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Estudos genéticos e funcionais sobre os genes VAPB e VRK1 em duas famílias portadoras de Esclerose Lateral Amiotrófica

Genetic and functional studies on the genes VAPB and VRK1 in two families carrying Amyotrophic Lateral Sclerosis

São Paulo
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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor Ciências, na Área de Biologia (Genética).

Orientador(a):
Profª Drª Mayana Zatz

São Paulo

2020

Ficha Catalográfica

Oliveira, Danyllo.
Estudos genéticos e funcionais sobre os genes *VAPB* e *VRK1* em duas famílias portadoras de Esclerose Lateral Amiotrófica / Danyllo Oliveira ; orientadora Mayana Zatz -- São Paulo, 2020.

110 f.

Tese (Doutorado) -- Instituto de Biociências da Universidade de São Paulo. Departamento de Genética e Biologia Evolutiva.

1. Células-tronco. 2. Neurônios motores. 3. Esclerose Lateral Amiotrófica. 4. Doenças Neurodegenerativas. I. Zatz, Mayana. II. Título.

Comissão Julgadora:

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Orientador(a)

Epígrafe

É meu propósito falar das metamorfoses dos seres
em novos corpos. Vós, deuses, que as operastes,
sede propícios aos meus intentos e acompanhai o meu poema,
que vem das origens do mundo até os meus dias.

Ovídio, *Metamorfoses*
(trad. Domingos Lucas Dias)

Agradecimentos

Durante esses anos, tive a oportunidade de aprender, discutir Ciência, compartilhar de momentos bons e difíceis. Foram quatro anos que, sem dúvida, ficarão marcados na minha história. Com eles, as pessoas que direta ou indiretamente contribuíram para que esse período se encerrasse com sucesso; passo a citá-las aqui:

Minha orientadora, Profa. Mayana Zatz, por ter me acolhido no seu laboratório, pela paciência e pelos ensinamentos. Sua dedicação à causa da ciência, determinação e trabalho são exemplos que levarei comigo por toda a vida.

Os pacientes estudados nessa tese, por tão bem ter nos recebido e colaborado em todos os nossos estudos. Sem esse apoio precioso, o presente trabalho não teria sido possível.

Ângela Vianna-Morgante por todos ensinamentos e colaborações desde o mestrado.

João Ricardo Mendes de Oliveira e Gabriela Ferraz Leal, que me iniciaram na Ciência e guiaram meus primeiros passos na Genética Humana.

Luciana Moura, pela avaliação clínica de todos os pacientes dessa tese, pela companhia nas viagens de coleta e as discussões que as acompanhavam.

Ao Professor Sergio Verjovski-Almeida e aos seus alunos, Murilo Sena e David Morales, pela ajuda com os experimentos de *RNA Sequencing*, fundamentais para a conclusão desse trabalho.

Os amigos do laboratório: Luiz Caires, Ernesto Goulart, Amanda Assoni, Carolini Kaid, Kayque Telles, Felipe Leite, Bruno Ghirotto, Uirá Souto, Thalita Figueiredo, Gerson Kobayashi, Luciano Brito, Letícia Nogueira, Camila Musso, Danielle Moreira, Lucas Alvizi, Bruno Araújo, Thiago Olávio, Melinda Beccari, por tudo o que aprendi com vocês, pela amizade, colaboração e companhia nos momentos fora da USP também. Muito obrigado!

Agnes Nishimura, pela paciência em me ensinar, mesmo de longe, a trabalhar com os neurônios motores.

O corpo técnico do Departamento de Biologia, em especial a Naila Lourenço, Monize Lazar e Silvia Costa, sempre dispostas a ajudar e ensinar, quando solicitadas.

Os meus familiares, por todo apoio, confiança e carinho durante esses anos longe de casa.

A FAPESP e ao CNPq pelo financiamento dos projetos de pesquisa (FAPESP: 2015/14821-1, 2017/16283-2; INCT – CNPq: 465355/ 2014- 5). O presente trabalho

foi também realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Nota do Autor

Este trabalho desenvolveu-se visando contribuir para a compreensão dos mecanismos moleculares da Esclerose Lateral Amiotrófica (ELA). Nesse sentido, inicialmente estudamos uma forma autossômica dominante de ELA, causada por mutações no gene *VAPB* (ELA8). Chamou-nos a atenção o fato de alguns indivíduos portadores da ELA8 desenvolverem uma progressão branda dos sintomas, enquanto outros, uma manifestação clínica típica para essa doença. Também apresentamos a caracterização fenotípica e os estudos genéticos em uma grande família consanguínea do interior da Bahia, portadora de um tipo autossômico recessivo de Esclerose Lateral Amiotrófica. Visando abordar todos os temas, dividimos esta tese em cinco capítulos.

