against a mean value of control samples while low ratio in a specific region is represented by a red dot in the third column, and in B) as relative fluorescence units (RFU) of each probe, where the reduced RFU peak is indicated by an arrow. Results from a standard sample (male control) are shown in C) as ratio of a control sample against a mean value of control samples and in D) as RFU peaks of each probe, where normal peak is indicated by an arrow.

Figure 3. DNA sequencing of novel sequence variation p.Val231Glyfs*2 identified in NPC1 gene. a Direct sequencing the forward primer. A) arrow indicates the beginning of the deletion of the variation B) the arrow shows where was deleted the nucleotide in the variation p.Val231Glyfs*2 (c.692_693del).

Figure 1

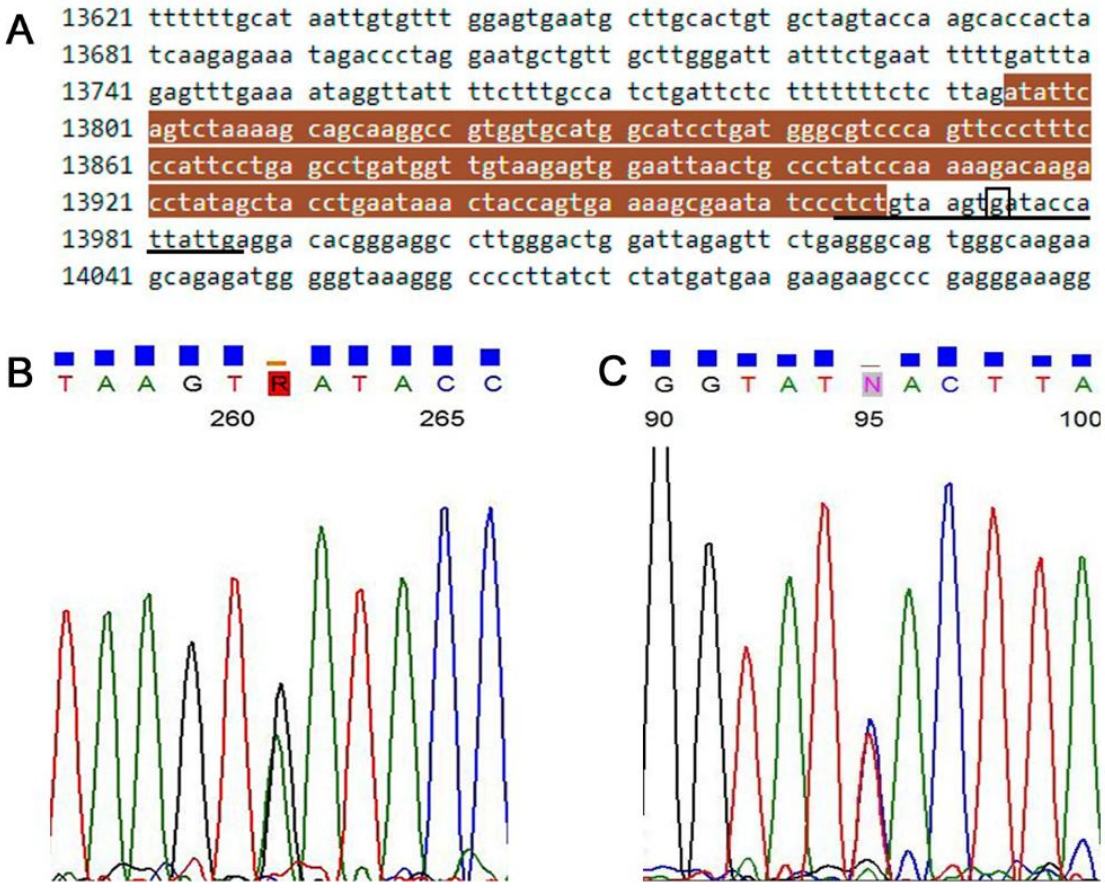

A
13621 ttttttgcat aattgtgttt ggagtgaatg cttgcactgt gctagtacca agcaccacta
13681 tcaagagaaa tagaccctag gaatgctgtt gcttgggatt atttctgaat ttttgattta
13741 gagtttgaaa ataggttatt ttctttgcca tctgattctc tttttttctc ttagatattc
13801 agtctaaaag cagcaaggcc gtggtgcatg gcatcctgat gggcgtccca gttccctttc
13861 ccattcctga gcctgatggt tgtaagagtg gaattaactg ccctatccaa aaagacaaga
13921 cctatagcta cctgaataaa ctaccagtga aaagcgaata tccctctgta agtggtacca
13981 ttattgagga cacgggaggc cttgggactg gattagagtt ctgagggcag tgggcaagaa
14041 gcagagatgg gggtaaaggg ccccttatct ctatgatgaa gaagaagccc gagggaaagg
B
T A A G T R A T A C C
260
265
C
G G T A T N A C T T A
90
95
100

Figure 2

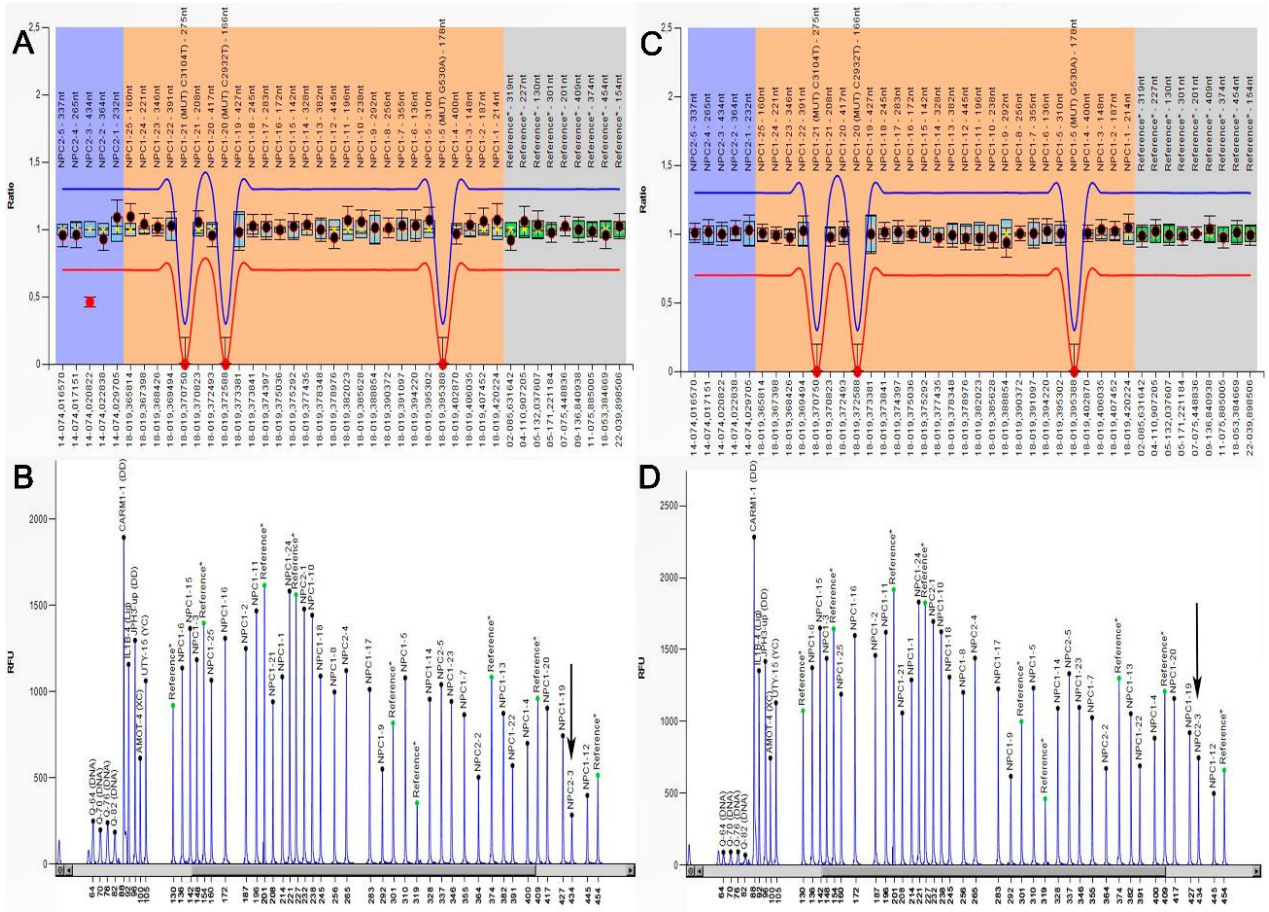
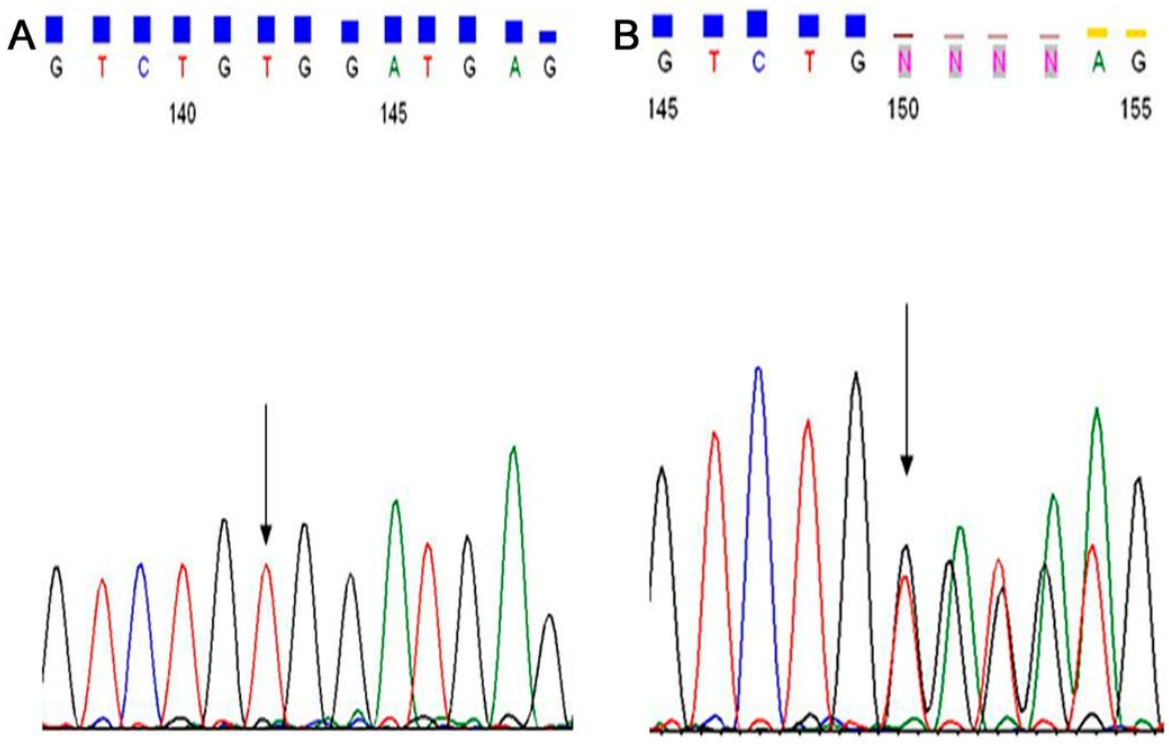


Figure 3



CAPÍTULO III

Manuscrito III - “Niemann-Pick type C: Analysis of variations in the sequences of CHIP and DNAJB6 genes as candidates to phenotype modifiers”

Artigo a ser submetido para a revista *Annals Human Genetics*

Niemann-Pick type C: Analysis of variations in the sequences of *CHIP* and *DNAJB6* genes as candidates to phenotype modifiers

Running Title: Modifiers genes to Niemann-Pick type C disease.

Márcia Polese-Bonatto[1,2]; Eduardo Preusser de Mattos[1,3]; Gabriel Vasata Furtado[1,3]; Giovana Bavia Bampi[1,3]; Maria Luiza Saraiva-Pereira[1,2,3,4,5]

[1]Laboratório de Identificação Genética – Centro de Pesquisa Experimental – Hospital de Clínicas de Porto Alegre – Porto Alegre, Rio Grande do Sul, Brazil;

[2]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica – Universidade Federal do Rio Grande do Sul - Porto Alegre, Rio Grande do Sul, Brazil;

[3]Programa de Pós-Graduação em Genética e Biologia Molecular – Universidade Federal do Rio Grande do Sul - Porto Alegre, Rio Grande do Sul, Brazil;

[4]Serviço de Genética Médica – Hospital de Clínicas de Porto Alegre - Porto Alegre, Rio Grande do Sul, Brazil;

[5]Departamento de Bioquímica – Universidade Federal do Rio Grande do Sul - Porto Alegre, RS, Brazil.

Corresponding author:

Maria Luiza Saraiva-Pereira, PhD

Laboratório de Identificação Genética - Centro de Pesquisa Experimental

Hospital de Clínicas de Porto Alegre

Ramiro Barcelos 2350

CEP 90035-903, Porto Alegre, Brazil

Phone: +55 51 33597689 e-mail: mlpereira@hcpa.edu.br

ABSTRACT

Niemann-Pick type C (NP-C) disease is an autosomal recessive disease caused by mutations in *NPC1* or *NPC2* genes, characterized by accumulation of non-esterified cholesterol in late endosome/lysosome, with classic clinical manifestations of hepatosplenomegaly and progressive neurodegeneration. Although genes associated with the disease are known, genotype-phenotype correlations are not straightforward in NP-C patients. This indicates a possible effects of phenotype modifiers. Different chaperones have been associated with expression of the *NPC1* gene. DNAJB6 is a chaperone of the Hsp40 family and CHIP a co-chaperone integral to the E3 ligase group, and both are involved in protein quality control. The aim of this study was to investigate specific polymorphisms in DNAJB6 and CHIP genes and evaluate a possible modifying effect in patients with NP-C. Specific SNPs of *DNAJB6* gene (rs9647660, rs12668448, rs4716707 and rs6459770) and rs6597 on *CHIP* gene were selected and genotyped. Allele and genotype frequencies were established and compared. rs6597 (*CHIP* gene) had the highest frequency T allele in the patient group. Considering the *DNAJB6* gene, a statistical difference was observed for the CT genotype in rs12668448. Haplotypes were established for the *DNJB6* gene and more frequents were ACAG and GTGA. In the present study, a single gene variation was found to be more frequent in patients than in controls. The significance of this variation has to be investigated to better define a possible role of this variation in NP-C.

KEYWORDS: *CHIP* gene, *DNAJB6* gene, modifiers genes, NP-C disease, *NPC1* gene, *NPC2* gene.

INTRODUCTION

Niemann-Pick C (NP-C) is a rare, autosomal recessive neurovisceral disease, caused by mutations in one of two genes, *NPC1* (in 95 % of cases) and *NPC2* (Vanier, 2015). *NPC1* gene is located on chromosome 18q11-q12, spans 57 kb and contains 25 exons (Carstea, 1997; Patterson et al., 2000). This gene encodes for a large glycoprotein of 1278 amino acid, including 13 transmembrane domains that reside in late endosomes (Carstea, 1997).

NP-C is a lysosomal storage disorder, the lipid trafficking defected result in intracellular accumulation of unesterified cholesterol and other compounds (Zech et al., 2013). Age of onset (AO) of the disease is variable, and non-visceral symptoms, such as neurological and psychiatric, can be present at different stages of disease (Patterson et al., 2012). The estimated incidence is 0.95/100,000 lives births in Western Europe (Vanier, 2015).

Although genes associated with the disease are known as stated above, genotype-phenotype correlations are not straightforward in NP-C patients. This indicates a possible effects of phenotype modifiers. Moreover, modifier genes that contribute to phenotypic variation in individual patients are largely unknown. The analysis of single nucleotide polymorphisms (SNPs) in candidate genes can be an option to uncover novel modifiers.

Currently, many studies related to quality control systems have shown promising.

Chaperones of both heat shock protein 70 family (Hsp70 - HSPA family) and heat shock protein 90 family (Hsp90 - HSPC family), heat shock protein 40 (Hsp40 - being DNAJB a member of this family), and co-chaperone Carboxy-

terminus of Hsc70-Interaction Protein (CHIP) have been associated with neurodegenerative diseases. Hsp70, Hsp90 and co-chaperone CHIP were identified in the control quality of NPC1 wild and mutant (p.I1061T) protein (Nakasone et al., 2014).

DNAJB6 is a class II Hsp40 member, which is involved in the blockade of beta-amyloid aggregation (Aβ), polyglutamine aggregates and cellular toxicity (Smith et al., 2015).

CHIP is a co-chaperone, belong E3 ligase family (Ballinger et al., 1999) binding to Hsp/c70 by C-terminal and Hsp90 by N-terminal (Matsumura et al., 2013). CHIP is involved in ubiquitination process of chaperones and to their address to the proteasome-ubiquitin pathway (Narayan et al., 2015).

In this work, *DNAJB6* and *CHIP* genes were chosen due to their association with neurodegenerative diseases. We have genotyped a group of 59 Brazilian NP-C patients for tag SNPs in *DNAJB6* and *CHIP* in order to test an association with mutation severity of these patients.

MATERIALS AND METHODS

2.1 DNA Isolation

Blood samples (5 mL) were collected in EDTA, and genomic DNA was isolated from peripheral blood leukocytes and kept at –20˚C. DNA was quantified with a NanoDrop ND-1000 Spectrophotometer.

2.2 Sample Collection

Fifty-nine unrelated NP-C patients that were referred to the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (HCPA) and confirmed by molecular analysis were included in this study. We have also genotyped unrelated healthy individuals as controls. This study was approved by our local Institutional Review Board (project number 05168).

2.3 SNP selection and genotyping

Four tagging (tag) SNPS of the DNAJB6 gene (rs9647660, rs12668448, rs4716707 and rs6459770) and one SNP of CHIP gene (rs6597) were selected. These polymorphisms were chosen through SNP tagging by HAPLOVIEW software, based on the populations evaluated in the HapMap Project (The International HapMap Consortium, 2005).

SNPs were genotyped by TaqMan® Allelic Discrimination Assays in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). Assays were performed following manufacturer instructions. In short, each reaction was performed in a final volume of 12 µl, containing 10 ng of genomic DNA, 0.3 µl of

TaqMan (40x), 6 µl of SNP genotyping assay, and 4.7 µl of water. TaqMan® assays used for genotyping were C__11156514_10 (Applied Biosystems) for rs9647660, C__442644_10 (Applied Biosystems) for rs12668448, C__230927_10 (Applied Biosystems) for rs4716707, C__505610_10 (Applied Biosystems) for rs6459770, and C_7576829_10 (Applied Biosystems) for rs6597. Allelic and genotypic frequencies were established. SNPs were tested for Hardy-Weinberg equilibrium through Arlequin Software v3.5.

Based on genotyping data, haplotypes were reconstructed using PHASE software v2.1 (Stephens et al., 2001).

2.4 Classification of the severity of mutations

Mutations carried by NP-C patients were classified by severity into three major groups: severe, mild and light. This classification was based on data from UniProt and *in silico* analysis using the following softwares: Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/),SNP3sD(http://www.snps3d.org), SHIFT (http://sift.bii.a-star.edu.sg), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph), Align GVGD (http://agvgd.iarc.fr/) and Mutation Taster (http:/mutationtaster.org/).

2.5 Statistical analyses

Statistical analyses were performed using SPSS 18.0 software and p values <0.05 were considered significant.

RESULTS

We have studied a total of 59 unrelated NP-C patients. Age at diagnosis (AD) of patients ranged from 2 months to 46 years, with average of 12.39 years. Allelic and genotypic frequencies of SNPs are shown in Tables 1 and 2.

No statistical difference was detected in allelic frequencies of DNAJB6 gene SNPs of NP-C patients when compared to controls (Table 1). However, higher frequency of T allele in rs6597 (*CHIP* gene) was shown in patients when compared to controls (Table 1). Similar analyses were also performed comparing genotypic frequencies of each SNP among groups, and no statistically difference was identified (Table 2). All SNPs were tested for HWE and values obtained can be found as supplementary material (Table S1).

Nine haplotypes were obtained (Table 3), and the most frequent was ACAG (34.3%), followed by GTGA (28.8%), and GCAG (11.9%). Curiously, two haplotypes (ACAA and ATGA) were only found in NP-C patients at very low frequencies (0.2% and 0.4%, respectively) (Table 3).

In order to correlate SNPs genotypes and mutation severity, patients were divided into 5 sub-groups according to their causing mutations at the *NPC1* gene as severe (S), mild (M) and light (L). Allelic frequencies in each category (S, M, and L) was 39.0%, 55.1%, and 5.9%, respectively. The majority of patients (47.5%) had two mild alleles (M/M), followed by patients with two severe alleles (S/S) (28.8%), and 15.2% showed one severe and one mild allele (S/M). Less represented groups were patients with one severe and one light allele (S/L), presented by 5.1% of patients and two light alleles (L/L) in 3.4%. No patient was found to carry one mild and one light allele (M/L). A statistically significant

association was found in the rs12668448 with mutation severity, with a much higher presence of heterozygous genotype (CT) in the M/M group (Table 5).