O capítulo 1 consiste numa revisão bibliográfica da literatura concernente à ELA. Procuramos descrever as alterações fenotípicas associadas a essa entidade, evidenciando a sua grande variabilidade de manifestações clínicas. Apresentamos também uma breve introdução sobre as principais estruturas do sistema nervoso central alteradas nessa doença. Estudos de anatomopatológicos e de neuroimagem foram descritos, com o objetivo de melhor classificar esse fenótipo num contexto complexo de doenças neurodegenerativas. Por fim, o estudo da etiologia da ELA foi apresentado, e os principais fatores genéticos já descritos para essa doença brevemente descritos.

Os capítulos 2 e 3 apresentam os resultados referentes ao estudo variabilidade clínica na ELA8. Ambos consistem em publicações submetidas às revistas *Human Molecular Genetics* e *Neural Regeneration Research*, respectivamente. Nessa vertente de nosso trabalho, procuramos explorar os mecanismos celulares e genéticos associados à variabilidade de início dos sintomas desse subtipo de ELA, visando identificarmecanismos atenuadores do processo neurodegenerativo.

O capítulo 4 apresenta o estudo da outra família por nós avaliada. Uma variante em homozigose, localizada no gene *VRK1*(p.R321C) foi identificada segregando com a ELA, e consiste, portanto, no fator etiológico mais provável nessa família. Um manuscrito foi preparado e encontra-se em fase de submissão

Os anexos de 1 a 5 apresentam publicações não relacionadas com o projeto principal, mas que foram derivadas de projetos desenvolvidos ao longo do doutorado. Dentre esses, destacamos estudos de correlação genótipo-fenótipo para formas autossômico dominantes de microcefalia (Oliveira et al., 2019 *Journal of Medical Genetics*) e a identificação de mecanismos de susceptibilidade à infecção pelo Zika vírus (Caires et al, 2018 *Nature Communications*).

A metodologia dos trabalhos encontra-se nos respectivos manuscritos, em cada capítulo. A bibliografia geral, por sua vez, em seção específica após o capítulo 5.

Abreviaturas

ELA – Esclerose Lateral Amiotrófica

DFT – Demência Fronto-Temporal

NMs – Neurônios Motores Superiores

NMIs – Neurônios Motores Inferiores

SNC – Sistema Nervoso Central

AD – Herança Autossômica Dominante

AR – Herança Autossômica Recessiva

L-X – Herança Ligada ao X

BMAA – Beta-Metil-Amino-L-Alanina

iPSC – Induced Pluripotent Stem Cells - céulas pluripotentes induzidas

GWAS – *Genome –Wide Association Studies* - Estudos de associação ampla do genoma

WES – *Whole Exome Sequencing* – Sequenciamento completo do exoma

WGS – *Whole Genome Sequencing* – Sequenciamento completo do genoma

MLPA – *Multiplex ligation probe amplification* – Amplificação da sonda de ligação múltipla

CB – Corpos de Bunina

VAPB – *Vesicle Associated Membrane Protein Associated Protein B/C*

VRK1 – *Vaccinia- Related Kinase 1*

C9orf72 – *Chromosome 9 Open Reading Frame 72*

TARDBP - *TAR DNA - binding protein 43*.

FUS – *Fused In Sarcoma*

SOD1 – Superóxido Dismutase 1

MSP – *Major Sperm Domain*

FXTAS – *Fragile X Associated Tremor/Ataxia Syndrome*

NMDA – Receptor ionotrófico do glutamato – N-Metil- D- Aspartato

AMPA – Receptor ionotrópico do glutamato – α – Amino – 3 – Hidroxi – Metil-5- 4 – Isoxazolpropiônico.