DISCUSSION

Neurodegenerative disease, such as Parkinson's disease and some lysosomal storage disorders, has been associated with quality control pathways dysfunction. Among these pathways, autophagy is a lysosomal-dependent degradation, and chaperone-mediated autophagy is one of proteolysis pathway (Osellame & Duchen, 2014). Identification of changes in these pathways may be a good explanation for different phenotypes observed in NP-C patients.

Chaperone/co-chaperone/client, proteins involved in quality control pathways, has been broadly associated to human biology. They are involved with many diseases, such as cystic fibrosis, cancer and neurodegeneration related in folding of specific proteins (Taipale et al., 2015). Many chaperones and co-chaperones are already known, among them we have the chaperone DNAJB6 (Hsp 40 family) and the co-chaperone CHIP, studied in this work.

Mutations identified in the *DNAJB6* is responsible for Limb-girdle muscular dystrophy (neurodegenerative disease) (Sarparanta et al., 2012; Smith et al., 2015), and it is also identified as a genetic modifier to human cardiomyopathy (Ding et al., 2016).

In this study, we used a strategy of group patients according to mutation severity, in order to overcome the lack of proper age of onset of the disease in some patients. In the association analysis, a high frequency of CT genotype for rs12668448 was detected. This association has to be further elucidated in order to confirm a possible role of DNAJB6 as a modifier of NP-C phenotype.

Previous published work identified CHIP with NPC1 protein by imunoprecipitation (Nakasone et al., 2014). The co-chaperone CHIP is the largest

proteasome degradation regulator, having numerous protein substrates as transcription factors, signaling intermediates and cytoskeletal or structural proteins. In this work, we have detected a statistically significant difference the ancestral allele (T allele) in rs6597 (within *CHIP* gene). We are aware that number of patients included in the study is limited, but it is unclear whether this result is just associated to sample representation.

Polymorphisms in *DNAJB6* and *CHIP* genes might be relevant for interactions of these protein with NPC1 that might have an effect in expression of mutations in the *NPC1* gene. The relevance of data presented here is still unclear. However, previous studies showed that chaperones, other than DNAJB6 can enhance the efficiency of mutant NPC1 through protein folding (Gelsthorpe et al., 2008; Nakasone et al., 2014).

Modifying factors in certain genes may influence possible interactions of their proteins with effect on mutant NPC1 protein expression. Molecular chaperones may increase efficiency and NPC1 proteins (Gelsthorpe et al., 2008; Nakasone et al., 2014). However preliminary, data presented here cannot rule out an association of polymorphisms in genes related to protein control quality in the expression of proteins associated to NP-C.

AUTHORS' CONTRIBUTIONS

Contribution of analytic tools and method development: M.P.B., E. P. M., G. V. F., G. B. B.; Data analysis: M.P.B., E. P. M., G. V. F., G. B. B.; Manuscript preparation: M.P.B., M.L.S.P.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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TABLE 1: Allelic frequencies and association analyses. Allelic frequencies of each SNP are shown for patients and controls. Association analysis were performed among groups.

SNPs ***DNAJB6*** **gene**	**Allele**	**Patients (n=118)**	**Local controls (n=100)**	**Controls from HapMap (n=330)**	***p***
rs9647660	A (%)	51 (43.2)	36 (36.0)	119 (36.1)	0.373
	G (%)	67 (56.8)	64 (64.0)	211 (63.9)	
rs12668448	C (%)	59 (50.0)	51 (51.0)	182 (55.2)	0.542
	T (%)	59 (50.0)	49 (49.0)	148 (44.8)	
rs6459770	A (%)	63 (53.4)	56 (56.0)	174 (52.7)	0.853
	G (%)	55 (46.6)	44 (44.0)	156 (47.3)	
rs4716707	A (%)	40 (33.9)	34 (34.0)	125 (37.9)	0.645
	G (%)	78 (66.1)	66 (66.0)	205 (62.1)	
SNP ***CHIP*** **gene**	**Allele**	**Patients (n=118)**	**Local controls (n=200)**	**Controls from HapMap (n=330)**	***p***
rs6597	T (%)	109 (92.4)	180 (90.0)	273 (82.7)	0.008*
	G (%)	9 (7.6)	20 (10.0)	57 (17.3)	

The HapMap population used was CEU (Utah residents with Northern and Western European ancestry). * represents value that is statistically significant (Pearson chi-square test).

TABLE 2: Genotypic frequencies and association analyses. Genotypic frequencies of each SNP in patients and controls are shown. Association analyses were performed among groups and of NP-C, controls and HapMap of the *DNAJB6* gene tag SNPs and the *CHIP* gene SNP.

SNPs *DNAJB6* gene	**Genotype**	**Patients (n=59)**	**Local controls (n=50)**	**Controls from HapMap (n=165)**	***p***
	AA (%)	10 (17.0)	7(14.0)	23 (14.0)	
rs9647660	AG (%)	32 (54.2)	22 (44.0)	73 (44.2)	0.512
	GG (%)	17 (28.8)	21 (42.0)	69 (41.8)	
	CC (%)	15(25.4)	11 (22.0)	50 (30.3)	
rs12668448	CT (%)	29 (49.2)	29 (58.0)	82 (49.7)	0.681
	TT (%)	15 (25.4)	10 (20.0)	33 (20.0)	
	AA (%)	19(32.2)	16 (32.0)	49 (29.7)	
rs6459770	AG (%)	25 (42.4)	24 (48.0)	76 (46.1)	0.950
	GG (%)	15 (25.4)	10 (20.0)	40 (24.2)	
	AA (%)	8 (13.5)	6 (12.0)	24 (14.5)	
rs4716707	AG (%)	24 (40.7)	22 (44.0)	77 (46.7)	0.886
	GG (%)	27 (45.8)	22 (44.0)	64 (38.8)	
SNP *CHIP* gene	**Genotype**	**Patients (n=59)**	**Local controls (n=100)**	**Controls from HapMap (n=165)**	
	TT (%)	50 (84.7)	81 (81.0)	113 (68.5)	
rs6597	TG (%)	9 (15.3)	18 (18.0)	47 (28.5)	0.058
	GG (%)	-	1 (1.0)	5 (3.0)	

TABLE 3: Haplotype distribution. The haplotype each group (NP-C patients, local controls and controls from HapMap) were inferred and distribution is presented below.

Haplotype	Patients (%) (n=118)	Controls (%) (n=100)	HapMap (%) (n=330)	Total (%)
ACAA	1 (0.8)	-	-	0.2
ACAG	44 (37.2)	33 (33.0)	111 (33.7)	34.3
ATAG	4 (3.4)	3 (3.0)	8 (2.4)	2.7
ATGA	2 (1.7)	-	-	0.4
GCAG	8 (6.8)	13 (13.0)	44 (13.3)	11.9
GCGA	6 (5.1)	5 (5.0)	27 (8.2)	6.9
GTAG	6 (5.1)	7 (7.0)	11 (3.3)	4.4
GTGA	31 (26.3)	29 (29.0)	98 (29.7)	28.8
GTGG	16 (13.6)	10 (10.0)	31 (9.4)	10.4

The HapMap population used was CEU (Utah residents with Northern and Western European ancestry).

TABLE 4: Association analysis of genotypic frequencies and mutation severity in NP-C patients.

SNPs *DNAJB6* gene	Genotype	S/S (n=17)	S/M (n=9)	S/L (n=3)	M/M (n=28)	L/L (n=2)	*p*
rs9647660	**AA (%)**	2 (3.4)	3 (5.1)	2 (3.4)	3 (5.1)	-	0.288
	AG (%)	9 (15.1)	3 (5.1)	1 (1.7)	18 (30.5)	1 (1.7)	
	GG (%)	6 (10.2)	3 (5.1)	-	7 (11.9)	1 (1.7)	
rs12668448	**CC (%)**	6 (10.2)	3 (5.1)	2 (3.4)	4 (6.8)	-	0.049*
	CT (%)	6 (10.2)	2 (3.4)	-	19 (32.0)	2 (3.4)	
	TT (%)	5 (8.5)	4 (6.8)	1 (1.7)	5 (8.5)	-	
rs6459770	**AA (%)**	7 (11.9)	4 (6.8)	2 (3.4)	6 (10.2)	-	0.196
	AG (%)	6 (10.2)	1 (1.7)	1 (1.7)	15 (25.2)	2 (3.4)	
	GG (%)	4 (6.8)	4 (6.8)	-	7 (11.9)	-	
rs4716707	**AA (%)**	2 (3.4)	3 (5.1)	-	3 (5.1)	-	0.272
	AG (%)	4 (6.8)	3 (5.1)	1 (1.7)	14 (23.7)	2 (3.4)	
	GG (%)	11 (18.6)	3 (5.1)	2 (3.4)	11 (18.6)	-	
SNP *CHIP* gene	**Genotype**	**S/S (n=17)**	**S/M (n=9)**	**S/L (n=3)**	**M/M (n=28)**	**L/L (n=2)**	
rs6597	**TT (%)**	13 (22.0)	8 (13.5)	2 (3.4)	26 (44.1)	1 (1.7)	0.164
	TG (%)	4 (6.8)	1 (1.7)	1 (1.7)	2 (3.4)	1 (1.7)	

*Statistically significant difference (Fisher's Exact Test)

TABLE S1: Data from Hardy-Weinberg equilibrium test.

SNPs *DNAJB6* gene	Patients (n=59)	Local controls (n=50)	Controls from HapMap (n=165)
rs9647660	0.78980	0.46545	0.61419
rs12668448	1.00000	0.50485	1.00000
rs6459770	0.29449	0.49778	0.34920
rs4716707	0.56023	0.45333	1.00000
SNP *CHIP* gene			
rs6597	1.00000	1.00000	1.00000

CAPÍTULO IV

Manuscrito IV – "Apolipoprotein E frequency in Brazilian population with Niemann-Pick type C disease"

Artigo a ser submetido para a revista *Journal of Human Genetics*

Apolipoprotein E frequency in Brazilian population with Niemann-Pick type C disease

Running Title: Apolipoprotein E frequency in NP-C patients

Márcia Polese-Bonatto[1,2]; Ana Carolina R. Farias[1]; Roberto Giugliani[3], Maria Luiza Saraiva-Pereira[1,2,3,4].

[1]Laboratório de Identificação Genética, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre – Porto Alegre, RS, Brazil;

[2]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[3]Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre RS, Brazil;

[4]Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Corresponding author:

Maria Luiza Saraiva-Pereira, PhD

Laboratório de Identificação Genética - Centro de Pesquisa Experimental

Hospital de Clínicas de Porto Alegre

Ramiro Barcelos 2350

CEP 90035-903, Porto Alegre, RS, Brazil

Phone: +55 51 33598011

e-mail: mlpereira@hcpa.edu.br

Abstract

Niemann-Pick type C (NP-C) disease is an autosomal recessive disorder caused by mutations in the *NPC1* or *NPC2* genes. Although, two genes are associated with the disease, there is still a lot to be uncover considering that phenotype of patient may vary in the presence of the same mutation in the causative gene. Several studies have looked for genes as modifiers of phenotype, among them the apolipoprotein E (*ApoE*) gene. Three alleles are identified in this gene: *ApoE ε2, ApoE ε3 and ApoE ε4*, resulting in three protein isoforms. The aim of this study was to identify a possible association between different APOE alleles and phenotype of NP-C patients. Samples from 60 unrelated patients and 60 healthy subjects were included in this study. Patients age at diagnosis varied from 2 months to 46 years, with an average of 11 years and 2 months. DNA was isolated and exon 4 of the *ApoE* gene was amplified by PCR followed by Sanger sequencing. *ApoE ε2, ApoE ε3* and *ApoE ε4* alleles were evaluated and 5 different genotypes were identified. Allelic frequencies of *ApoE ε2, ApoE ε3* and *ApoE ε4* in the patient group were 6.3%, 82.9% and 10.8%, respectively. The most frequent genotype was *ApoE ε3/ε3* with 63.8% and genotypes *ApoE ε2/ε2* and *ApoE ε4/ε4* were not identified in this group. No statistical differences were observed in the analysis performed. However, we cannot rule out the effect of ApoE on the phenotype of patients considering that this protein is present in cholesterol metabolism and has been shown to be associated with several neurodegenerative diseases.

Introduction

Niemann-Pick type C (NP-C) disease is an autosomal recessive disease caused by mutations in one of two genes: the *NPC1* gene or the *NPC2* gene. The disease is characterized by accumulation of unesterified cholesterol and glycosphingolipids in lysosomes and late endosomes due to failure of intracellular lipid trafficking (1).

Age of onset of the disease is variable, ranging from childhood to adulthood. Clinical manifestations are quite heterogeneous, that makes a major difference between groups (2–5). Three main types of signs and symptoms observed in NP-C patients are visceral, neurological, and psychiatric. However, patients with similar symptoms can have different genotypes, which can delay the diagnosis (6). Patients lifespan may range from few days to more than 60 years; but in most cases death occurs between 10 and 25 years of age (2).

Although, two genes are associated with the disease as described above, there is still a lot to be uncover considering that phenotype of patient may vary in the presence of the same mutation in the causative gene. Phenotypic variation in inherited disorders can be due modifiers acting along with the genetic background. And modifier genes can frequently contribute to the phenotype in some autosomal recessive disorders.

In order to address modifier genes in NP-C, *apolipoprotein E* (*ApoE*) has been appointed as a potential modifier due to the association with cholesterol pathway. Cholesterol and other lipids are transported by ApoE protein in plasma and the central nervous system, binding to ApoE receptors in the cellular surface (7, 8). ApoE is a lipoprotein that has 299 amino acid with high expression in brain

and plays a major role in central nervous system cholesterol homeostasis. N-terminal region (residues 136-150) of the protein interacts with ApoE receptors while binding of lipids occurs in the C-terminal region (residues 244-272) (9, 10).

Two specific polymorphisms (rs429358 and rs7412) present in the human *ApoE* gene results in three alleles (ε2, ε3 and ε4) (Fig 1A), and six different genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4). *ApoE ε3* allele is the most frequent (77.9%), and *ApoE ε2* allele is rare (8.4%) (8, 10, 11). Frequency of *ApoE ε4* allele has been reported to be 13.7% in the general population, but frequency of this allele can reach 40% in patients with Alzheimer Disease (AD) (10).

The three major isoforms (ApoE ε2, ApoE ε3, and ApoE ε4) differ from each other only by a Cys to Arg amino acid substitution at positions 112 or 158 (Fig 1B) (7, 9, 11). These differences among isoforms interfere in the structure and influence the association with lipids as well as the binding with receptors (9). *ApoE ε2* is low effective to transport lipids, being related to hyperlipoproteinemia type III and, in a couple of populations, this allele has been identified as having a protective effect in AD (10, 12). *ApoE ε4* is a risk factor for several diseases, including neurological disorders such as tauopathies and Lewy body disease, Parkinson's disease (10). *ApoE ε3* is considered the "neutral" ApoE genotype (13).

The different isoforms of ApoE modulate β-amyloid metabolism (Aβ) and can contribute to Aβ toxicity (14). Neurofibrillary tangle and decreased levels of Aβ40 and Aβ42 are observed in NP-C. Considering that *ApoE ε4* allele have been previously associated to early onset of NP-C and *ApoE ε2* allele with later onset

of the disease in a small cohort (13), we have genotyped a group of 60 Brazilian NP-C patients in order to test for this association.

Material and Methods

Sample Collection and DNA Isolation

We have studied 60 unrelated NP-C patients confirmed by molecular analysis, from different regions of Brazil, that were referred to the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (HCPA). We have also enrolled 60 unrelated healthy individuals as controls. This study was approved by our local Institutional Review Board (project #05168).

Blood samples (5 mL) were collected in EDTA, and genomic DNA was isolated from leukocytes as previously described (15) and kept at –20ºC. DNA was quantified using NanoDrop 1000 (Thermo Scientific) equipment.

Amplification of the *ApoE* Gene

Polymerase chain reaction (PCR) was used to amplify fragment of interest of *ApoE* gene. Exon 4 of the gene were amplified using specific primers ApoE_E4F (5' GACCATGAAGGAGTTGAAGGCCTAC 3') and ApoE_E4R (5' CACGCGGCCCTGTTCCACCAG 3'), which generates a fragment of 373 bp that includes both SNPs. The amplification reaction was performed in a final volume of 25 µL containing 50 ng genomic DNA, 200 mM of each dNTP, 5 µM of each primer (forward and reverse), 1.0 mM of $MgCl_2$, 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, and 1 U of AmpliTaqGold® DNA Polymerase (Thermo Fischer Scientific). Cycling conditions were initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 67°C for 30 s, and extension at 68°C for 1 min, followed by final extension at 68°C for 10 min.

Each PCR product was verified by electrophoresis on a 1.5 % (w/v) agarose gel and visualization under UV light.

Genotyping of *ApoE* alleles

Amplicons were purified using PEG 50% (polyethylene glycol and 2.5 M NaCl) as follows: 20 µL PEG 50% was added to 20 µL the PCR product and incubated at 37°C for 15 min, the mixture was centrifuged at 15.000 g at room temperature for 15 min. Pellet was then washed using cold ethanol and amplicon was resuspended in 8 µL. DNA sequencing was performed using BigDye® Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) from forward and reverse primers, following the manufacturer's instructions. Sequences were analyzed with DNA Sequencing Analysis software v. 5.2 (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Bioinformatic tools

Severity of mutations in NP-C patients were determined using the following bioinformatic tools: PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/), SNPs3D (http://www.snps3d.org/), Align GVGD (http://agvgd.iarc.fr/), Mutation Taster (http://www.mutationtaster.org/)

Results

SNPs of interest (rs429358 and rs7412) were genotyped by Sanger sequencing and figure 2 shows representative profiles of 5 out of 6 possible genotypes. *ApoE ε2*/*ε2* was not found in any samples neither from patients nor controls.

Allelic frequencies of *ApoE ε2*, *ApoE ε3*, and *ApoE ε4* alleles in NP-C patients were 8.3%, 80.8%, and 10.8%, respectively. Similar distribution was also observed in controls, although a slightly higher frequency of *ApoE ε2* allele was observed in NP-C patients. No statistically significant difference was found among groups. These data are summarized in Table 1.

Patients were further divided into 4 groups according to age at diagnosis: (i) early infant (diagnosis up to 2 years old), (ii) late infant (diagnosis from 3 to 5 years old), (iii) juvenile (diagnosis from 6 to 15 years old) and, adolescent/adult (diagnosis from 16 years old). Distribution of patients in each group was 30.00% (18), 23.33% (14), 20.00% (12) and 26.67% (16), respectively. Allelic and genotypic frequencies were established in each group (Table 1 and Table 2), but no statistically difference was seen.

In the group of patients, *ApoE ε3*/*ε3* was the most frequent genotype (68.3%), followed by *ApoE ε3*/*ε4* (15.0%), *ApoE ε2*/*ε3* (10.0%), and *ApoE ε2*/*ε4* (6.7%). *ApoE ε4*/*ε4* genotype was just identified in the one sample in the control group and *ApoE ε2*/*ε2* was not present in any group (Table 2).

Patients' mutations were subdivided into three main cluster as follows: severe (S), mild (M) and light (L). Therefore, six groups could be potentially find within our cohort: patients with two severe alleles (S/S), two mild alleles (M/M), two light alleles (L/L), one severe and one mild allele (S/M), one severe and one

light allele (S/L), and one mild and one light allele (M/L). The latter (M/L) was not detected among patients included in this study, and no statistically significant difference was found among groups, indicating no correlation between genotype and phenotype (Table 2).

Analysis of presence and absence of *ApoE ε2* and *ApoE ε4* and association to early or late diagnosis was also performed. However, no correlation was observed in this analysis (Fig 3).

Discussion

The lifespan of NP-C patients varies from months until over 60 years of age, according to severity of symptoms. However, in the majority of cases, death occurs between 10 to 25 years of age. NP-C clinical presentation is heterogeneous, and age of onset can be highly variable (2, 3, 16–18). This heterogeneous clinical presentation can delay diagnosis and increased vigilance combining characteristic clinical disease manifestation with biomarkers and genetic screening assay is suggested to reduce time between age of onset and age at diagnosis (19).

Patients in our cohort were from different regions of Brazil, as previously stated. Therefore, as age at onset was not available from all patients, we classified patients according to age at diagnosis. In the present cohort, age at diagnosis varied from 2 months to 46 years of age, with a median of 11.13 years, being 27 males and 33 females. This observation emphasizes a great variation of age of diagnosis of NP-C cases, especially in patients lacking organomegaly in neurological and psychiatric cases, as previously reported (2). Patients were classified into four general categories, early-infantile, late-infantile, juvenile and adolescent/adult-onset based on age of neurological onset (20). However, poor genotype-phenotype correlation is commonly observed even in affected siblings (21). Unfortunately, allelic and genotypic frequencies (Tables 1 and 2) of *ApoE* alone do not seem to be responsible for this clinical variation.

Some previous work has been shown association between *ApoE* gene and onset of Alzheimer disease, and likely with other neurodegenerative diseases (22). Early neurological disease onset and more severe

neuropathological findings are associated with *ApoE ε4* allele in AD (23). ApoE was suggested to be a modifier gene that contribute but does not determine an earlier onset of neurological symptoms in a cohort with fifteen NP-C patients (13).

In the Azores Island, allelic frequencies obtained for *ApoE ε2*, *ApoE ε3*, and *ApoE ε4* were 6.75%, 83.73%, and 9.52%, respectively (24). These frequencies are similar to the data reported in this work. This is not surprising considering the etnic background of the Brazilian population.

In the group of patients, *ApoE ε3*/*ε3* genotype was the most frequent (68.3%), followed by *ApoE ε3*/*ε4* (15.0%), *ApoE ε2*/*ε3* (10.0%), and *ApoE ε2*/*ε4* (6.7%) (Table 2). The most frequent genotype in the Azores population was also *ApoE ε3*/*ε3* and with a similar frequency (69.84%) as in this present cohort. And a very low frequency was shown for *ApoE ε4*/*ε4* genotype (0.79%), and no patients carrying *ApoE ε2*/*ε2* genotype was reported (24). In a different work, similar genotypic frequencies distribution was identified in patients with Machado-Joseph disease (MJD), another neurodegenerative disorder (25).

As stated before, a protect effect of *ApoE ε2* was reported in AD (10, 12) and *ApoE ε4* was associated to an increased cardiovascular risk (10). These data indicate a possible effect of these allele in the NP-C patients. We are aware that classifying patients according to age at diagnosis might be a limitation to evaluate modifiers. However, in infant cases, age at diagnosis may be closer to age of onset. Thereby, patients were also analysed based only in the presence or absence of *ApoE ε2* and presence or absence of *ApoE ε4*, but no association was observed. These results can be explained by: i) age at diagnosis is not a good criterion to this evaluation; ii) patients of our coorte may have a different

genetic background from previous studied populations or; iii) *ApoE ε2* and *ApoE ε3* have no effect on NP-C patients.

ApoE ε2 allele has been associated to earlier onset in Parkinson disease (22, 26), and in Huntington disease (27). And an increased risk for earlier onset was reported in MJD (25) and in frontotemporal dementia (28). Studies with more homogeneous results of *ApoE* allele are related to AD, where *ApoE ε4* is strongly associated with increased risk for the disease, and *ApoE ε2* with lower risk for AD. Therefore, it is required additional studies with other neurodegenerative disease to further understand the correlation of *ApoE* gene and clinical outcomes.

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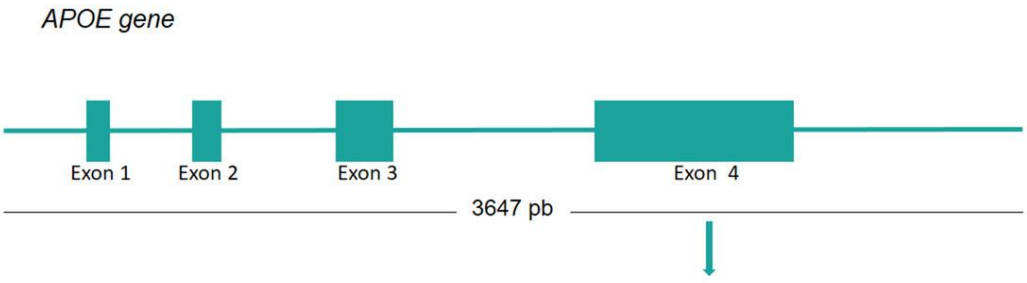
Figure Legends

Fig. 1 Schematic diagram of the human *ApoE* gene and protein topology. A) Schematic diagram of the human *ApoE* gene, showing single nucleotide polymorphisms (rs429358 and rs7412) that are responsible for three different alleles (*ApoE ε2*, *ApoE ε3*, and *ApoE ε4*). The three isoforms differ from each other only by a Cys to Arg amino acid substitution at positions 112 (or 130) and/or 158 (or 176); numbers within brackets relate to position including signal peptide.

Fig. 2 DNA sequencing of rs429358 and rs7412. Nucleotide sequences presented in this figure represents 5 out of 6 possible genotypes in the *ApoE* gene.

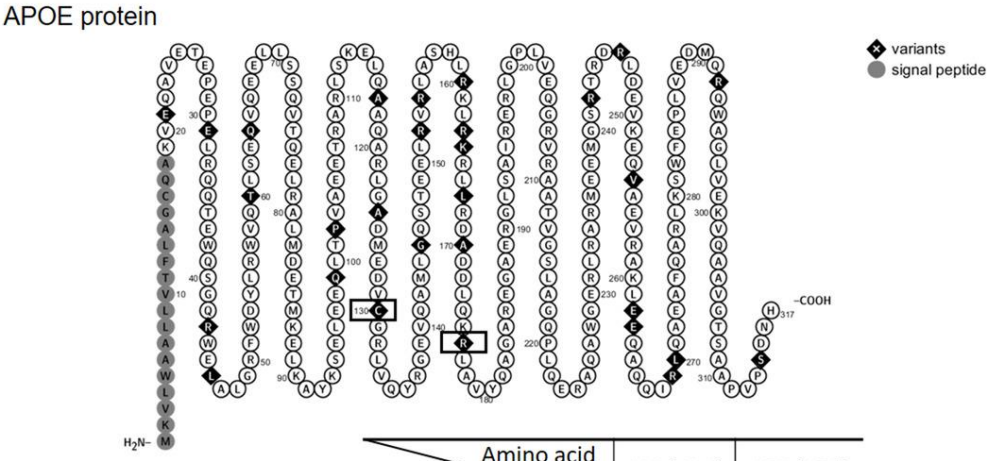
Fig. 3 Comparison of presence or absence of *ApoE ε2* or *ApoE ε4* alleles and age at diagnosis. Each bar represents mean age at diagnosis in 4 different groups of patients that were divided according to *ApoE ε2* or *ApoE ε4* alleles. Data was analyzed using Fisher's exact test.

Figure 1



a

Allele \ SNP	rs429358	rs7412
ApoE2	**T**GC	**T**GC
ApoE3	**T**GC	**C**GC
ApoE4	**C**GC	**C**GC



b

Isoform \ Amino acid	112 (130)	158 (176)
ApoE2	Cys	Cys
ApoE3	Cys	Arg
ApoE4	Arg	Arg

Figure 2

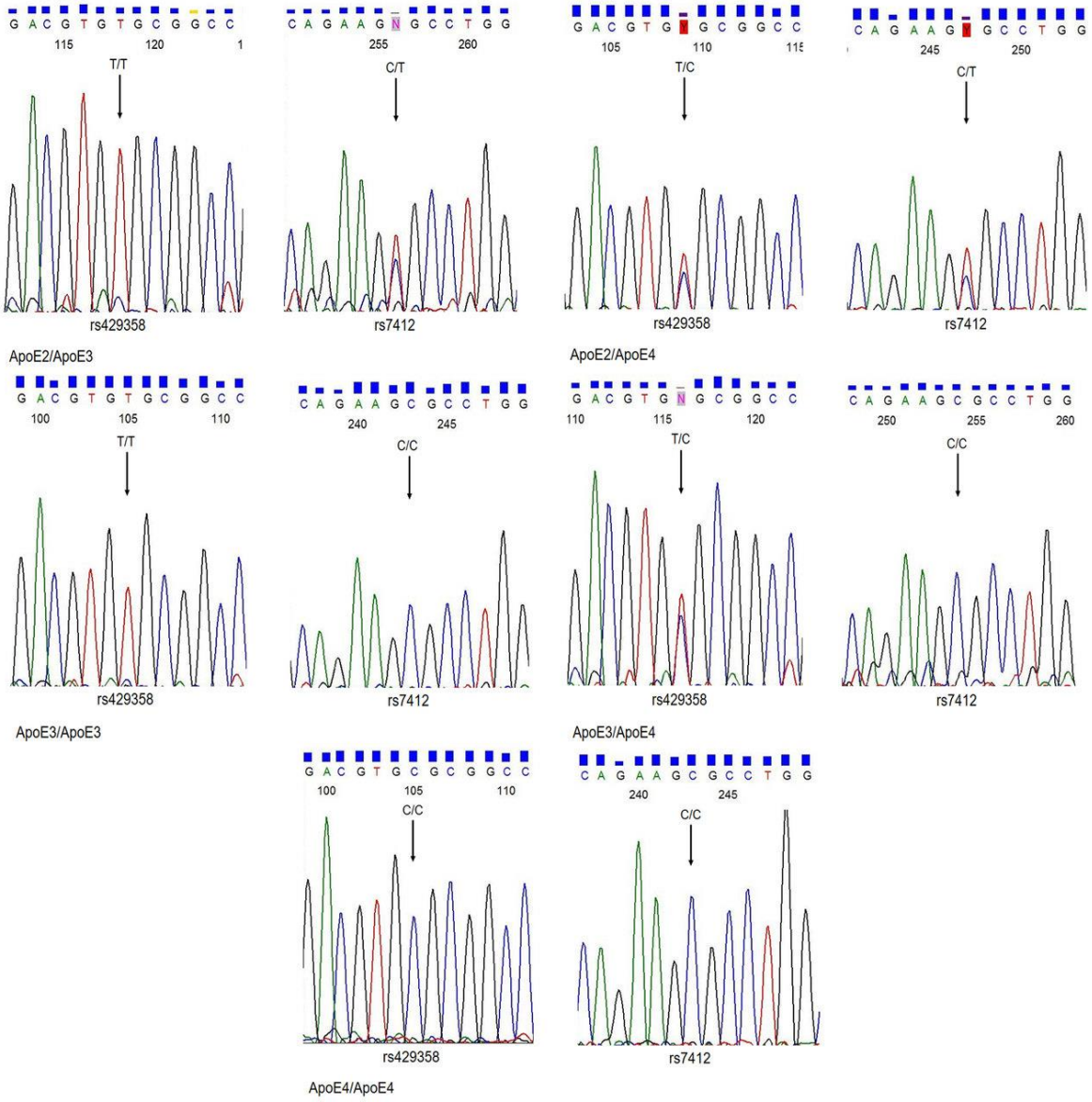

T/T
rs429358
C/T
rs7412
ApoE2/ApoE3
T/C
rs429358
C/T
rs7412
ApoE2/ApoE4
T/T
rs429358
C/C
rs7412
ApoE3/ApoE3
T/C
rs429358
C/C
rs7412
ApoE3/ApoE4
C/C
rs429358
C/C
rs7412
ApoE4/ApoE4

Figure 3

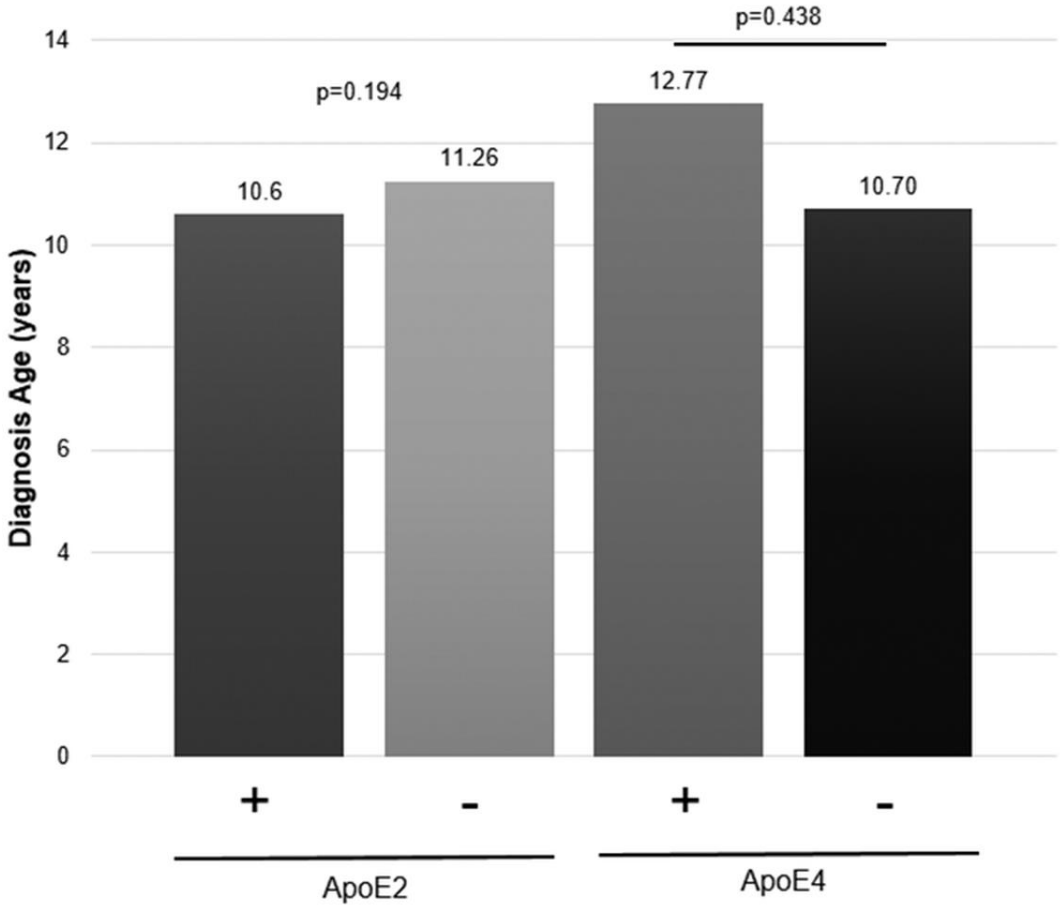

p=0.438
p=0.194
12.77
11.26
10.6
10.70
Diagnosis Age (years)
14
12
10
8
6
4
2
0
+
-
+
-
ApoE2
ApoE4

Table 1 Frequencies of *ApoE* alleles and association analysis among different age groups in NP-C patients.

Allele	Patients (n=120)	Controls (n= 120)	*p**	*early infantile* (n=36)	*late infantile* (n=26)	*juvenile* (n=26)	*adult* (n=32)	*p***
***ApoE ε2* (%)**	10 (8.3)	5 (4.2)		2 (1.7)	2 (1.7)	4 (3.3)	2 (1.7)	
***ApoE ε3* (%)**	97 (80.8)	102 (85.0)	0.446	32 (26.6)	20 (16.7)	19 (15.8)	26 (21.7)	0.679
***ApoE ε4* (%)**	13 (10.8)	13 (10.8)		2 (1.7)	4 (3.3)	3 (2.5)	4 (3.3)	

* Pearson's chi-squared test; **Fisher’s exact test.

Table 2 Genotype frequency and association analysis among groups and the *ApoE* gene. Genotype frequency and association analysis among the *ApoE* gene and mutation severity: severe (S), mild (M) and light (L).

Genotype	Patients (n=60)	Controls (n=60)	*p**	S/S	S/M	S/L	M/M	L/L	*p**	early infant	late infant	juvenile	adult	*p**
ApoE ε2/ε3 (%)	6 (10.0)	5 (8.3)	0.260	2 (3.3)	-	-	4 (6.7)	-	0.485	1 (1.7)	-	3 (5.0)	2 (3.3)	0.374
ApoE ε2/ε4 (%)	4 (6.7)	-		-	1 (1.7)	-	3 (5.0)	-		1 (1.7)	2 (3.3)	1 (1.7)	-	
ApoE ε3/ε3 (%)	41 (68.3)	43 (71.7)		15 (25.0)	5 (8.3)	03 (5.0)	17 (28.3)	1 (1.7)		15 (25.0)	9 (15.0)	7 (11.6)	10 (16.7)	
ApoE ε3/ε4 (%)	9 (15.0)	11 (18.3)		1 (1.7)	3 (5.0)	-	4 (6.7)	1 (1.7)		1 (1.7)	2 (3.3)	2 (3.3)	4 (6.7)	
ApoE ε4/ε4 (%)	-	1 (1.7)		-	-	-	-	-		-	-	-	-	

*Fisher's exact test.

PARTE III

DISCUSSÃO

A crescente descrição de mutações vem auxiliando o diagnóstico da doença de NP-C. No ano de 2000, havia menos de 100 mutações identificadas no gene *NPC1* (Yamamoto *et al.*, 2000). Atualmente, esses números ultrapassam 470 mutações no gene *NPC1* e 27 no gene *NPC2*, dados do *The Human Gene Mutation Database* (HGMD®), mas constantemente esses números são atualizados através de novas publicações. No primeiro capítulo deste trabalho há cinco novas mutações identificadas e, que também farão parte deste banco.

No nosso estudo, entre os 54 pacientes diagnosticados com NP-C, 96,3% dos casos as mutações foram identificadas no gene *NPC1*, corroborando com os dados da literatura que inferem valores próximos a 95% (Vanier, 2010). A mutação mais frequente identificada na nossa população, p.Ala1035Val, difere-se da mutação mais frequente do Oeste Europeu e de famílias dos Estados Unidos, de origem europeia, sendo a mutação p.Ile1061Thr a mais frequente nestas localidades. Talvez essa variação ocorra pela população brasileira apresentar suas peculiaridades de miscigenação.

A segunda mutação mais frequente na nossa amostra, p.Pro1007Ala, também foi observada como segunda mais frequente em países da Europa (Jahnova *et al.*, 2014). Uma característica interessante desta mutação é que em testes de Filipin, podem ser observados resultados classificados como fenótipos variantes ou negativos (Fernandez-Valero *et al*., 2005) característica essa também observada em algumas amostras desse trabalho. Dessa forma, é interessante considerar que o perfil dessa mutação é recorrente em diferentes populações.

A frequência de algumas mutações foram maiores em algumas regiões específicas, como exemplo a p.Ala1035Val foi mais frequente em São Paulo, a p.Pro1007Ala no Paraná e a p.Phe1221Serfs*20 em Pernambuco. Isso pode ser o reflexo de um país considerado continental e, que apresenta uma vasta diversidade na sua formação ética.

A região sudeste, foi a que apresentou maior número de casos de NP-C, possivelmente por tratar-se da região mais populosa do país e com mais acesso a saúde. Na região norte, nenhum caso foi identificado. O diagnóstico é uma difícil tarefa, pois as manifestações clínicas e idade de início dos sintomas são variadas, combinado com os difíceis testes laboratoriais (testes bioquímicos e genéticos), exigindo acesso a centros especializados (Vanier, 2016).

Além das variações já descritas, 5 novas foram identificadas neste trabalho. As alterações foram avaliadas por programas de bioinformática, os quais, atualmente, são ferramentas que permitem realizar diferentes análises biológicas e inferir o efeito da alteração no produto final (proteína) e, de forma rápida avaliar o impacto sobre o fenótipo. As ferramentas de bioinformáticas utilizadas para predição *in silico*, cada vez mais tem se tornado confiáveis a través da análise e combinações de algoritmos, aumentando a fidedignidade dos resultados (Ghosh *et al.,* 2017) Testes *in vivo* não devem ser descartados, porém, a quantidade de novas mutações que atualmente são descritas, retardaria o diagnóstico e as condutas clínicas para NP-C assim como para outras patologias.

As variações p.Lys38_Tyr40del, p.Asn195Lysfs*2 e p.Cys238Arg estão localizadas no lúmen A enquanto as variações p.Ser365Pro e p.Val694Met estão em regiões de transmembrana da proteína NPC1. A localização da variação na

proteína é um fator importante para inferir se a alteração levará a modificação de fenótipo ou não, bem como a gravidade da mesma. As alterações que levam a mudança do quadro de leitura, tendem a terem efeitos no fenótipo, uma vez que a estrutura da proteína é afetada. Nas alterações do tipo *missense*, a troca de um único nucleotídeo, pode não ser o suficiente para afetar a estrutura e/ou função da proteína, por este motivo mais ferramentas de bioinformática devem ser utilizadas.

A identificação de mutações específicas se faz necessário para uma futura compreensão do comportamento das mesmas, o que permitirá o desenvolvimento de tratamentos mais específicos e eficientes para casos de NP-C (Rosenbaum & Maxfield, 2011).

Muitos casos de pacientes com suspeita de NP-C continuam sem diagnóstico, porém sabe-se que isso pode ocorrer devido às alterações patogênicas estarem acontecendo em regiões que ainda não se compreende qual é a efetiva contribuição sobre o fenótipo.