Eph – Receptor de Efrina

Índice

Capítulo 1

I. Introdução

Esclerose Lateral Amiotrófica

A Esclerose Lateral Amiotrófica (ELA), também chamada de Doença de Lou Gherig, é uma doença neurodegenerativa fatal, caracterizada pela morte celular seletiva de neurônios motores superiores (NMS) e inferiores (NMI) (Coatti et al., 2015). Pacientes portadores de ELA normalmente referem fraqueza, perda muscular e fasciculações, que inevitavelmente progridem para comprometimentos físicos mais graves, como a perda da deambulação e da capacidade de deglutição, por exemplo. A morte em média ocorre cerca de três anos após o início dos sintomas, e decorrem de insuficiência respiratória, na maioria dos casos (Coatti et al., 2015; Taylor et al., 2016).

Os primeiros trabalhos descrevendo pacientes com ELA foram realizados por Jean Martin Charcot (1825- 1893), em uma série de publicações, a partir de 1865 (Katz et al., 2015). Nesses relatos, não passaram despercebidas por Dr. Charcot as alterações neuropatológicas subjacentes a essa enfermidade. A própria designação que a doença viria a receber, nomeada por ele, derivava desses achados histológicos. Sendo assim, segundo o eminente neurobiologista, a "esclerose lateral" e a "amiotrofia" consistiriam na degeneração de duas subestruturas da medula espinhal, as colunas laterais e os cornos anteriores, respectivamente. Na perspectiva por ele traçada para o processo patológico da doença, a "esclerose" nas colunas laterais seria a lesão primária, e a perda neuronal nos cornos anteriores a secundária (Takeda et al., 2020). Essa hipótese foi posteriormente invalidada, visto que são os neurônios motores os principais tipos celulares afetados na ELA. As colunas laterais, por sua vez, nada mais são do que tratos descendentes de NMS localizados em regiões superiores do Sistema Nervoso Central (SNC). Apesar disso, o nome esclerose lateral amiotrófica permaneceu até os dias de hoje (Goetz, 2000).

Estudos populacionais sobre a ELA têm ocorrido principalmente em países europeus. A incidência dessa doença na Suécia e na Escócia, por exemplo, tem estimativas similares de 3,8: 100 000. Por outro lado, estudos epidemiológicos em populações do leste da Ásia, como China e Coreia apontam para uma frequência menor,

chegando a 1,2:100 000 e 0,8:100 000 (Longinetti & Fang, 2019) enquanto Rose et al (2019) descrevem uma incidência de 0,6:100 000 no Canadá.

Embora ainda não se saiba a causa dessa variação, é provável que fatores ambientais, genéticos, demográficos ou até mesmo metodológicos possam ser responsáveis pela aparente discordância. Entre esses, poder-se-ia citar a frequência elevada das formas causadas por repetições patogênicas do C9orf72 (um fenômeno genético-populacional eminentemente europeu), e as diferenças entre os protocolos na condução de pacientes com provável Esclerose Amiotrófica. Apesar disso, a ELA é a doença de neurônio motor mais comum, e uma das doenças neurodegenerativas mais frequentes, atrás apenas de Doença de Alzheimer e Parkinson (Martin, 2010).

1.1 Aspectos Clínicos

Devido à ausência de biomarcadores para a Esclerose Lateral Amiotrófica, o seu diagnóstico é principalmente clínico. O El Escorial, elaborado pela *World Federation of Neurology*, consiste no protocolo mais aceito atualmente para avaliação de pacientes com doença de neurônio motor (Brooks et al., 2000; Boekestein et al., 2010). Segundo os seus critérios, o portador de ELA deve ser avaliado tanto fisicamente quanto por eletroneuromiografia (Brooks et al, 2000). Exames de neuroimagem também são preconizados na maioria dos casos, de modo a excluir alterações estruturais ou lesões que poderiam explicar o quadro clínico (Brooks et al., 2000).

Fenotipicamente, a Esclerose Lateral Amiotrófica é extremamente heterogênea. Pacientes portadores dessa doença podem variar quanto à idade de início, extensão do curso clínico, presença ou não de alterações comportamentais, sítio de início dos sintomas, bilateralidade ou não dos fenótipos e tipo de neurônio motor afetado por ocasião do início da doença (Swinnen & Robberecht, 2014). Dessa maneira, acredita-se não na existência de uma entidade nosológica específica, mas sim em um espectro que varie quanto à apresentação dessas alterações clínicas. A grosso modo pode-se, portanto, separar os sintomas associados à ELA em motores e extra-motores (Takeda et al, 2019).