A utilização de múltiplas técnicas se faz necessário devido as limitações presentes em cada uma delas, como exemplo, teste positivo de Filipin pode tratar-se de NP-A ou NP-B e ser diagnosticado erroneamente como NP-C. A técnica de sequenciamento também pode não detectar algumas alterações, como deleções de maior grandeza ou até mesmo do gene inteiro, sendo necessário neste caso o uso da técnica de MLPA. O alto custo para o diagnóstico, com o uso de múltiplos testes atualmente é uma realidade, por este motivo há procura de novas metodologias e biomarcadores.

Para NP-C, o qual é uma doença autossômica recessiva, dois alelos mutados devem ser identificados para confirmação do diagnóstico. Nas 265

amostras testadas, 54 delas dois alelos mutados foram identificados (dados do Capítulo I deste trabalho). Porém, houveram critérios que tornaram as amostras que não tiveram seu diagnóstico concluído, elegíveis para serem testadas pela técnica de MLPA antes de serem liberadas como negativas para NP-C. Os critérios foram os seguintes: amostras com forte suspeita clínica e/ou teste de Filipin positivo ou variante; identificação de um ou nenhum alelo mutado; se enquadrando nestes critérios 34 amostras.

Das 34 amostras elegíveis, em 18 amostras foram identificados apenas um alelo mutado. Sabemos que grandes deleções, inserções e até deleções de genes inteiros podem passar despercebidos pelo sequenciamento. Entre as 18 amostras, foram identificadas 12 variações distintas, 11 delas já descritas no HMGD e/ou em bancos de dados (1000genomes, ExAC, genomAD). A variação p.Val231Glyfs*2 não foi identificada em nenhum dos bancos de dados utilizados, possivelmente tratando-se de uma nova variação.

A p.Val231Glyfs*2 é uma alteração que leva a produção de uma proteína truncada e com efeito deletério, visto que NPC1 é uma proteína de 1278 amino ácidos e, o produto dessa mutação será uma proteína de 233 amino ácidos. Como exemplo, a falta significativa de regiões funcionais como a SSD (regiões de detecção de esterol) indica a perda de função da proteína (Davies et al., 2000).

Atualmente, se considera diagnóstico de NP-C quando ocorre a identificação dos dois alelos mutados. A identificação de apenas um alelo mutado nas 18 amostras anteriormente citadas não permitiu a completa elucidação destes casos. Alguns trabalhos vêm apresentando desfechos que podem instigar sobre a necessidade da identificação dos dois alelos mutados.

Dados apresentados em um artigo, trouxe o caso de uma família de quatro irmãos, três irmãos que foram a óbito com 7, 9 e 11 anos e a quarta irmã, relatada como viva na publicação do artigo, uma mulher de 55 anos que apresentou hepatoesplenomegalia até os 13 anos de idade mas que não foi o suficiente para leva-la a óbito. Análises posteriores identificaram apenas variação em um alelo, p.Ile1061Thr, sendo levantada a hipótese de que a presença de um alelo mutado pode ser o suficiente para algumas manifestações mais brandas de fenótipo (Harzer *et al.,* 2014).

A variação c.363+7G>A identificada no gene *NPC2*, foi detectada primeiramente pela técnica de MLPA e posteriormente confirmada por sequenciamento de Sanger. Na análise dos resultados de MLPA, foi observada uma redução de 50% da intensidade da sonda específica, que indica uma possível deleção da região em análise. A técnica de Sanger evidenciou a troca de um nucleotídeo (G>A) na posição 7 a jusante do exon 3, permitindo concluir que a diminuição do sinal foi devido à falta de ligação da sonda em um dos alelos por causa do polimorfismo identificado. Análise *in silico* indicou que essa alteração não tem efeito sobre o *splicing*, não sendo uma variação significativa para alteração de fenótipo. Este dado nos mostra que, as técnicas devem ser complementares, pois apenas o MLPA poderia inferir uma falsa deleção nesta amostra. Não ter sido identificada nenhuma alteração sugere que a variação do número de cópias, deleções e duplicações são eventos menos frequentes nos genes *NPC1* e *NPC2*.

O conhecimento sobre NP-C tem avançado significativamente, porém ainda está longe da compreensão completa de sua fisiopatologia, por esse motivo a busca incessante de modificadores se fazem necessário. Genes

modificadores são aqueles que, juntamente com o gene responsável pela doença e fatores ambientais podem estar associados ao fenótipo da doença (Patterson et al., 2012; Vanier et al., 2016).

Neste trabalho foi possível identificar o perfil das mutações em pacientes brasileiros com NP-C, utilizar múltiplas técnicas e avaliar candidatos elencados como possíveis modificadores após consideráveis revisões bibliográficas. Achados em genes modificadores poderia elucidar, em parte, a diferença de fenótipo observada em pacientes que apresentam a mesma mutação.

A seleção de rotas relacionadas com as proteínas NPC1 e NPC2 foi o ponto de partida para a escolha dos genes que seriam elencados como possíveis modificadores. Considerou-se a hipótese de que os alvos selecionados poderiam ter efeito sobre a fisiopatologia da doença, e a análise de SNPs foi a ferramenta utilizada para essa análise.

Distintas rotas têm se apresentado alteradas em doenças lisossômicas de depósito (DLD), entre elas NP-C, destacando-se a via proteossomal e autofágica. Estas rotas são ativadas devido à instabilidade de proteínas e enzimas o que resulta em rápida degradação e perda de função. Devido ao lisossomo ser uma das mais importantes organelas celulares, tem sido um dos maiores alvos de pesquisa nos últimos 20 anos (Suzuki, 2014).

O acúmulo de proteínas mal enoveladas, possivelmente devido a alterações em proteínas conhecidas como chaperonas, pode ser citotóxico e estar relacionada a patologias, incluindo doenças lisossômicas e neurodegenerativas (Smith *et al.*, 2015; Osellame & Duchen, 2014). As chaperonas são moléculas que não se encontram no produto final, mas que

interagem com as proteínas de maneira que estas alcance a conformação funcional (Ellis, 1987).

As famílias das chaperonas *heat shock protein 70* (Hsp70 – família HSPA), *heat shock protein 90* (Hsp90 – família HSPC), *heat shock protein 40* (DNAJB membro da família Hsp40) e a co-chaperona *carboxy-terminus of Hsc70-interaction protein* (CHIP) estão sendo associadas a doenças neurodegenerativas. No trabalho de Nakasone *et al*. (2014) foram identificadas as chaperonas Hsp70, Hsp90 e co-chaperona CHIP no controle de qualidade de NPC1 selvagem e mutada (p.I1061T). Estes achados têm redobrado a atenção para as chaperonas em NP-C.

Na realização deste trabalho foram selecionados 5 SNPs, um no gene *CHIP* (rs6597) e quatro do gene *DNAJB6* (rs9647660, rs12668448, rs4716707 e rs6459770), com o objetivo de correlacionar variações entre esses genes e o efeito sobre o fenótipo dos pacientes NP-C.

A frequência alélica e a análise de associação dos SNPs (CHIP e DNAJB6) demostrou diferença significativa em CHIP, porém o alelo mais frequente observado na população de pacientes NP-C foi o alelo ancestral (T), nos demais nenhuma correlação foi identificada. A frequência genotípica também foi avaliada, mas nenhuma diferença estatística foi observada. Estas análises podem ser comprometidas devido ao baixo número de casos utilizados nesse estudo levando a diminuição do poder estatístico.

Com o objetivo de buscar uma correlação entre genótipo e a gravidade da mutação, estas foram classificadas em leve, média e severa. O genótipo CT do SNP rs12668448 do gene *DNAJB6*, apresentou diferença significativa, porém o

fato de haver mais genótipos M/M (M=médio) pode ter sido o responsável por esse resultado.

Para a análise de frequência alélica e genotípica, a informação da idade de início das manifestações clínicas seria de extrema valia para a correlação (idade de início x alelo ou genótipo), mas este dado não foi encontrado na grande maioria das fichas dos pacientes, por este motivo utilizou-se para a análise a idade de diagnóstico. A idade de diagnóstico é variável, com atraso ou nunca realizado se carecer de manifestações clínicas como organomelagia, sintomas neurológicos e casos psiquiátricos (Vanier, 2010).

Estudos têm demonstrado que pelo menos em algumas mutações no gene *NPC1* a utilização de terapias com chaperonas podem levar a recuperação do fenótipo (Zampiere *et al.*, 2012). Em várias DLDs, com Doença de Fabry, Doença de Gaucher Doença de Pompe entre outras, tem sido demonstrado a possibilidade da utilização de chaperonas como terapia (Suzuki, 2014).

Outro modificador que parece estar associado a NP-C é a ApoE, o gene apresenta dois polimorfismos (rs429358 e rs7412), que resultam em três isoformas (E2, E3 e E4) as quais se diferem na habilidade de redistribuição do colesterol. As diferenças entre as isoformas interferem na estrutura e influenciam a associação com lipídios e a ligação com receptores (Hatters *et al*., 2006). Sabe-se atualmente que a isoforma E4 está associada como um possível fator de risco para a doença de Alzheimer, enquanto a isoforma E2 apresenta efeito protetivo (Mahley & Rall, 2000). Devido a sua relação direta com o metabolismo do colesterol, a ApoE se torna um forte candidato a modificador da doença de NP-C.

No primeiro trabalho, onde os autores buscaram uma associação deste alelo com a gravidade da doença de NP-C, os resultados apontaram como um possível modificador, mesmo com um número significativamente pequeno de pacientes (n=15) (Fun *et al*., 2012). Por esse motivo, os dois SNPs foram selecionados para teste em uma população maior.

Para análise, a amostra foi dividida em dois momentos, as que tinham ou não o alelo ApoE *ε*2 e em seguida a amostra foi novamente dividida entre as que tinham ou não o alelo ApoE *ε*4. Nenhuma correlação foi identificada, possíveis justificativas para o resultado: a idade de diagnóstico não é um bom critério para a avaliação; a diversidade da população brasileira pode não ser similar a outras populações ou; os alelos ApoE *ε*2 e ApoE *ε*4, não tem nenhum efeito sobre NP-C.

A fisiopatologia de NP-C não está completamente estabelecida. Com isso, nenhuma hipótese deve ser considerada como nula. Muitas rotas além das mencionadas neste trabalho podem estar envolvidas nesta patologia. Alterações lisossomais, devido a sua importância celular, devem ser consideradas não só como uma consequência, mas também como causa de outras alterações fisiológicas no organismo.

CONCLUSÃO

O desenvolvimento do presente trabalho permitiu avaliar, por meio de análises moleculares, amostras de pacientes brasileiros com suspeita da doença de Niemann-Pick tipo C e identificar o espectro de mutações nos genes *NPC1* e *NPC2* associados a esta patologia.

Foi possível identificar as mutações p.Ala1035Val, p.Pro1007Ala e p.Phe1221Serfs*20 como sendo as mais comuns da coorte brasileira. Além disso, elas também apareceram com maior frequência em estados específicos como São Paulo, Paraná e Pernambuco, respectivamente.

Para a investigação de possíveis ocorrência de rearranjos gênicos nos genes *NPC1* e *NPC2* em pacientes com suspeita clínica de NP-C, foi utilizada a técnica de MLPA a qual não evidenciou nenhuma alteração na população elegível para esta análise. Rearranjos gênicos parecem não ser alterações frequentes nesses genes.

Na investigação de nucleotídeo único nos genes *DNAJB6* e *CHIP* como possíveis modificadores de fenótipo, não foi encontrada correlação significativa entre estes genes e pacientes NP-C. Frente aos resultados, os genes *DNAJB6* e *CHIP* aparentam não ter efeito de modificador de fenótipo.

Outro candidato a modificador, a apolipoproteina E, selecionada devido a sua correlação com o metabolismo do colesterol e com outras doenças neurodegenerativas, determinou-se as isoformas através da combinação de alelos, porém não houve nenhuma correlação com o fenótipo da coorte de pacientes NP-C.

A fisiopatologia de NP-C não está completamente estabelecia. A continuidade da identificação de mutações e de genes modificadores auxiliará na compreensão desta patologia.

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ANEXOS

ANEXO I – Artigo. “Clinical and molecular characterization of hereditary spastic paraplegias: A next-generation sequencing panel approach.”

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Clinical and molecular characterization of hereditary spastic paraplegias: A next-generation sequencing panel approach

Daniela Burguez[a,c], Márcia Polese-Bonatto[c,e], Laís Alves Jacinto Scudeiro[h], Ingemar Björkhem[l], Ludger Schöls[m], Laura Bannach Jardim[a,c,f,h,i], Ursula Matte[d,f,k], Maria Luiza Saraiva-Pereira[a,c,e,f,j], Marina Siebert[d,g], Jonas Alex Morales Saute[a,b,c,h,i,*]

[a] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil
[b] Neurology Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil
[c] Genetics Identification Laboratory (Experimental Research Center), Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil
[d] Unit of Molecular and Protein Analysis (Experimental Research Center), Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil
[e] Postgraduate Program in Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[f] Postgraduate Program in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[g] Postgraduate Program in Gastroenterology and Hepatology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[h] Postgraduate Program in Medicine: Medical Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[i] Department of Internal Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[j] Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[k] Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[l] Karolinska University Hospital Huddinge, Karolinska Institute, Stockholm, Sweden
[m] Center for Neurology, Hertie Institute for Clinical Brain Research, Eberhard-Karls-University, Tübingen, Germany

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ABSTRACT

Background: Molecular diagnosis of hereditary spastic paraplegias (HSP) is a difficult task due to great clinical and genetic heterogeneity. We aimed to characterize clinical and molecular findings of HSP families from Rio Grande do Sul, Brazil; and to evaluate the diagnostic yield of a next-generation sequencing (NGS) panel with twelve HSP-related genes.

Methods: A consecutive series of HSP index cases with familial recurrence of spasticity, consanguinity or thin corpus callosum (TCC) were included in this cross-sectional study.

Results: Among the 29 index cases, 51.7% (15/29) received at least a likely molecular diagnosis, and 48.3% (14/29) a defined diagnosis. NGS panel diagnostic yield was 60% for autosomal dominant HSP (6/10, all SPG4), 47.4% for autosomal recessive HSP (9/19: 5 SPG11, 2 SPG7, 1 SPG5 and 1 cerebrotendinous xanthomatosis), and 50% for patients with TCC (3/6, all SPG11). Remarkably, 2/6 SPG11 patients presented keratoconus, and tendon xanthomas were absent in the patient with cerebrotendinous xanthomatosis.

Conclusion: A likely molecular diagnosis was obtained for more than half of families with the NGS panel, indicating that this approach could be employed as a first-line investigation for HSP. SPG4 is the most frequent form of autosomal dominant and SPG11 of autosomal recessive HSP in Southern Brazil.

1. Introduction

Hereditary spastic paraplegias (HSP) are a group of heterogeneous genetic disorders caused mainly by degeneration of the corticospinal tract longest axons [1,2]. HSP are clinically classified as pure or complicated forms, with ages at onset varying from early childhood to late adulthood [1–4]. An isolated pyramidal syndrome with predominance at lower limbs with or without vibration sense impairment and urinary urgency defines pure HSP; whereas complicated HSP presents a more complex clinical picture with additional neurological findings, such as ataxia, epilepsy, and cognitive decline [3].

All forms of inheritance are known to cause HSP with > 80 published genes or loci [5,6]. However, a smaller number of genes are responsible for most cases [1,5,7,8]. Most HSP-related proteins will affect axon and vesicle transport, control of endoplasmic reticulum morphology, mitochondrial quality control, myelination, protein folding/degradation, or lipid and purine nucleotide metabolism [1,4].

HSP are rare diseases that represent a significant burden to affected

* Corresponding author at: Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos 2350, 90.035-903 Porto Alegre, Brazil.
E-mail address: jsaute@hcpa.edu.br (J.A.M. Saute).

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individuals and families. A recent systematic review of studies from European, Northern African and Japanese populations reported different prevalence estimations ranging from 0.1 to 9.6 per 100.000 individuals [7]. There is no prevalence study of HSP in Latin America.

Due to the great clinical and genetic heterogeneity of HSP, achieving a genetic diagnosis can be a difficult task. Overall, > 50% of autosomal dominant HSP (AD-HSP) cases and 70% of autosomal recessive HSP (AR-HSP) cases never receive a genetic diagnosis by conventional sequencing methods [7,8]. With the advent of next generation sequencing (NGS), simultaneous sequencing of even thousands genes is now possible, faster and at lower cost [8,9], decreasing the diagnostic odyssey and the need for additional tests.

Our aim was to characterize clinical and molecular findings of HSP families from Rio Grande do Sul, Brazil, and to evaluate the diagnostic yield of a NGS panel with twelve HSP-related genes.

2. Materials and methods

2.1. Design and subjects

Index cases from consecutive families with clinical suspicion of HSP were recruited from April 2011 to November 2014 at Neurogenetics outpatients' clinics, Hospital de Clinicas de Porto Alegre, in a cross-sectional study. Eligibility was suspicion of HSP according to clinical diagnosis criteria [10] and presence of at least one of the following criteria: familial recurrence, consanguinity or thin corpus callosum (TCC) on magnetic resonance imaging (MRI). Age was not an exclusion criterion. The study was approved by the Ethics in Research Committee of our institution (GPPG-HCPA/14-0695), which follows the Declaration of Helsinki. Informed written consent was obtained from all individuals' prior participation.

2.2. Neurological and genetic evaluation

Severity of disease was evaluated with the Spastic Paraplegia Rating Scale (SPRS, range: 0–52, crescent in severity) [10]. Peripheral polyneuropathy was defined on clinical grounds by abnormal tactile-algesic and/or thermal discrimination, and decreased distal deep tendon reflexes and, when available, by nerve conduction studies and electromyography. The mode of inheritance was classified as apparently autosomal dominant when HSP was reported in > 1 generation. Families with several affected members in only one generation, those from consanguineous marriages, and simplex cases with TCC were classified as apparently autosomal recessive.

For simplex cases irrespective of consanguinity we excluded structural/inflammatory lesions by MRI of brain/spinal cord and examined vitamin B12, cooper, lipid profile and lactate blood levels; thyroid and hepatic function and HIV-1/2, HTLV-1/2 and Lues serology. For simplex cases or patients with suspected AR inheritance, we systematically screened for deficiency of lysosomal enzymes (arylsulfatase A, β-galactosidase, hexosaminidase A/B and galactocerebrosidase) and performed urine sulfatides chromatography, plasma amino acid analysis by tandem mass spectrometry and GC/MS urinary organic acid analysis. For simplex cases and patients with possible X-linked inheritance we screened for elevated levels of very long chain fatty acids. Patient's with AD inheritance were screened for SCA1, SCA2, SCA3, SCA6 and SCA7 by PCR using specific fluorescent primers followed by capillary electrophoresis of respective genes.

2.3. Genetic analysis

A customized AmpliSeq™ panel (Thermo-Fisher-Scientific) was designed using Ion AmpliSeq™ designer software in order to target all coding DNA sequences and flanking regions of the 11 HSP genes *ATL1, BSCL2, CYP7B1, KIAA0196, KIF5A, NIPA1, REEP1, SPAST, SPG7, SPG11* and *ZFYVE26* plus *CYP27A1,* related to cerebrotendinous xanthomatosis (CTX). This panel consisted of two primer pools with 257 different amplicons. NGS was performed using the Ion Torrent Personal Genome Machine (Ion-Torrent™). For detailed NGS procedures see Appendix 1. The raw data generated from NGS run was processed by Torrent Suite Software v5.0 (Thermo-Fisher-Scientific). After sequencing, reads were mapped to hg19 using Torrent Mapping Alignment Program (TMAP). Coverage assessment was performed using the Coverage Analysis plugin available in the Torrent Browser. Variants were annotated using Ion Reporter (Thermo-Fisher-Scientific), and Enlis Genome Research software (Enlis-LLC). Integrative Genomics Viewer was used for variant visualization. All genes were considered for analysis, regardless inheritance pattern classification.

2.4. Variant analysis

Sequences were searched for using the National Center for Biotechnology Information (NCBI) protein database, and variants are described with reference to the following transcripts: *ATL1* (NM_015915.4), *BSCL2* (NM_001122955.3), *CYP7B1* (NM_004820.3), *CYP27A1* (NM_000784.3), *KIAA0196* (NM_014846.3), *KIF5A* (NM_004984.2), *NIPA1* (NM_144599.4), *REEP1* (NM_022912.2), *SPAST* (NM_014946.3), *SPG7* (NM_003119.3), *SPG11* (NM_025137.3) and *ZFYVE26* (NM_015346.3). Sequence variations were compared to data available in the Human Gene Mutation Database (HGMD®). Mutalyzer 2.0 [11] was used for checking variants' nomenclature.

PolyPhen-2 [12], SIFT [13], M-CAP [14], Mutation Taster [15], Human Splicing Finder v3.0 (HSF3.0) [16] and ESEfinder v.3.0 (ESE3.0) [17] were used for *in silico* analysis. Phylogenetic conservation was estimated with Genomic Evolutionary Rate Profiling (GERP + +) [18]. Allele frequencies were searched on ExAC [19], gnomAD [20] and 1000 genomes browser [21]. Variants were classified according to American College of Medical Genetics and Genomics criteria [22].

2.5. Confirmation of disease-causing variants

Sanger sequencing was used to confirm known or likely disease-causing variants found by NGS, for affected relatives of index cases and for segregation analysis. Polymerase chain reaction (PCR) was used to selective exon amplification. Annealing temperatures and primer sequences are given in Supplemental Table 1 and sequencing details in Appendix 1.

2.6. Statistical analysis

All variables in the study showed normal distribution on one-sample Kolmogorov-Smirnov test. Quantitative features are reported as mean and standard deviation (SD). Age at onset, disease duration, and SPRS were compared between patients with pure and complicated HSP and between patients with and without a probable molecular diagnosis using two-tailed unpaired Student's *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Clinical and genetic classifications

Twenty-nine unrelated index cases were analyzed by the NGS-HSP panel (19 females, mean [SD] age at onset, 27.1 [14.7] years; disease duration, 17 [10.3] years). Twelve (41.4%) index cases presented with pure and 17 (58.6%) with complicated HSP. Pedigrees suggested AD-HSP in 10/29 (34.5%) and AR-HSP in 19/29 (65.5%) index cases, including two isolated cases with TCC and complicated HSP. Six (20.6%) index cases had TCC on MRI.

Pure-HSP: 8/12 (66.7%) index cases were classified as AD-HSP; mean (SD) age at onset was 29.7 (17.2) years, disease duration 14.5 (9.3) years and SPRS 20.9 (6.7) points. Complicated-HSP: 15/17

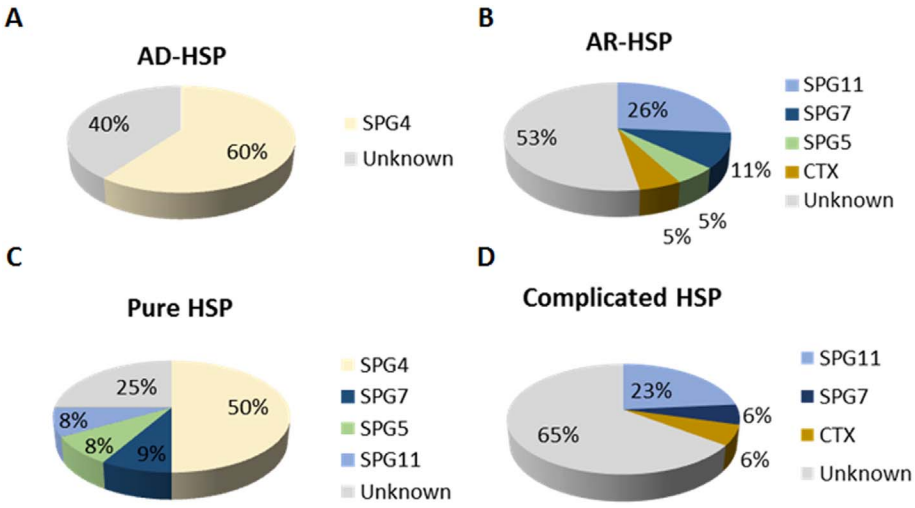

Fig. 1. Diagnostic yield of HSP-NGS panel according to genetic and clinical classification. AD, autosomal dominant; AR, autosomal recessive; CTX, cerebrotendinous xanthomatosis.

(88.2%) index cases were classified as AR-HSP; mean (SD) age at onset was 25.2 (12.9) years, disease duration 17.06 (2.2) years and SPRS 28 (9.1) points. Complicated-HSP were more frequently associated to autosomal recessive inheritance ($X^2 = 9.38$, $p = 0.002$) and higher SPRS scores than pure-HSP, but without statistical significance ($p = 0.061$); age at onset and disease duration were similar between groups.

Fifty-two individuals from these 29 families were evaluated in total (26 with pure and 26 with complicated-HSP). Most frequent additional feature for complicated-HSP was ataxia 14/26 (53.8%), followed by intellectual disability 13/26 (50%), dysarthria 9/26 (34.6%), Parkinsonism 8/26 (30.8%, mainly bradykinesia) and signs of peripheral neuropathy 7/26 (26.9%, without considering vibratory sense abnormalities). See Supplemental Table 2.

3.2. Next generation sequencing

NGS-HSP panel mean coverage on target was 116.13 reads. A total of 94.3% of NGS fragments had at least 30-fold coverage and passed quality control. Overall the NGS-HSP panel diagnostic yield was 51.7% (15/29 patients) for at least a likely molecular diagnosis, and 48.3% (14/29 patients) for definitive molecular diagnosis. Ages at onset, disease duration and severity were similar between patients with and without at least a probable diagnosis (data not shown).

Diagnostic yield of the NGS-HSP panel for AD-HSP was 60% (6/10, Fig. 1A), 47.4% (9/19) for AR-HSP (Fig. 1B) and 50% (3/6) for HSP patients with TCC. The panel diagnostic yield for pure-HSP was 75% (9/12, Fig. 1C) and for complicated-HSP was 35.3% (6/17, Fig. 1D).

Among index cases with a confirmed genetic diagnosis, eight presented with pure (6 SPG4, 1 SPG7, 1 SPG5) and six with complicated HSP phenotypes (4 SPG11, 1 SPG7, 1 CTX). In a single case with pure HSP, a probable diagnosis was indicated by the presence of a homozygous likely pathogenic variant on *SPG11*.

3.3. Sanger sequencing

Sanger sequencing confirmed all variants classified at least as likely pathogenic on NGS. Sanger sequencing of affected relatives diagnosed 15 additional cases, totaling 30 patients; 15 patients (6 families) with SPG4, 6 patients (5 families) with SPG11, 4 patients each with SPG7 (2 families) and SPG5 (1 family), and one patient with CTX. Detailed

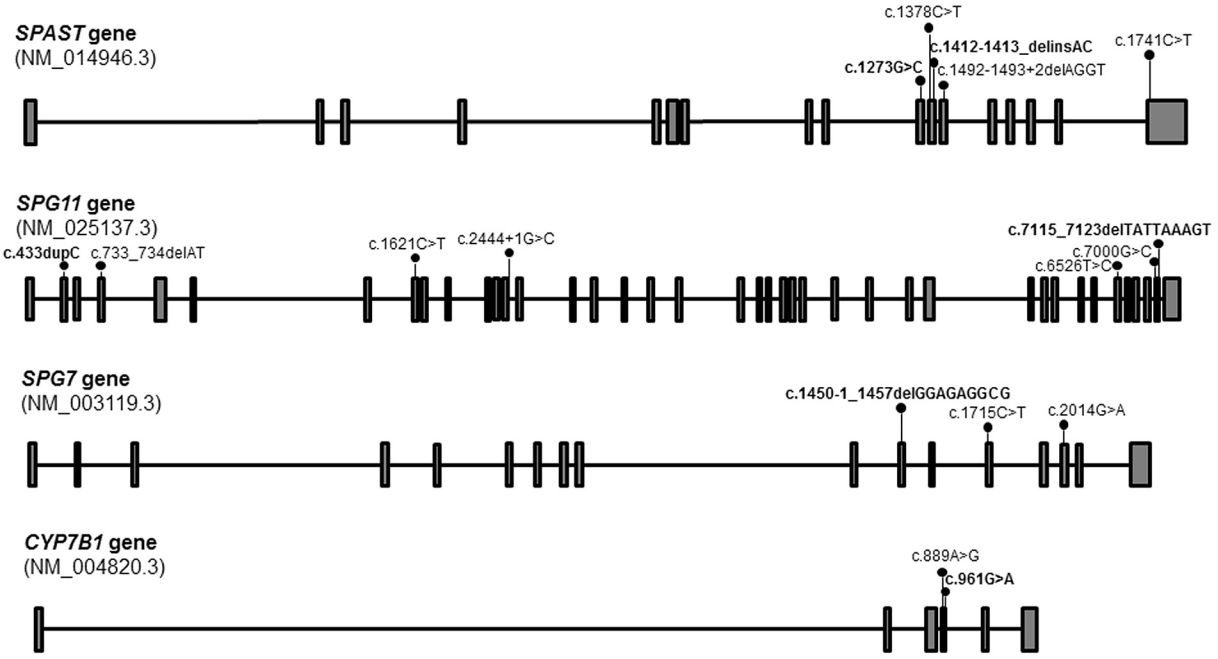

Fig. 2. Schematic representations of mutations in *SPAST, SPG11, SPG7* and *CYP7B1* genes. Novel mutations are depicted in bold.

Table 1
Novel variants in HSP genes.

Gene	*Nucleotide change*	*Predicted AA change*	*Mutation type*	*AF (ExAC)*	*AF (gnomAD)*	*AF (1000 genomes)*	*SIFT*	*Polyphen2*	*Mutation taster*	*MCAP*	*GERP + +*	*Segregation*	*Functional evidence*	*De Novo*	*N families*	*Classification*
SPAST	c.1273G > C	p.Ala425Pro	Missense	0	0	0	0	1	1	0.270	5.62	Yes	NA	Yes	1	Pathogenic
SPAST	c.1412_1413delinsAC	p.Gly471Asp	Missense	0	0	0	0	1	0.999	NA	4.6 (0.2)	Yes	NA	NA	1	Pathogenic
SPG11	c.7115_7123delTATTAAAGT	p.Leu2372_Ser2375delinsSer	Indel	0	0	0	NA	NA	0.999	NA	1.55 (3.9)	NA	NA	NA	1	Likely pathogenic
SPG11	c.433dupC	p.Gln145Profs*18	Frameshift	0.000008	0.000004	0	NA	NA	1	NA	6.1	Yes	NA	No	1	Pathogenic
SPG7	c.1450-1_1457delGGAGAGGCG	p.Glu484Glyfs*4	Deletion	0.0003	0.0002	0	NA	NA	1	NA	4.02 (1.5)	NA	NA	No	2	Pathogenic
CYP7B1	c.961G > A	p.Glu321Lys	Missense	0	0	0	0	1	0.999	0.297	5.93	Yes	Yes	No	1	Pathogenic

AA, amino acid; AF, allele frequency; NA not available. For GERP + + data is shown as mean (standard deviation) or raw value.

individual data is given in Supplemental Table 2.

3.4. Novel variants

Of the 18 at least likely pathogenic variants identified, 6 (identified in 7 families) were novel (Fig. 2). All novel variants were predicted to be pathogenic by *in silico* analysis and all occurred at conserved residues, see Table 1. Variant p.Ala425Pro in *SPAST* segregated with disease phenotype in the index case and in three affected sons, and occurred *de novo* in the index case. A homozygous *indel* in *SPG11*, c.7115_7123delTATTAAAGT was identified in one index case with pure phenotype, this variant was predicted to activate an exonic cryptic acceptor site and to alter an exonic splicing enhancer site on HSF3.0, and to lead to the loss of the splice enhancer site SRSF2 on ESE3.0 prediction, segregation analysis of this variant; however, was not possible. A small deletion in *SPG7* (c.1450-1_1457delGGAGAGGCG) was identified in two unrelated families (HSP41 and HSP42). In HSP41, both c.1450-1_1457delGGAGAGGCG and the previously reported pathogenic missense variant p.Gly672Arg [23] were found in *SPG7*; but segregation analysis was not possible. In HSP42, segregation analysis confirmed that c.1450-1_1457delGGAGAGGCG was in *trans* with the previously reported pathogenic missense variant p.Ala572Val [24]. These variants segregated with phenotype in three siblings. Variant p.Glu321Lys in *CYP7B1* occurred in *trans* with the pathogenic missense variant p.Thr297Ala [25]. These variants segregated with the phenotype in four affected siblings. We measured 27-hydroxycholesterol (27-OHC) levels as previously described [26]. Mean 27-OHC levels were 7-fold higher in plasma (mean ± SD 1177.2 ± 178.6 ng/ml; normal range: 89–243 ng/ml) and 30-fold higher in cerebrospinal fluid (CSF) (15.2 ± 2.6 ng/ml; normal range: 0.5–0.8 ng/ml) in the affected siblings when compared to healthy controls, providing functional evidence of pathogenicity.

We also classified variants with at least 30-fold coverage and with allele frequency of < 1% on ExAC. The list of these rare variants that were classified from benign to uncertain significance are detailed on Supplemental Table 3.

3.5. Autosomal dominant mutations

3.5.1. SPG4

All AD-HSP (6 families, 15 patients, 10 females) were a molecular diagnosis was reached carried variants in *SPAST*; all of them presented as pure-HSP (Table 2). Age at onset was highly variable varying from 1 to 73 years of age. Use of canes/walkers was required by 40% of SPG4 patients and only a single patient required a wheelchair. Two missense variants were associated with early-onset. Pyramidal involvement started in the first year of life in all carriers of p.Ala425Pro variant, and at 4 and 7 years of age in carriers of c.1412_1413delinsAC (p.Gly471Asp) variant. Interestingly, p.Gly471Asp amino acid change due to a nucleotide substitution (c.1412G > A) was also previously associated to childhood-onset HSP phenotype [27]. No SPG4 index case carried the p.Ser44Leu disease modifying polymorphism in *SPAST*.

3.6. Autosomal recessive mutations

Patients with AR-HSP are shown in Table 3.

3.6.1. SPG11

Mutations in *SPG11* were identified in 5 families (6 patients, 4 females); all patients presented with a complicated-HSP phenotype except for patient HSP8 (homozygous for the novel *SPG11* indel c.7115_7123delTATTAAAGT). One patient (HSP45) carried 3 *SPG11* previously reported variants: c.1621C > T [28], c.6526T > C [28], and c.7000G > C [29]. On segregation analysis, paternal allele carried both c.1621C > T and c.6526T > C variants in *cis* while the maternal one carried the c.7000G > C mutation. Both paternal variants

Table 2
Clinical and molecular findings of families with defined autosomal dominant HSP diagnosis.

Fam	*Subject*	*Gene*	*Nucleotide change*	*Predicted AA change*	*Mutation type*	*Phenotype*	*AO*	*DD*	*SPRS*	*Canes/ walker*[a]	*Wheel-chair*[a]	*Additional features*
2	HSP5	*SPAST*	c.1267G > T	p.Val423Leu	Missense	Pure	23	8	20	28	No	No
9	HSP12	*SPAST*	c.1492_1493 + 2delAGGT	p.Arg498Alafs*13	Deletion	Pure	30	11	22	43	No	No
9	HSP13	*SPAST*	c.1492_1493 + 2delAGGT	p.Arg498Alafs*13	Deletion	Pure	48	20	37	NA	63	No
9	HSP14	*SPAST*	c.1492_1493 + 2delAGGT	p.Arg498Alafs*13	Deletion	Pure	41	3	22	44	No	No
12	HSP18	*SPAST*	c.1273G > C	p.Ala425Pro	Missense	Pure	1	27	10	No	No	Dysarthria, mild intellectual disability
12	HSP19	*SPAST*	c.1273G > C	p.Ala425Pro	Missense	Pure	1	7	19	7	No	Mild intellectual disability
12	HSP20	*SPAST*	c.1273G > C	p.Ala425Pro	Missense	Pure	1	3	8	No	No	No
12	HSP21	*SPAST*	c.1273G > C	p.Ala425Pro	Missense	Pure	1	2	18	No	No	No
15	HSP25	*SPAST*	c.1412_1413delinsAC	p.Gly471Asp	Missense	Pure	7	26	31	24	No	No
15	HSP26	*SPAST*	c.1412_1413delinsAC	p.Gly471Asp	Missense	Pure	4	2	5	No	No	No
20	HSP37	*SPAST*	c.1378C > T	p.Arg460Cys	Missense	Pure	55	4	20	No	No	No
20	HSP38	*SPAST*	c.1378C > T	p.Arg460Cys	Missense	Pure	45	11	13	55	No	No
20	HSP39	*SPAST*	c.1378C > T	p.Arg460Cys	Missense	Pure	73	10	20	No	No	No
20	HSP40	*SPAST*	c.1378C > T	p.Arg460Cys	Missense	Pure	62	0	8	No	No	No
24	HSP46	*SPAST*	c.1741C > T	p.Arg581*	Nonsense	Pure	45	10	14	No	No	No

AO, age at onset; DD, disease duration; NA, not available; SPRS, Spastic Paraplegia Rating Scale.
[a] Age in years at loss of independent walking or wheelchair dependency.

(c.1621C > T and c.6526T > C) were reported in a single patient with amyotrophic lateral sclerosis phenotype, but authors were unable to evaluate if variants were in *cis* or *trans* [28]. Our data suggest that c.1621C > T and c.6526T > C variants are linked and that at least the proximal nonsense variant c.1621C > T (p.Gln541*) is pathogenic. Most frequent complicating features in SPG11 were intellectual disability/dementia, 5/6 (83.3%); motor neuron involvement, 4/6 (66.7%); dysarthria, 3/6 (50%); and keratoconus, 2/6 (33.3%). Only the patient with pure HSP phenotype was not wheelchair dependent. TCC and the "ears of the lynx" sign was present in 3/4 (75%) SPG11 patients (Table 3). The remaining 2/6 patients did not perform brain MRI.

3.6.2. SPG7

Mutations in *SPG7* were identified in 2 families (4 patients, 2 females); one patient with pure and 3 with complicated forms. Most frequent complicating features in SPG7 were dysarthria, 4/4 (100%); ataxia, 3/4 (75%); ptosis, 2/4 (50%); and dysphagia, 2/4 (50%). We had an unsolved case with SPG7 suspicion where the index patient (HSP36) presented a single heterozygous pathogenic variant in *SPG7* (p.Leu78*) that was previously reported to segregate in both recessive and dominant manners [30,31]. The reported family with presumed dominant segregation of p.Leu78* was of gipsy ancestry. The index case presented the *SPG7* variant in heterozygous state (age at onset of 20 years). Contrastingly, patient's mother and aunt, who were also affected by a similar disorder but with later age at onset (32 and 48 years of age), presented p.Leu78* in both alleles [30]. Copy number variations of *SPG7* were not planned in our study and this case remained unsolved.

3.6.3. SPG5 and CTX

Mutations in *CYP7B1* (SPG5) were identified in 1 family (4 patients, 2 females), where 3 cases showed pure HSP and the other had ataxia, dysphagia and parkinsonism as complicating symptoms. Three out of the four patients required canes/walkers for locomotion at mean disease duration of 15.5 years. Pathogenicity of the new p.Glu321Lys variant in *CYP7B1* was confirmed by segregation analysis and by increased levels of 27-hydroxycholesterol detected in both CSF and plasma [26]. Two patients presented dyslipidemia and were treated with 20 mg/day simvastatin for 2 years. Both reported symptoms stabilization during this period. Mutation in *CYP27A1* (CTX) was identified in a single female patient with HSP-phenotype complicated by ataxia, dysarthria, extrapyramidal findings and cognitive impairment. She presented a complicated-HSP phenotype. Later, a follow-up brain MRI depicted new cerebellar white matter lesions (Supplemental Fig. 1) suggestive of CTX. Plasma cholestanol levels were elevated, 1.64 mg/dl (normal range: 0.17 ± 0.12 mg/dl, cut-off for CTX diagnosis > 1 mg/dl, OSHU, Oregon, USA) and both Sanger sequencing and NGS-HSP panel revealed a homozygous p.Arg474Gln mutation in *CYP27A1* [32] confirming CTX. Bilateral subcapsular cataract was present at age 47. There was no clear xanthoma, and her spine MRI was unremarkable.

4. Discussion

HSP are a heterogeneous group of rare and neglected diseases in which molecular analysis is essential to establish diagnosis and to provide adequate genetic counselling. NGS panel of 12 HSP-related genes was used as an initial molecular diagnostic approach. Genes have been selected to represent the most prevalent forms of HSP in different populations, including two potentially treatable conditions, SPG5 and CTX. With this strategy, > 50% of HSP families from Rio Grande do Sul, Brazil received at least a likely molecular diagnosis, allowing the diagnosis of additional 15 individuals by target mutation analysis.

Several studies in the last years addressed different molecular strategies for the diagnosis of HSP; most using NGS technologies, as whole-exome (WES), targeted exome and panel-based sequencing [5,6,8,31,33,34]. Most of these studies adopted multiple molecular approaches with an overall diagnostic yield varying from 25.0%–52.5% [5,8,31,33,34]. Mixed molecular approaches and absence of pre-specified selection criteria limit previous information on diagnostic yield for HSP. An exception is a large series of consanguineous AR-HSP where the genetic basis was identified in approximately 75% of cases (33% known and 42% new genes) with WES of 2 or 3 individuals and a sophisticated confirmation approach [6]. With this exception, our selection of HSP genes and patients turned out to be among the most successful approaches published so far.