Os sintomas motores implicam em alterações na capacidade de produzir movimento, e esses se traduzem em uma miríade de fenótipos. Na maioria dos casos, os pacientes com ELA manifestam fraqueza muscular, fasciculações e atrofia da

musculatura esquelética. Tais sintomas são consistentes com um início dito "espinal" da doença, e envolveria eminentemente a perda neuronal de NMIs (Rowland & Schneider, 2001) Este grupo celular é diretamente associado às fibras musculares através de junções neuromusculares, formações sinápticas específicas da interação neurônio: músculo (Davis-Dusenbery et al., 2014) Por outro lado, esses indivíduos também podem manifestar, associados ou não aos sintomas já descritos, hiperreflexia e clônus, fenótipos característicos da perda de NMSs (Rowland & Schneider, 2001; Chió et al., 2011). Estes últimos são neurônios responsáveis por controlar, através de sinapses com os NMIs ou neurônios de circuito local na medula espinal, o movimento voluntário dos membros (Ravitz & La Spada, 2009; Davis-Dusenbery et al., 2014). Os sinais de Babinski e de Hoffman, indicativos de lesão em NMSs, frequentemente são avaliados em pacientes portadores de ELA, para caracterizar o tipo de neurodegeneração em curso (Chió et al., 2011).

Em cerca de 20% dos pacientes, entretanto, o início dos sintomas se dá por deficiência na deglutição (disfagia), disartria, fasciculações na língua, hiperreflexia na mandíbula e riso e/o choro descontrolado (sinais "pseudobulbares"). Tais características correspondem a um início bulbar da Esclerose Lateral Amiotrófica (Chió et al., 2011; Miller et al., 2014). Esse subtipo de manifestação também possui uma progressão frequentemente mais severa que os subtipos espinais, levando a óbito num prazo mais curto a partir do início dos sintomas. Tal fenômeno se deve ao rápido comprometimento dos centros respiratórios localizados no tronco encefálico (Chió et al., 2009).

Curiosamente, análises epidemiológicas da ELA bulbar mostram uma razão de 3:1 em relação ao número de mulheres e homens afetados, respectivamente. Um possível efeito modulador de hormônios sexuais, no processo neurodegenerativo, foi postulado para explicar essa discrepância. Entretanto, essa hipótese ainda não foi propriamente avaliada (Turner et al., 2010).

Em outros subtipos de ELA, os pacientes são caracterizados como portadores de Esclerose Lateral Primária ou Atrofia Muscular Progressiva. A base neuropatológica para essa variabilidade é consistente com o tipo neuronal envolvido (Cervenakova et al, 2000; Gordon et al., 2006). Assim, a Esclerose Lateral Primária é causada pela morte seletiva de neurônios motores superiores, e a Atrofia Muscular Progressiva pelos neurônios motores inferiores. A ocorrência de manifestações do outro subtipo neuronal

em cada uma delas é um fenômeno frequente, o que acaba por tornar o quadro clínico típico da Esclerose Lateral Amiotrófica (D'Amico et al., 2013).

Outras entidades têm sido descritas como variações da Esclerose Lateral Primária e da Atrofia Muscular progressiva, a saber: ELA pseudopolineurítica, "*Flail Arm"Syndrome* (Síndrome de Vulpian- Bernhardt) e "*Flail leg" Syndrome* (Síndrome de Marie-Patrikios) e ELA hemiplégica (Wijesekera et al., 2009; Kobayashi et al., 2010; Chug et al., 2013). Os três primeiros subtipos consistem em manifestações típicas de morte dos neurônios motores inferiores, distinguindo-se umas das outras a partir do conjunto de membros afetados. Nesse sentido, a ELA pseudopolineurítica caracteriza-se pelo comprometimento apenas das extremidades dos membros, enquanto nas Síndromes de Vulpian-Bernhardt e de Marie-Patrikios os sinais de degeneração motora ocorrem apenas nos membros superiores e inferiores, respectivamente (Wijesekera et al., 2009; Kobayashi et al., 2010). Por fim, a ELA hemiplégica consiste na degeneração unilateral de neurônios motores superiores de ambos os membros (Chugh et al., 2013).