Our study described a systematical evaluation of the mutation spectrum of HSP in Rio Grande do Sul, adding information to previous reports on SPG4 [35] and to case series of SPG11 [36] from southeast and southern regions of Brazil. Rio Grande do Sul is the southernmost state of Brazil, and is characterized by a major European background, as 83% of its population is self-identified as being from European descent [37]. Six novel disease-related variants (5 with confirmed pathogenicity), and novel clinical findings, such as the presence of keratoconus in SPG11, were reported. We have also strengthened evidence that CTX should be included in the differential diagnosis of HSP, regardless the presence of xanthomas.

Table 3
Clinical and molecular findings of families with at least a probable diagnosis of autosomal recessive HSP.

Fam	*Subject*	*Gene*	*Nucleotide change*	*Predicted AA change*	*Zygosity*	*trans*	*Classification*	*Phenotype*	*AO*	*DD*	*SPRS*	*TCC*	*Canes/ walker*[a]	*Wheel-chair*[a]	*Additional features*
1	HSP1	*CYP7B1*	c.889A > G/c.961G > A	p.Thr297Ala/ p.Glu321Lys	Compound heterozygous	Yes	Pathogenic	Pure	30	20	30	0	40	No	No
1	HSP2	*CYP7B1*	c.889A > G/c.961G > A	p.Thr297Ala/ p.Glu321Lys	Compound heterozygous	Yes	Pathogenic	Pure	30	10	17	0	No	No	Gaze-evoked nystagmus
1	HSP3	*CYP7B1*	c.889A > G/c.961G > A	p.Thr297Ala/ p.Glu321Lys	Compound heterozygous	Yes	Pathogenic	Complicated	35	26	36	0	40	No	Abnormal stance, dysphagia, bradykinesia, abnormal tactile-algesic discrimination
1	HSP4	*CYP7B1*	c.889A > G/c.961G > A	p.Thr297Ala/ p.Glu321Lys	Compound heterozygous	Yes	Pathogenic	Pure	36	12	28	0	45	No	No
5	HSP8	*SPG11*	c.7115_7123delTATTAAAGT	p.Leu2372_Ser 2375delinsSer	Homozygous	NA	Likely pathogenic	Pure	45	6	23	0	No	No	No
7	HSP10	*SPG11*	c.2444 + 1G > C	NA	Homozygous	NA	Pathogenic	Complicated	15	15	44	+	NA	25	Ataxia, parkinsonism, dysarthria, intellectual disability. Brain WML (ears of the lynx sign) and atrophy
8	HSP11	*CYP27A1*	c.1421G > A	p.Arg474Gln	Homozygous	NA	Pathogenic	Complicated	22	27	24	0	NA	41	Ataxia, parkinsonism, dysarthria, peripheral neuropathy, intellectual disability, dystonia, cataract. Cerebellar WML
21	HSP41	*SPG7*	c.1450-1_1457delGGAGAGGCG/ c.2014G > A	p.Glu484Glyfs*4/ p.Gly672Arg	Compound heterozygous	NA	Pathogenic	Pure	34	20	NA	0	No	No	Pes cavus, mild ptosis, mild dysarthria
22	HSP42	*SPG7*	c.1450-1_1457delGGAGAGGCG/ c.1715C > T	p.Glu484Glyfs*4/ p.Ala572Val	Compound heterozygous	Yes	Pathogenic	Complicated	22	39	NA	0	No	No	Ataxia, nystagmus, broken-up smooth pursuit, hypermetric saccades, dysarthria, dysphagia, abnormal tactile-algesic sensitivity, ptosis
22	HSP43	*SPG7*	c.1450-1_1457delGGAGAGGCG/ c.1715C > T	p.Glu484Glyfs*4/ p.Ala572Val	Compound heterozygous	Yes	Pathogenic	Complicated	40	28	NA	0	NA	60	Ataxia, dysarthria, psychiatric symptoms
22	HSP44	*SPG7*	c.1450-1_1457delGGAGAGGCG/ c.1715C > T	p.Glu484Glyfs*4/ p.Ala572Val	Compound heterozygous	Yes	Pathogenic	Complicated	22	48	NA	0	NA	69	Ataxia, dysarthria, dysphagia
23	HSP45	*SPG11*	c.1621C > T/c.7000G > C	p.Gln541*/ p.Ala2334Pro	Compound heterozygous	Yes	Pathogenic	Complicated	20	9	41	+	24	29	Intellectual disability, keratoconus, signs of motor neuron disease. Brain WML (ears of the lynx sign) and atrophy
25	HSP47	*SPG11*	c.733_734delAT	p.Met245Valfs*2	Homozygous	NA	Pathogenic	Complicated	14	16	35	NA	NA	27	Intellectual disability, keratoconus, signs of motor neuron disease
28	HSP50	*SPG11*	c.433dupC	p.Gln145Profs*18	Homozygous	NA	Pathogenic	Complicated	18	20	28	+	NA	30	Intellectual disability, dysarthria, pes cavus, signs of motor neuron disease. Brain WML (ears of the lynx sign) and atrophy.
28	HSP51	*SPG11*	c.433dupC	p.Gln145Profs*18	Homozygous	NA	Pathogenic	Complicated	20	24	38	NA	NA	34	Intellectual disability, dysarthria, signs of motor neuron disease

AO, age at onset; DD, disease duration; NA, not available; SPRS, Spastic Paraplegia Rating Scale; TCC, thin corpus callosum; WML, white matter lesions.
[a] Age in years at loss of independent walking or wheelchair dependency.

4.1. Autosomal dominant HSP

Point mutations in *SPAST* are the most common cause of AD-HSP in Rio Grande do Sul, which is consistent with studies from other countries [5,7,8]. *SPAST* mutations were found in 60% of AD-HSP families, a higher relative frequency than reported in a recent collaborative Brazilian study [35]. Although one could argue that our results might be biased by a smaller sample of families, our results were very similar to the largest cohort of HSP patients published so far (222 individuals with AD-HSP). This German study found that 61% of AD-HSP were due to *SPAST* mutations [5]. We adopted the same inheritance pattern criteria as this study. Previous studies suggested that SPG3A is the main cause of AD-HSP with childhood onset [38]. In our cohort, 3/10 index patients started symptoms with ≤ 10 years-old, 2/3 had mutations in *SPAST* and the other remained without diagnosis. These results were similar to the German cohort were SPG4 was twice as common in this subgroup as SPG3A [5]. All Brazilian SPG4 families in our cohort carried different mutations (Fig. 1). No other genetic diagnosis was found for AD-HSP in our study.

4.2. Autosomal recessive HSP

This is the first systematic assessment of AR-HSP in Brazil to date. We have obtained a higher diagnostic yield (47.4%) than previous studies using Sanger sequencing of target genes [7]; and similar yield to recent studies using NGS technologies [8,33,34]. SPG11 was the most common form of AR-HSP in our region being responsible for 26.3% families, followed by SPG7 that was diagnosed in 10.5%, and SPG5 and CTX that were diagnosed in 5.2% of families each. Similar to previous studies [5], SPG11 patients presented a more severe disease than other genotypes. TCC and “ears of the lynx” sign were frequent findings on brain MRI of SPG11 patients. Interestingly, 2/6 SPG11 patients (2 families) presented bilateral keratoconus, an extra-neurological feature that has not been previously associated with SPG11, broadening the spectrum of complicating symptoms going along with this genotype. SPG7 was the second most frequent form of AR-HSP in our population. Patients with SPG7 had long disease durations (> 30 years) with relatively few handicap.

4.3. Treatable forms of HSP

We found a single family with four individuals with SPG5, which, similarly to the German cohort [5], presented a more unfavorable disease course than SPG4 and SPG7. SPG5 is caused by the loss of function of oxysterol-7 α-hydroxylase, an enzyme involved in the degradation of cholesterol into primary bile acids. Recent reports suggest that statins might be effective in reducing the levels of the accumulated toxic oxysterol 27-hydroxycholesterol, being a potential therapy for the disease [39]. In our cohort, one case of CTX that did not present tendon xanthomas was found. In agreement with CTX patients' reports with spinal xanthomatosis [40] and of pure-HSP without xanthomas [41], our study clearly indicates that CTX should be considered in the differential diagnosis of HSP regardless the presence of xanthomas, especially as being a treatable disorder of bile acid synthesis.

4.4. Study limitations

The eligibility criteria of our study may have selected a population with high risk of an inherited disorder. The enriched population might have increased the diagnostic yield of our approach. However, it does not influence the general genotype distribution that can be expected within populations with comparable ethnicity and family history. On the other hand, our approach may have underestimated the real frequency of genotypes as MLPA analysis concerning genomic deletions/duplications was not performed. The diagnostic yield of the NGS panel in isolated cases of Brazilian population needs to be addressed in future studies as well as the proportion of HSP forms in Brazilian states from northern and northeast regions, which present greater African and Native Americans background and lower European descent, from 23 to 30% [37].

5. Conclusion

The targeted gene panel with twelve HSP genes seems to be an adequate strategy for HSP diagnosis that could be employed as a first-line investigation of patients with suspicion of HSP. Harder to interpret and more expensive NGS approaches, as WES, could be used as the next step for patients with negative results in the panel analysis. Due to the great proportion of SPG4 among AD-HSP, another possible strategy would be to perform Sanger sequencing of the 17 exons of *SPAST* for patients with clear AD inheritance, followed by similar targeted panel if negative results were found. SPG4 is the most frequent form of AD-HSP and SPG11 of AR-HSP in Rio Grande do Sul, Brazil.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2017.10.010.

Conflict of interest

All authors report no conflict of interest related to the study.

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ANEXO II – Artigo. “Molecular and biochemical biomarkers for diagnosis and therapy monitorization of Niemann-Pick type C patients.”

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Molecular and biochemical biomarkers for diagnosis and therapy monitorization of Niemann-Pick type C patients

Tatiane Grazieli Hammerschmidt[a,c,*], Graziela de Oliveira Schmitt Ribas[c,d], Maria Luiza Saraiva-Pereira[b,c], Márcia Polese Bonatto[b], Rejane Gus Kessler[b,e], Fernanda Timm Seabra Souza[b,d], Franciele Trapp[d], Kristiane Michelin-Tirelli[b,d], Maira Graeff Burin[b,d], Roberto Giugliani[b,d,e], Carmen Regla Vargas[a,b,c,d,*]

[a] Departamento de Análises, Faculdade de Farmácia, UFRGS, Avenida Ipiranga, 2752, CEP 90610-000, Porto Alegre, RS, Brazil
[b] Programa de Pós-Graduação em Ciências Biológicas, Bioquímica, UFRGS, Rua Ramiro Barcelos, 2600, CEP 90035-003, Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Ciências Farmacêuticas, UFRGS, Av. Ipiranga, 2752, CEP 90610-000, Porto Alegre, RS, Brazil
[d] Serviço de Genética Médica, HCPA, Rua Ramiro Barcelos, 2350, CEP 90035-003, Porto Alegre, RS, Brazil
[e] Departamento de Genética, IB, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

Background: Niemann-Pick type C (NP-C), one of 50 inherited lysosomal storage disorders, is caused by NPC protein impairment that leads to unesterified cholesterol accumulation in late endosomal/lysosomal compartments. The clinical manifestations of NP-C include hepatosplenomegaly, neurological and psychiatric symptoms. Current diagnosis for NP-C is based on observation of the accumulated cholesterol in fibroblasts of affected individuals, using an invasive and time expensive test, called Filipin staining. Lately, two metabolites that are markedly increased in NP-C patients are arising as biomarkers for this disease screening: 7-ketocholesterol and cholestane-3β,5α,6β-triol, both oxidized cholesterol products.

Objective: In this work, we aimed to evaluate the performance of cholestane-3β,5α,6β-triol analysis for the screening and monitoring of NPC patients, correlating it with chitotriosidase levels, Filipin staining and molecular analysis. It was investigated 76 non-treated individuals with NP-C suspicion and also 7 patients with previous NP-C diagnosis under treatment with miglustat, in order to verify the cholestane-3β,5α,6β-triol value as a tool for therapy monitoring.

Results: Considering molecular assay as golden standard, it was verified that cholestane-3β,5α,6β-triol analysis presented 88% of sensitivity, 96.08% of specificity, a positive and negative predictive value calculated in 91.67% and 94.23%, respectively, for the diagnosis of NP-C. Chitotriosidase levels were increased in patients with positive molecular analysis for NP-C. For Filipin staining, it was found 1 false positive, 7 false negative and 24 inconclusive cases, showing that this assay has important limitations for NP-C diagnosis. Besides, we found a significant decrease in cholestane-3β,5α,6β-triol concentrations in NP-C patients under therapy with miglustat when compared to non-treated patients.

Conclusion: Taken together, the present data show that cholestane-3β,5α,6β-triol analysis has a high potential to be an important NP-C screening assay, and also can be used for therapy monitorization with miglustat in NP-C patients.

1. Introduction

Niemann-Pick type C (NP-C) is a lysosomal lipid storage disease (LSD) with autosomal recessive inheritance, caused by mutations in NPC1 or NPC2 genes and mainly characterized by unesterified cholesterol accumulation in late endosomal/lysosomal (LE/L) compartments (Vanier and Millat, 2003). As a result of this genetic defect, there is an accumulation of other lipids, such as glucosylceramide, GM1 and GM2

Abbreviations: NP-C, Niemann-Pick type C; LSD, lysosomal storage disorder; CNS, central nervous system; 7-KC, 7-ketocholesterol; GC/MS, gas chromatography/mass spectrometry; LC/MS-MS, liquid chromatography/tandem mass spectrometry; LDL, low density lipoprotein; LE, late endosomal; ROS, reactive oxygen species; CSF, cerebrospinal fluid; HSEM, horizontal saccadic eye movement

* Corresponding authors at: Serviço de Genética Médica, HCPA, Rua Ramiro Barcelos, 2350, CEP 90035-003, Porto Alegre, RS, Brazil.
E-mail addresses: tatiane.hammerschmidt@gmail.com (T.G. Hammerschmidt), crvargas@hcpa.ufrgs.br (C.R. Vargas).

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gangliosides in peripheral tissues (liver, spleen and lungs) and in central nervous system (CNS) of the affected individuals (Patterson, 2003). In this way, clinical presentation is extremely heterogenous and includes hepatosplenomegaly, neonatal jaundice, dysarthria, dysphagia, vertical supranuclear gaze palsy, psychiatric and/or cognitive dysfunction and it may vary between patients in terms of age-onset and disease severity, delaying the recognition of the disease (Patterson, 2003). Regarding its rarity, NP-C incidence is estimated in 1/89,000, but this data may be significant uncertain because of a late-onset NPC1 phenotype, with a markedly higher incidence, on the order of 1/19,0001/36,000. (Wassif et al., 2016).

Despite there is no cure for NP-C, the management of symptoms is an important goal in therapy for these patients (Patterson et al., 2012). Miglustat, a small iminosugar molecule able to cross the blood-brain barrier and to reversibly inhibit glucosylceramide synthase (the first enzyme in glycosphingolipid synthesis) was proposed for the treatment of the disease (Fecarotta et al., 2015). The efficacy of miglustat on neurological manifestations progression has been studied in NPC patients enrolled in international clinical trials and observational studies. Data from one-year treatment of juvenile and adult NPC patients suggested that miglustat improves or stabilizes several neurological manifestations (Fecarotta et al., 2015; Patterson et al., 2007). Cyclodextrins are also showing some promising results in several studies, but the mechanisms are not yet completely established (Atger et al., 1997; Aqul et al., 2011). Treatment with subcutaneously hydroxypropyl-β-cyclodextrin (HPβCD) of a NP-C murine model shows an improvement in cholesterol metabolism in the liver and the most other organs, as well as ameliorates cerebellar neurodegeneration (Ramirez et al., 2010; Nusca et al., 2014). Administration of intracisternal HPβCD to NP-C cats with ongoing cerebellar dysfunction slowed disease progression, increased survival time, and decreased the accumulation of brain gangliosides (Vite et al., 2015). Recent phase I/II clinical trial showed that patients with NPC1 treated with intrathecal HPβCD had slowed disease progression with an acceptable safety profile (Ory et al., 2017).

Due to its heterogeneity in symptoms and clinical nature, prompt diagnosis for NP-C is a challenge. Once considered standard gold assay for NP-C diagnosis, Filipin staining is based in a coloration using a fluorescent antibiotic, which binds to cholesterol accumulated in fibroblasts from NP-C patients. However, a variant profile in fluorescent pattern can cause doubts in assay interpretation. Besides, Filipin test is an invasive and expensive procedure, requiring a specialized center to perform it (Vanier et al., 2016). Fluorescence microscopy is a valuable tool for studying intracellular transport processes, but this method can be challenging for lipid molecules, such as cholesterol (Maxfield and Wüstner, 2012). Alternatively, accumulated cholesterol can be also visualized by immunofluorescence using a cholesterol-binding bacterial toxin, perfringolysin O (Kwiatkowska et al., 2014).

Determination of chitotriosidase is also used as a general and potential indicator of LSD, including NP-A, NP-B and NP-C. However, normal levels of this enzyme may occur in these patients, showing a lack of sensitivity and specificity of this assay (Vanier et al., 2016). Therefore, definitive diagnosis depends on molecular analysis of NPC1 and NPC2 genes for most cases.

In NPC deficient cells, there is an association between oxidative stress and accumulated cholesterol by increased production of reactive oxygen species and oxidative damage (Ribas et al., 2012). Cholesterol can suffer oxidation in different ways, what could be mediated by enzymes or through non-enzymatic reactions (Fig. 1). Oxidized cholesterol products, specifically cholestane-3β,5α,6β-triol (3β,5α,6β-triol) and 7-ketocholesterol (7-KC), are markedly increased in plasma of NP-C patients and in animal models, whereas remain normal in other LSD (Jiang et al., 2011). These findings indicate that 3β,5α,6β-triol and 7-KC are NPC1 disease-specific biochemical markers and suggest a possible utility of these markers in diagnosis and therapeutic evaluation of NPC1 disease (Jiang et al., 2011). Determination of these metabolites can be performed using gas chromatography/mass spectrometry (CG/MS) or by liquid chromatography/tandem mass spectrometry (LC–MS/MS) methods (Boenzi et al., 2016; Porter et al., 2010). Therefore, oxysterols analysis by LC–MS/MS became an alternative and non-invasive assay to screen potential NP-C patients, as well as a tool for treatment monitoring. However, its correlation with tests currently used for NP-C diagnosis must be better investigated.

In order to evaluate the 3β,5α,6β-triol measurement for NP-C therapy monitorization and also as a biomarker for NP-C diagnosis, in this work we analyzed 3β,5α,6β-triol and chitotriosidase levels, Filipin staining and mutations in NPC genes in biological samples from patients with NP-C suspicious and in treated NP-C patients referred to our specialized center in South Brazil.

2. Materials and methods

2.1. Samples

Skin biopsy and blood samples were obtained from 76 individuals with suspicious of NP-C disease in Medical Genetics Service of *Hospital de Clínicas de Porto Alegre*, Brazil. Additionally, 7 blood samples from patients with previous diagnosis of NP-C under miglustat therapy (therapeutic regime: 200 mg thrice a day) were collected. These blood samples were collected in tubes with EDTA as anticoagulant, centrifuged for five minutes at 3000 rpm and plasma was frozen at −80 °C. The clinical features presented by these patients included dystonia, dysphagia, seizures, vertical supranuclear palsy and psychiatric disorders.

This work was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All subjects in this study signed an informed consent, and this project was approved by the Ethics Committee of *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil under the registration number 13-0239.

2.2. Cholestane-3β,5α,6β-triol analysis

Levels of triol were determined by LC–MS/MS in EDTA-plasma, using cholestane-3β,5α,6β-triol D7 as internal standard and derivatization with dimetylglycine, according to Jiang et al. (2011), with some modifications. The chromatographic separation was performed on a column ACE 3 C18 (4.6 × 150 mm, 3 μm) using a gradient of mobile phase A (0.1% formic acid + 1 mM ammonium acetate in water) and mobile phase B (0.1% formic acid + 1 mM ammonium acetate in methanol). Detection was performed with a Waters Quattro Micro API tandem mass spectrometer in positive atmospheric-pressure chemical ionization (APCI) and multiple reaction monitoring (MRM) mode. The optimized MS/MS conditions were as follows. APCI probe temperature and source temperature were 500 °C and 120 °C, respectively; cone voltage and coll energy were 30 V and 20 eV, respectively; desolvation gas flow and cone gas flow were 600 L/h and 50 L/h, respectively; monitored mass transitions were 591.5 → 104 for the triol and 598.8 → 103.8 for the internal standard; retention time was 5.5 min and quantification was based on standard curve ranging from 2 to 400 ng/mL for the triol (Ribas et al., 2016).

2.3. Chitotriosidase assay

Plasma enzyme determination was performed according to Hollak et al. (Hollak et al., 1994), using 4-methylumbelliferylβ-DN,N′,N″-triacetylchitotrioside as reaction substrate. The mixture for the enzyme assay was composed by 5 μL of acidified plasma and 26 μM of substrate dissolved in 100 mM citrate plus 200 mM phosphate buffer (pH 5.2), obtaining a total volume of 105 μL. This mixture was incubated for 15′ at 37 °C. Glycine-sodium hydroxide buffer (0.5 M, pH 10.3) was used as stop solution for the reaction and the fluorescence was determined with a Hitachi F2000 spectrofluorometer (λ excitation 365 nm and emission 450 nm). Normal range was considered between 8.8 and 132.0 nmol/h/

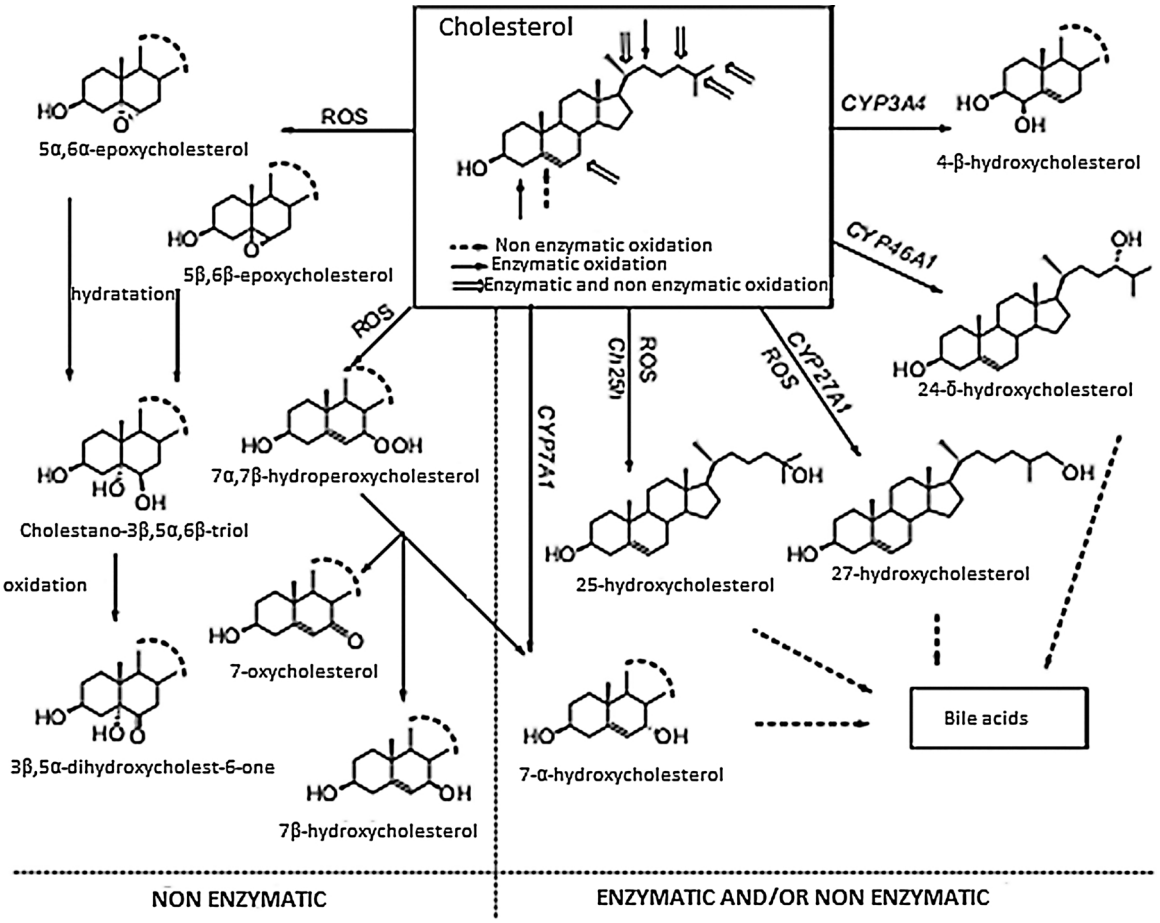

Fig. 1. Different pathways for oxysterols production. Oxysterols can be formed enzymatically, such as by oxidoreductases from cytochromes P450 or by hydrolases as cholesterol-25-hydroxylase (Ch25 h). They also can be generated through non enzymatic oxidation reactions mediated by reactive oxygen species (ROS), such as the hydroxyl radical (OH·), which promote oxidation especially on the carbons 5 and 7 from cholesterol structure.

mL.

2.4. Filipin staining

Skin biopsy samples were used for fibroblasts culture with HAM-F-10 medium and 10% of Fetal Calf Serum. After the cells reached confluence, Low Density Lipoprotein (LDL) was added to the culture. After two days, cells were transferred to slides and stained with Filipin reagent for histological examination in a fluorescent microscope. Intracellular lipid accumulation was determined as described in Blanchette-Mackie et al., (1988); 'classical' pattern of cholesterol accumulation showed a strong fluorescence in perinuclear vesicles (positive). The pattern of cells samples was categorized in normal (clear, no fluorescence), inconclusive or variant (moderated fluorescence) or typical or "classical" (high fluorescence).

2.5. Molecular analysis

Mutation analysis was performed using DNA isolated by standard method from patients' blood samples. Coding sequences and flanking regions of the NPC1 and the NPC2 genes were amplified with PCR, purified and submitted to direct DNA sequencing using the BigDye1 Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Products were then submitted to capillary electrophoresis in an ABI PRISM1 3130xl Genetic Analyzer, and sequences were analyzed with DNA Sequencing Analysis software v. 5.2 (Applied Biosystems). Mutations were confirmed by sequencing an independent DNA sample with both forward and reverse primers. Molecular analyses were conducted in blood of all patients of this study.

2.6. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Unpaired Student's *t*-test was used for groups comparison, and difference was considered significant when $p < 0.05$. All analysis and graphs were performed using the software GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA – version 7.0 for Windows®) in a compatible PC.

3. Results

Subjects were segregated in 2 different groups: patients with 3β,5α,6β-triol levels higher than 100 ng/mL (group A) and lower than 100 ng/mL (group B). This separation was established according to the cut-off value founded for this analyte in our laboratory. Table 1 shows that individuals from group A also presented higher chitotriosidase activity compared to group B, although this biomarker is not specific for NP-C, and also can be found increased in others LSD, such as Gaucher disease and NP-A/B (Sheth et al., 2010). Molecular analysis showed that 2 patients from group A did not have any mutation in NPC gene, excluding the existence of the disease. On the other hand, 3 individuals from group B presented positive molecular analysis for NPC, despite low levels of 3β,5α,6β-triol. Results of molecular analysis are summarized in Table 2. Considering these data and applying MedCalc software, the analysis of 3β,5α,6β-triol levels presented 88% of

Table 1
Results of Filipin staining, chitotriosidase activity and molecular analysis in patients with cholestane-3β,5α,6β-triol levels higher than 100 ng/mL (Group A) and lower than 100 ng/mL (Group B).

	3β,5α,6β-triol concentration (ng/mL; mean ± SD)	[a]CT activity (nmol/h/mL; mean ± ± SD)	Positive cases by Filipin staining	Negative cases by Filipin staining	Inconclusive cases by Filipin staining	Positive cases by molecular analysis	Negative cases by molecular analysis
Group A (n = -24)	164.6 ± 10.67	929.3 ± 346.5	10	0	2	22	2[b]
Group B (n = -52)	34.7 ± 3.803	174.5 ± 49.73	1	7	24	3	49

[a] Chitotriosidase.
[b] Patients later diagnosed as Niemann-Pick A/B by measurement of sphingomyelinase activity using a radioactive method adapted from Pentchev et al. (Pentchev et al., 1980).

Table 2
Results for molecular analysis.

Molecular analysis	Patients
Heterozygous for mutations p.D945N and p.F1221SfsX20	1
Homozygous for mutation p.A1035 V	5
Heterozygous for mutations p.I923 V and p.A1035 V	1
Heterozygous for mutations p.N195Kfsp*2 and p.F1221SfsX*20	1
Heterozygous for mutations c.114-122del19 and p.F1221SfsX20	2
Homozygous for mutation p.V694M	2
Homozygous for mutation p.F1221SfsX20	2
Heterozygous for mutations p.R1186H and p.F1221SfsX20	1
Heterozygous for sequence variation p.G992R and heterozygous for mutation p.G1140 V.	1
Heterozygous for mutations p.A1035 V and p.E1166 K	1
Heterozygous for mutations p.S151Ffs*70 and p.F1221SfsX20	1
Heterozygous for variations p.G910S and p.G992W	1
Homozygous for mutation p.Q710Rfs*27	1
Homozygous for mutation p.P1007 V	1
Homozygous for mutation p.P1007A.	1
Homozygous for mutation p.R518 M	1
Heterozygous for variation p.G992R and heterozygous for mutations p.A1035 V.	1
Heterozygous for mutations p.P1007A V and p.E1166 K	1
No pathogenic mutations in the NPC1/NPC2 genes	51

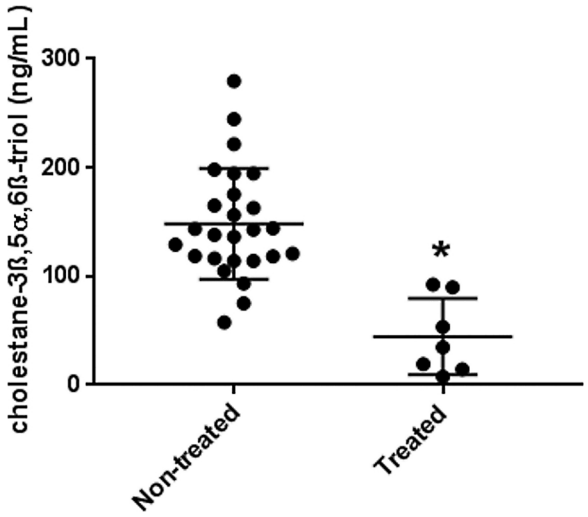

Fig. 2. Cholestane-3β,5α,6β-triol concentration in treated and non-treated patients with NP-C. Data is represented as mean ± SD. Student's *t* test was performed, and *p < 0.0001 when compared with non-treated individuals.

sensitivity, 96.08% of specificity, a positive and negative predictive value calculated in 91.67% and 94.23%, respectively. For these calculations, it was used molecular analysis as gold standard, since Filipin staining could not be performed in all individuals.

The therapy with miglustat and its effect in 3β,5α,6β-triol levels was also evaluated. It can be observed in Table 3 and Fig. 2 that 3β,5α,6β-triol levels are significantly lower in treated patients when compared to non-treated individuals, showing that this metabolite could be used not only for screening but also for therapy monitorization in NP-C.

4. Discussion

NP-C is a LSD currently conceived as a lipid trafficking disorder. Impaired egress of cholesterol from the late endosomal/lysosomal compartments is a specific key element of the pathogenesis (Vanier, 2015). Accumulated cholesterol in viscera and CNS can be oxidized by reactive oxygen species (ROS) in a nonenzymatic reaction forming oxysterols, mainly 3β,5α,6β-triol and 7-KC, which can be measured in plasma, contributing therefore for the disease investigation (Jiang et al., 2011). In this work, we determined 3β,5α,6β-triol levels, chitotriosidase activity, Filipin staining and NPC gene mutations from 76 individuals with NP-C suspicion, in order to investigate the potential of 3β,5α,6β-triol analysis for NP-C screening. The current method used for diagnosis in NP-C is Filipin staining, although this analysis shows a difficult interpretation and a variant presentation that can confuse the analyst (Vanier and Latour, 2015). For Filipin analysis, it was found 1 false positive, 7 false negative and 24 inconclusive cases, showing the limitations of this assay. The sensitivity (88%) and specificity (96.08%) of 3β,5α,6β-triol analysis verified by this work is consistent with previous studies (Jiang et al., 2011; Ribas et al., 2016; Reunert et al., 2016), and reaffirm the high potential of this metabolite for screening and its importance in NP-C diagnosis, especially when Filipin cannot be performed or is inconclusive. Even so, considering that the predictive positive value of 3β,5α,6β-triol was 91%, these data reinforce the crucial role of molecular analysis for definitive diagnosis, that should be performed in all individuals with a strong clinical suspicion, independent of 3β,5α,6β-triol concentrations.

Table 3
Cholestane-3β,5α,6β-triol concentration, age of NP-C patients and therapy duration.

	3β,5α,6β-triol concentration (ng/mL; mean ± SD)	Age (years; mean ± SD)	Treatment duration (years; mean ± SD)
Treated (n = 7)	44.47 ± 13.3	23.44 ± 11.95	4.29 ± 1.80
Non-treated (n = 25)	148.5 ± 10.0	12.5 ± 13.63	–

Progressive neurological manifestations in NP-C have a profound effect on life's quality of patients and their families. The correct and early identification of NP-C, as well as the appropriate use of symptomatic and disease-specific therapies can dramatically improve life quality for all those affected. Currently therapy for NP-C patients consists in a symptomatic treatment together with the use of miglustat to reduce neurological impairment. Miglustat is a small molecule that can cross the blood-brain barrier and acts as an inhibitor for the glucosylceramide synthase enzyme, decreasing glycosphingolipids, GM2 and GM3 levels in NP-C patients (Lyseng-Williamson, 2014). Besides, miglustat improves the traffic lipids in lymphocytes type B of NP-C patients (Lachmann et al., 2004), and also decreases lipid peroxidation and increases antioxidant status in NP-C1 patients (Ribas et al., 2012). In this context, our study found significantly 3β,5α,6β-triol decreased levels in patients treated with miglustat when compared to non-treated patients, probably caused by reduced cholesterol availability for oxidation (Lachmann et al., 2004), and also providing a less oxidative environment at a cellular level of these patients. Currently, monitorization of miglustat efficacy in NP-C patients consists in clinical evaluation of neurologic symptoms (e.g. ambulation, manipulation, language and swallowing) as well as horizontal saccadic eye movement velocity (HSEM) (Lyseng-Williamson, 2014). In this way, our data shows that miglustat provides an improvement in biochemical status in treated individuals by reducing 3β,5α,6β-triol levels, what reinforces the potential use of this metabolite for the therapy monitorization of NP-C patients.

Porter et al. found an increase of 3β,5α,6β-triol levels in mice brain tissue and in NP-C patients cerebrospinal fluid (CSF) (Porter et al., 2010), showing a possible role of this metabolite in neurological manifestations of these individuals. Considering that miglustat therapy can slow down, or even decrease neurodegeneration in NP-C by mechanisms not completely understood, if the reduction of 3β,5α,6β-triol observed in plasma of miglustat-treated NP-C patients of this study also occur in the CNS, the results of this work allow us to hypothesize that 3β,5α,6β-triol reduction could be associated with the neurological improvement by miglustat. However, further studies are necessary to explore how this mechanism works in NP-C disease.

5. Conclusion

Taken together, our presented data shows that the cholestane-3β,5α,6β-triol analysis by LC–MS/MS can be used for Niemann-Pick type C disease diagnosis and screening with good sensitivity and specificity. This biomarker emerges as a potential candidate to substitute the current test for NP-C: the Filipin staining. In the other hand, the 3β,5α,6β-triol analysis also can be used for therapy monitoring, since a significant decrease in this metabolite levels was observed in patients treated with miglustat when compared to non-treated patients. Although the mechanism of this effect is not completely clear, it may be related to improve in lipid traffic and decrease in oxidative stress caused by this treatment.

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Declaration of interest

The authors declare that there is no conflict of interests.

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ANEXO III – Artigo. “Are Cognitive Changes in Hereditary Spastic Paraplegias Restricted to Complicated Forms?”

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Are Cognitive Changes in Hereditary Spastic Paraplegias Restricted to Complicated Forms?

Laís Alves Jacinto-Scudeiro[1], Gustavo Dariva Machado[2], Annelise Ayres[3], Daniela Burguêz[2], Marcia Polese-Bonato[4], Carelis González-Salazar[5], Marina Siebert[6,7], Marcondes Cavalcante França Jr.[8], Maira Rozenfeld Olchik[9] and Jonas Alex Morales Saute[1,2,10,11]*

[1] Graduate Program in Medicine: Medical Sciences, Universidade Federal Rio Grande do Sul, Porto Alegre, Brazil, [2] Medical Genetics Service, Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil, [3] Postgraduate Program in Health Sciences, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil, [4] Graduate Program in Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [5] Graduate program in Medical Physiopathology, School of Medical Science, Universidade Estadual de Campinas, Campinas, Brazil, [6] Graduate program in Gastroenterology and Hepatology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [7] Unit of Laboratorial Research/Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil, [8] Department of Neurology, School of Medical Science, Universidade Estadual de Campinas, Campinas, Brazil, [9] Department of Surgery and Orthopedics, Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [10] Department of Internal Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [11] Neurology Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil

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Christos Proukakis,
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***Correspondence:**
Maira Rozenfeld Olchik
mairarozenfeld@hotmail.com

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Background: Little is known about the cognitive profile of Hereditary Spastic Paraplegias (HSP), where most scientific attention has been given to motor features related to corticospinal tract degeneration.

Objectives: We aimed to perform a broad characterization of the cognitive functions of patients with pure and complicated HSP as well as to determine the frequency of abnormal cognitive performances in the studied subtypes.

Methods: A two-center cross-sectional case-control study was performed. All individuals underwent cognitive assessment through screening tests (Mini Mental State Examination—MEEM and Montreal Cognitive Assessment—MOCA) and tests to assess specific cognitive functions (Verbal fluency with phonological restriction—FAS; Verbal categorical fluency—FAS-cat and Rey's Verbal Auditory Learning Test -RAVLT).

Results: Fifty four patients with genetically confirmed HSP diagnosis, 36 with spastic paraplegia type 4 (SPG4), 5 SPG11, 4 SPG5, 4 cerebrotendinous xanthomatosis (CTX), 3 SPG7 and 2 SPG3A, and 10 healthy, unrelated control subjects, with similar age, sex, and education participated in the study. SPG4 patients had worse performances in MOCA, FAS, FAS-cat, and RAVLT when compared to controls. Most SPG4 patients presented cognitive changes not compatible with dementia, performing poorly in memory, attention and executive functions. SPG5 patients scored lower in executive functions and memory, and SPG7 patients performed poorly on memory tasks. All evaluated cognitive functions were markedly altered in CTX and SPG11 patients.

Conclusions: Cognitive abnormalities are frequent in HSP, being more severe in complicated forms. However, cognitive impairments of pure HSPs might impact patients' lives, decreasing families' socioeconomic status and contributing to the overall disease burden.

Keywords: hereditary spastic paraplegia, HSP, SPG, cognitive profile, memory, executive function

INTRODUCTION

Hereditary Spastic Paraplegias (HSP) is a heterogeneous group of neurodegenerative genetic diseases (> 80 *loci* have been described) with spastic gait as the core feature (1, 2). HSP subtypes are clinically classified into pure forms—in which only a pyramidal syndrome is found (although changes in vibratory sensation and neurogenic bladder are accepted)—and complicated forms in which pyramidal findings are accompanied by dysfunction in other neurological systems (e.g., ataxia, parkinsonism, cognitive impairment, etc.) or by systemic involvement (3).

Although HSP are rare conditions, recent studies suggest that its prevalence (2–10 per 100,000) is similar to or higher than that of more studied conditions such as hereditary ataxias (4). Spastic paraplegia type 4 (SPG4) is the most frequent autosomal dominant subtype, whereas SPG11 and SPG7 are the most frequent autosomal recessive subtypes of HSP worldwide (4, 5).

There are few studies that have investigated the cognitive profile of HSP patients. Most of then focused on complicated forms, reporting intellectual disability and cognitive deficits perceptible by physician's clinical evaluation in up to 100% of SPG11 patients (6, 7). On the other hand, the cognitive characterization of the pure forms of HSP remains unclear, with some reports describing mild cognitive changes without the use of tests to evaluate specific cognitive functions or only being described through the physician's clinical impression (2, 6–11). Thus, the objective of this study was to characterize the cognitive functions of memory, orientation, verbal fluency, language, attention, and executive functions in HSP patients, to determine the frequency of cognitive changes in the different subtypes, and to correlate these findings with disease severity variables.

MATERIALS AND METHODS

Design and Subjects

We performed a two-center, exploratory, cross-sectional, case-control study conducted at two teaching hospitals in the Brazilian cities of Porto Alegre and Campinas. We included consecutive patients followed at the Neurogenetics outpatient clinics of these hospitals, from December 2016 to August 2018, and who presented genetically confirmed diagnosis of HSP or genetic or biochemical diagnosis of CTX, which can be considered as a complicated form of HSP (5). Healthy, unrelated subjects, with similar sex, age, and education characteristics were recruited from the local community of Porto Alegre as the control group for the adult population. Concomitant neurological or systemic conditions that could present cognitive alterations were exclusion criteria. The project was approved by the institutions' ethics committees under review numbers 170012 and 62653816.7.0000.5404, which follows the Declaration of Helsinki. Informed written consent was obtained from all individuals' prior participation.

Cognitive Evaluation

Cognitive assessments were performed by a single evaluator (LAJS) through standardized tests validated for the Brazilian population, which analyze a range of competencies, namely:

2.1 Mini Mental State Examination (MMSE): screening test for cognitive function evaluation. The maximum score is 30 points and, in the Brazilian population, a score of 28 points or more is indicative of normal cognitive function for individuals who have been formally educated for >8 years (12).

2.2 Montreal Cognitive Assessment (MoCA): screening test for cognitive function evaluation. The maximum score is 30 points and a score of 26 point or more is indicative of normal cognitive function (13).

2.3 Verbal fluency with phonological restriction (FAS): it consists in naming words beginning with the letters F, A, and S, respectively, and assesses executive function, language and semantic memory. Performance can be affected by education and by age (14).

2.4 Verbal categorical fluency (animals) (FAS-cat): this measure is a variation of the verbal fluency test and it is restricted to a semantic category. The score may be affected by education (15).

2.5 Rey's Verbal Auditory Learning Test (RVALT): is a tool to assess immediate memory (RVALT) as well as short (A6) and long-term (A7) retention. The test involves five consecutive repetitions and retrievals of stimuli from a list of 15 words (16).

Cognitive performance for the study population under 17 years-old was evaluated with the Wechsler Intelligence Scale for Children (WISC III) (17). This test can be used as an IQ (Intelligence Quotient) test for children and it is most often used as a clinical tool to measure individual's cognitive abilities. We used the Cubes and Vocabulary subtests to evaluate children's performance. Dementia diagnosis was based on Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (18) criteria.

Evaluation of Depressive Symptoms

In order to verify if depressive symptoms could act as a confounding factor for cognitive performance, the Beck Depression Inventory (BDI) was applied. BDI is a self-reported

TABLE 1 | Demographics of the enrolled individuals.

	Controls (*N* = 10)	SPG4		SPG11 (N=5)	CTX (*N*= 4)	SPG5 (*N* = 4)	SPG7 (*N* = 3)	SPG3A (*N* = 2)
		Adult (*N* = 31)	Children (*N* = 5)					
Female sex	6 (60%)	17 (47%)	2 (40%)	4 (80%)	2 (50%)	2 (50%)	1 (33%)	2 (100%)
Age	46.5 (12.3)	45 (18)	9 (2.9)	36.4 (5.7)	47.5 (10.5)	54 (6.5)	53.3(25.6)	38.5 (17.7)
Educational level	9.4 (3.1)	7.16 (3.9)	3.8 (3.11)	10 (2.8)	8 (4.8)	4.5 (1)	16.3 (1.6)	9 (4.2)
Age at onset	–	34.7 (16.8)	1.75 (1.30)	17.4 (2.8)	25.7 (20.2)	34 (4.9)	21.7(12.5)	11.5 (0.7)
Disease duration	–	16.4 (11.3)	8.2 (2.77)	20.4 (4.2)	22 (16.9)	21.2(5.4)	32.3(17.5)	27 (18.4)
SPRS	–	18.7 (9.8)	11.4 (5.9)	37.2 (6.1)	27 (16.1)	33.2 (11.2)	22.6(10.6)	8.5 (3.5)
Disease stage	–	0–0 (0%)	0–0 (0%)	0–0 (0%)	0–0 (0%)	0–0 (0%)	0–0 (0%)	0–0 (0%)
		1–1 (3%)	1–2 (40%)	1–0 (0%)	1–1 (25%)	1–0 (0%)	1–0 (0%)	1–1 (50%)
		2–5 (16%)	2–3 (60%)	2–0 (0%)	2–1 (25%)	2–0 (0%)	2–0 (0%)	2–0 (0%)
		3–8 (26%)	3–0 (0%)	3–0 (0%)	3–0 (0%)	3–1 (25%)	3–1(33%)	3–1 (50%)
		4 –15(49%)	4–0 (0%)	4–1 (20%)	4–1 (25%)	4–3 (75%)	4–2(66%)	4–0 (0%)
		5–2 (6%)	5–0 (0%)	5–4 (80%)	5–1 (25%)	5–0 (0%)	5–0 (0%)	5–0 (0%)
Cross-sectional disease progression	–	1.13	1.39	1.82	1.22	1.56	0.48	0.31
BDI	–	8.6 (5.9)	NA	NA	9 (1)	4.75 (3.6)	6.3 (5.5)	7 (1.4)

Data are shown as means in years (standard deviation), except for sex and disease stage that are shown as frequencies. CTX, cerebrotendinous xanthomatosis; SPRS, Spastic Paraplegia Rating Scale; BDI, Beck Depression Inventory; NA, not available—patients unable to perform this test.

questionnaire composed of 21 questions that assess the intensity of depressive symptoms. Scores range from 0 to 63 points that are crescent in severity (19).

Motor Neurological Evaluation

Neurological severity was assessed by the Brazilian Portuguese version of the Spastic Paraplegia Rating Scale (SPRS). SPRS scores ranges from 0 to 52 and are crescent in severity (20). Disease stage was classified as: (0) asymptomatic; (1) no functional handicap, but signs at examination (slight gait stiffness); (2) mild gait stiffness, walking unlimited, and running still possible; (3) moderate gait stiffness, limited walking without aid, and running impossible; (4) moderate to severe gait stiffness, walking possible with aid; and (5) walking impossible, wheelchair bound. We also estimated the cross-sectional disease progression as the cross-sectional quotient of disease severity (SPRS) and disease duration, as previously established (2). Disease duration and age of onset of the first motor symptom were reported by patients and their relatives.

Statistical Analysis

Statistical tests were selected according to the distribution of data given by Shapiro-Wilk test and histograms. Descriptive analysis of cognitive assessment scores was carried out based on the tests cut-offs for normal performance according to age and education level in the Brazilian population. Comparisons between SPG4 and controls individuals' scores and between SPG4 patients with truncating and non-truncating variants scores were performed by Student's *t*-test or Mann–Whitney *U*-test. The 95% confidence interval (CI) for differences in means between groups was also provided. Correlations of cognitive performance scores with independent variables (age, education level, age at onset, SPRS, disease stage, disease duration, cross-sectional disease progression) were performed with the Pearson or Spearman correlation tests. A linear regression model was built with independent variables that presented $P < 0.2$ in the simple correlation test, where only variables that maintained $P < 0.05$ after adjustment for covariates were kept in the final model. Statistical significance was defined as $P < 0.05$.

RESULTS

A total of 54 patients with HSP were enrolled in the study, including 36 (5 children) SPG4 (17 families), 5 SPG11 (4 families), 4 SPG5 (1 family), 4 CTX (4 families), 3 SPG7 (3 families), and 2 SPG3A (1 family) patients and 10 healthy control subjects. See **Table 1** for the clinical and demographical characterization of the study population and **Supplementary Table 1** (21–26) for detailed individual data of subjects.

Patients' Performance in Relation to Cognitive Test Scores

Table 2 details the cognitive performance of HSP patients and controls. Most SPG4 patients presented cognitive changes not compatible with dementia, performing poorly in memory, attention and executive function. SPG5 patients scored lower in executive functions and memory, and SPG7 patients performed poorly on memory tasks, also presenting cognitive changes not compatible with dementia. All evaluated cognitive functions were altered in patients with CTX (2/4 with dementia) and SPG11 (all with dementia) patients. The 2 patients with SPG3A performed within normal limits on cognitive tests. Of note,

TABLE 2 | Group performances in cognitive tests.

	Controls (*N* = 10)	SPG4 (*N* = 31[a])	SPG5 (*N* = 4)	SPG7 (*N* = 3)	SPG11 (*N* = 5)	CTX (*N* = 4)	SPG3 (*N* =)
MMSE	25	26	24	28	18	20	29.5
	(25–27.25)	(23–27)	(22.5–27)	(27.5–28.5)	(14–19)	(17.5–25)	(29–30)
	40%	64%	50%	66%	100%	75%	0%
MOCA	24.9	19.1	20.75	22.33	6.4	18.5	27
	(±2.84)	(±4.81)	(±4.42)	(±0.57)	(±3.91)	(±3.05)	(±1.41)
	40%	87%	100%	100%	100%	75%	0%
FAS	31.3	18.67	20	21	6.6	23	31
	(±9.15)	(±9.47)	(±9.76)	(±5.56)	(±3.97)	(±8.96)	(±8.48)
	0%	45%	50%	66%	100%	25%	0%
FAS-cat	18.1	11.77	13.5	14	4.8	11.5	21
	(±5.64)	(±4.71)	(±2.38)	(±2)	(±2.86)	(±3.31)	(±0)
	0%	41%	0%	33%	100%	25%	0%
RAVLT	39.3	24.22	30.75	25.66	11.4	23	40.5
	(±7.9)	(±8.49)	(±5.43)	(±2.51)	(±10.89)	(±8.08)	(±0)
	60%	83%	75%	66%	100%	100%	0%
A6	8.2	3.93	6.5	4	0.2	2.75	9
	(±2.86)	(±2.26)	(±4.12)	(±1.73)	(±0.44)	(±2.98)	(±1.41)
	40%	83%	75%	100%	100%	75%	0%
A7	8	3	2.5	4	0	2.5	9
	(6–10.25)	(1.5–4)	(2–**7**.5)	(3–4)	(0)	(1–4.5)	(0)
	40%	96%	75%	66%	100%	75%	0%

Data are shown as means (standard deviation), except for MMSE and A7 that are shown in median (interquartile range), and percentages of altered performances according to the normal standards for the given test. CTX, cerebrotendinous xanthomatosis; FAS, verbal fluency with phonological restriction; FAS-cat, verbal categorical fluency (animals); MMSE, Mini Mental State Examination; MOCA, Montreal Cognitive Assessment; RVALT, A6 and A7, Rey's Verbal Auditory Learning Test.

[a]*Only adult patients with SPG4 were considered.*

SPG11 patients were unable to respond to BDI, because of cognitive impairment.

Comparative Analysis Between SPG4 and Control Subjects

While the performance of SPG4 patients was similar to controls in the MMSE ($P = 0.800$, **Figure 1A**), their scores were lower than those of the control subjects in all other cognitive tests: MOCA (−4.93, 95% CI, −8.20 to −1.66, $P = 0.004$, **Figure 1B**), FAS (−12.62, 95% CI, −19.54 to −5.70, $P = 0.001$, **Figure 1C**), FAScat (−6.32, 95% CI, −9.96 to −2.68, $P = 0.001$, **Figure 1D**), RAVLT (−15.05, 95% CI, −21.22 to −8.92, $P < 0.001$, **Figure 1E**), A6 (−4.26, 95% CI, −6.04 to −2.48; $P < 0.001$, **Figure 1F**) and A7 ($P < 0.001$, **Figure 1G**).

Which Factors Correlated to Cognitive Decline in SPG4 Patients?

Although no statistically significant difference was found between SPG4 patients and control subjects in MMSE, both disease duration (Beta = −0.351, 95% CI, −0.170 to −0.014, $R^2 = 0.116$, $P = 0.022$, **Figure 2A**) and education years (Beta = 0.492, 95% CI, 0.152 to 0.614, $R^2 = 0.227$, $P = 0.002$) were independently correlated with MMSE in the linear regression model. For each additional year of disease duration, there was a decrease of 0.092 points in MMSE, and for each additional education year there was an increase of 0.383 points in the MMSE results. Education and disease duration were also the only variables independently correlated in the linear regression model with MOCA. For each additional year of disease duration there was a decrease of 0.162 points in MOCA (Beta = −0.382; 95% CI, −0.294 to −0.030, $R^2 = 0.137$, $P = 0.018$, **Figure 2B**), and for each additional education year there was an increase of 0.525 points in MOCA (Beta = 0.416, 95% CI, 0.134 to 0.916, $R^2 = 0.163$, $P = 0.01$). Age was the only variable correlated with FAS ($R = -0.520$, $P = 0.003$) and RAVLT ($R = -0.428$, $P = 0.016$), and neither significant correlations were found between the independent variables and performances in the FAScat, nor A6 and A7. BDI scores did not correlate with cognitive tests performance ($P > 0.2$ for all comparisons), making it very unlikely that depressive symptoms were influencing the results of the cognitive evaluation.

Genotype-Phenotype Correlation in SPG4

Variants in *SPAST* were classified as truncating (nonsense and frameshift variants, N=11 individuals) and non-truncating (missense and in-frame insertion, N=20 individuals). Clinical, demographical and cognitive findings of patients with truncating and non-truncating variants did not differ; see **Supplementary Table 2** for details.

Cognitive Performance in Children

The WISC-III results showed low average IQ scores in 60% (3/5) of the children with SPG4 and average IQ in the others. There were no children in the other HSP subtypes. Mean cubes subtest (executive subscale) performance was 9.2 (4.16) and mean

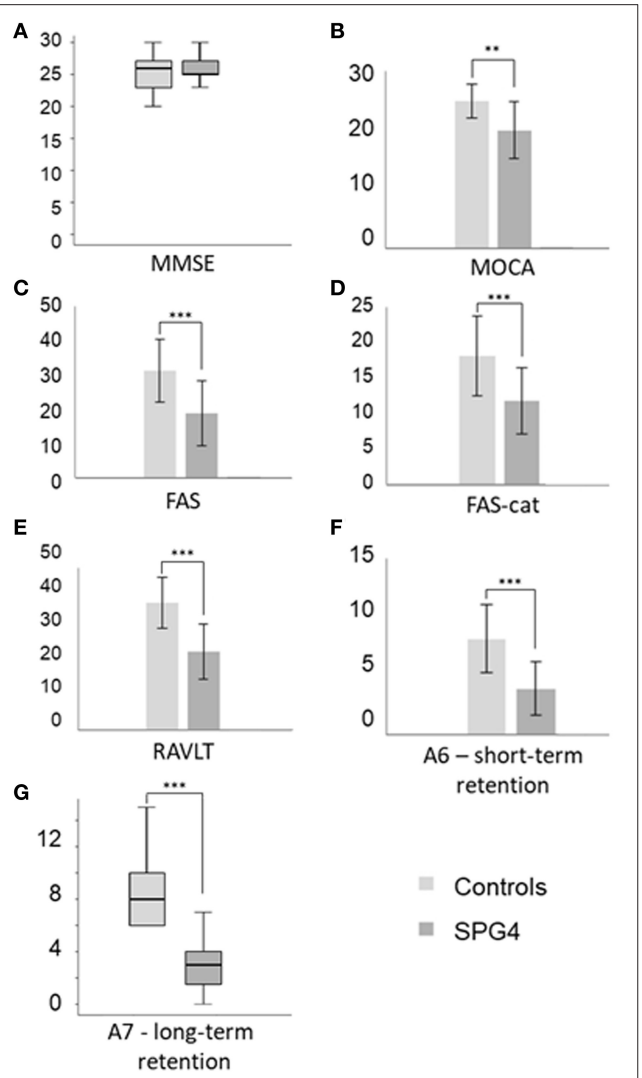

FIGURE 1 | Comparison between control and SPG4 subjects performances in cognitive tests. Bars represent mean values and standard deviation. Box-plots represent median values and interquartile ranges. $^{**}P < 0.01$ e $^{***}P \leq 0.001$. FAS, verbal fluency with phonological restriction; FAS-cat, verbal categorical fluency (animals); MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment; RVALT, A6 and A7, Rey's Verbal Auditory Learning Test. **(A)** MEEM, **(B)** MOCA, **(C)** FAS, **(D)** FAS-cat, **(E)** RVALT, **(F)** A6 - short-term retention, **(G)** A7 - long-term retention.

vocabulary test (verbal subscale) was 7.2 (2.51), which are low average values. All evaluated children had missense pathogenic variants in *SPAST*.

DISCUSSION

Over the last years, non-motor symptoms of movement disorders have received greater attention (27, 28), albeit in a more timid manner for hereditary spastic paraplegias (29). Classically the pure forms of HSP have been described as solely motor syndromes in which only pyramidal signs are found. However, these descriptions are generally based on the neurologist's impression, without a more formal assessment of non-motor symptoms, such as subtle cognitive changes (2). In this study, we found impairments in memory, attention, executive functions and verbal fluency in SPG4 patients, the most prevalent HSP subtype worldwide and the prototype of pure forms of the disease, and we confirmed the more severe cognitive dysfunction of complicated forms of HSP.

We found cognitive changes not compatible with dementia in most evaluated SPG4 patients, with abnormal cognitive performances ranging from 41 to 96%, depending on the utilized test and domain, with memory (immediate and recent) being the most frequently altered cognitive function. Our results are partially in agreement with descriptions of subtle cognitive deficits in these patients in a few case series (2, 7, 8, 10, 11). The inverse correlation between MMSE and MOCA with disease duration and the normal, albeit low average, performance in children on intelligence tests might suggest that cognitive dysfunction in SPG4 is progressive and worsens with disease progression. SPG4 patients with truncating and with non-truncating variants had similar cognitive performances, which is in agreement with a previous study (8).

Neuroimaging findings from previous studies (30, 31) are in agreement with the cognitive changes we have found, suggesting a more widespread central nervous system involvement in SPG4. Extensive fractional anisotropy reduction in non-motor regions (posterior cingulate gyri and splenium of the corpus callosum) were found in SPG4 patients on magnetic resonance (MRI) diffusion tensor imaging (30), as well as decreased brain activity in the left insular cortex in functional MRI (31), which regulates a wide range of cognitive and emotional functions (32).

Despite the cognitive impairment of SPG4 patients in the overall evaluation, no differences to controls were found with MMSE screening test. On the other hand, most SPG4 patients (87%) scored below normal thresholds for MOCA. The discrepancy of MMSE and MOCA was also verified among patients with SPG5 and SPG7 and was suggested by a previous study, even though this particular tool was not used by that authors (10). Therefore, MOCA is likely to be a more sensitive screening tool for cognitive changes in HSP, especially for pure forms.

Most SPG11 and CTX patients presented major cognitive deficits and scores well below what would be expected for all cognitive functions tested (not only memory). These results corroborate previous findings of severe intellectual disability in this population confirming the higher prevalence and severity of cognitive dysfunction when compared to pure HSP forms (33, 34).

Due to the exploratory nature of the study we neither performed sample size calculation nor definition of the study power and main outcome. Nevertheless, statistically significant differences between SPG4 and controls were found in all tests, except MMSE. There was no trend for lower MMSE scores in SPG4 ($P = 0.800$) when compared to controls and therefore there is a low chance of Type 2 error. Finally, we should mention that we were unable to compare the cognitive performances of children and adults due to the different nature of cognitive tests according to age.

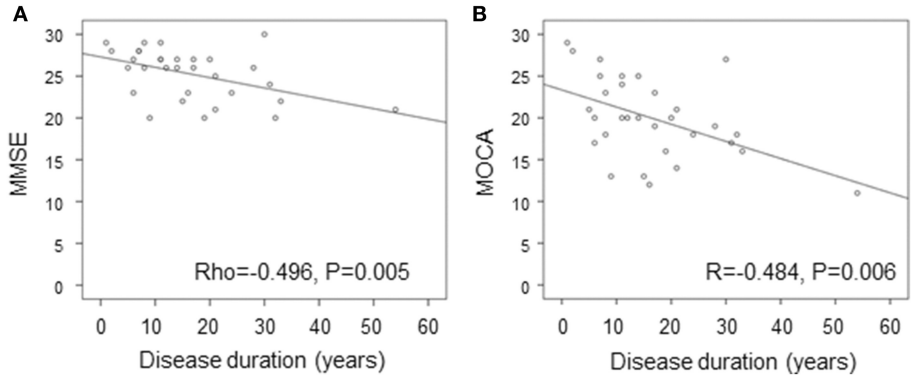

FIGURE 2 | Correlation of **(A)** MMSE and **(B)** MOCA with disease duration in SPG4. Simple correlation of MMSE and MOC with disease duration in SPG4. MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment.

CONCLUSION

Cognitive abnormalities are frequent in HSP, with dementia being commonly observed in complicated forms and cognitive changes not compatible with dementia in pure forms of the disease. SPG4, the most frequent and the prototype of pure HSP, present multiple cognitive abnormalities that might impact patients' lives and result in difficulties at school, on their careers and, consequently, might decrease patients' and families' socioeconomic status. Longitudinal studies are needed to assess the rate of progression of cognitive changes and to verify whether motor and cognitive functions have similar or different patterns of progression and thus different pathophysiological processes.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Comitê de Ética e Pesquisa-Hospital de Clínicas de Porto Alegre (CEP-HCPA) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Comitê de Ética e Pesquisa (CEP-HCPA).

AUTHOR CONTRIBUTIONS

LJ-S, MO, and JS conception and design of the research. LJ-S, AA, GD, DB, MP-B, MS, CG-S, MC, and JS recruitment of patients and data collection. LJ-S and JS tabulation, statistical analysis, creation of tables and figures. LJ-S, MO, and JS writing the text. LJ-S, GD, AA, DB, MP-B, CG-S, MS, MC, MO, and JS review of text and addition of significant parts.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00508/full#supplementary-material

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