Os sintomas extra-motores da ELA são variáveis, sugerindo um amplo número de subestruturas do SNC fora das regiões motoras envolvidas no seu processo patológico. Dentre eles, a Demência Fronto-temporal (DFT) notabiliza-se pela sua alta recorrência nesses pacientes, quando comparado aos demais fenótipos (Ripon et al., 2006; Mioshi et al., 2014). Embora já se especulasse sobre uma origem biológica comum entre ELA e DFT, foi apenas a partir da descoberta da expansão de hexanucleotídeos em *C9orf72* que, finalmente, se estabeleceu uma correlação essas entidades. Hoje considera-se que ambas são parte de um espectro clínico, onde a ELA e a DFT seriam seus extremos opostos (De Jesus-Hernandez, et al., 2011; Renton et al., 2011).

Análises epidemiológicas têm observado que cerca de 50% dos pacientes diagnosticados com ELA apresentam comprometimento cognitivo e comportamental. Nessas mesmas análises, 15% apresentaram também sintomas consistentes com um diagnóstico clínico de DFT (Pulkan et al., 2007). Não está claro, entretanto, se esses fenótipos são decorrentes de um efeito primário da degeneração neuronal, ou derivado de alterações psicológicas ocasionadas pela progressão da doença no indivíduo. A ausência de um protocolo clínico para DTF no El Escorial também pode concorrer para inconsistências no seu diagnóstico. Apesar disso, é consenso que indivíduos com

Demência Fronto-Temporal tipicamente manifestam alterações comportamentais, de personalidade e de linguagem, que progridem ao ponto de levar ao mutismo. No início da doença, as habilidades de memória, praxia e gnosia estão relativamente preservadas, tornando-se, portanto, em ponto de referência para o diagnóstico diferencial (Elahi & Miller, 2017; Mossevelde et al., 2018). Em casos de ELA-DFT no mesmo indivíduo, os sintomas associados à Demência comumente manifestam-se anteriormente aos danos motores, podendo ser esses de natureza espinal ou bulbar.

Outros sintomas extra-motores associados a ELA incluem a ataxia, atrofia palidonigroluisiana e parkinsonismo. Os primeiros têm sido pincipalmente associados a formas de Ataxias Espinocerebelares do tipo 2, onde expansões de CAG do gene *ATXN2* são a etiologia molecular da doença. Curiosamente, também foi verificado que as repetições subclínicas desse gene consistiam em fatores de risco para a ELA (Braga-Neto et al., 2011; Elden et al, 2010; Swinnen & Robberecht, 2014). As atrofias palidonigroluisinas constituem em alterações apresentando coreia, tremor, parkinsonismo e distonia. Tratam-se, portanto de fenótipos associados à degeneração nos gânglios da base. Nesses casos, extremamente raros e que podem vir ou não acompanhados de ELA, os indivíduos têm sido classificados dentro de outro espectro fenotípico, que também inclui as Paralisias Supranucleares Progressivas (Uchino et al., 2018; Ito et al., 2020).

A Esclerose Lateral Amiotrófica associada ao parkinsonismo tem uma origem diversa. Em alguns casos, a ELA e a Doença de Parkinson (DP) manifestam-se conjuntamente, podendo ser acompanhadas ou não de Demência Fronto-Temporal. Esses indivíduos são normalmente responsivos ao levodopa e têm uma manifestação típica da Esclerose Lateral Amiotrófica, que normalmente surge posteriormente aos sintomas da DP (Desai & Swash, 1999; Manno et al., 2013). Como trata-se de uma entidade rara; tais indivíduos têm sido considerados casos de comorbidades, embora mais recentemente mutações em *DJ1* tenham sido descritas em pacientes ELA-DP-DFT, o que sugere mecanismos comuns de neurodegeneração para as três doenças (Annesi et al., 2005). Casos inespecíficos de parkinsonismo não responsivo ao levodopa são também reportados em casos de Esclerose Lateral Amiotrófica, podendo estar presente em até 15% destes (Manno et al., 2013). Nesses indivíduos, tremor e alterações de postura são os achados clínicos extra-piramidais mais frequentes. Casos

específicos de ELA e parkinsonismo também têm sido descritos na Ilha de Guam, um território americano no Oceano Pacífico (Steele & McGeer, 2008).

Embora associadas a sistemas neurais distintos dos comumente comprometidos na Esclerose Lateral Amiotrófica, alterações sensoriais e incontinência urinária neurogênica têm sido descritos nesses pacientes (Baltadzhieva et al., 2005; Isaacs et al., 2007). Os casos de perda sensorial são particularmente recorrentes em indivíduos com mutações no gene *SOD1,* e frequentemente são seguidos de perda neuronal nos cornos posteriores da medula espinal (Shibata et al., 1996). As alterações urinárias neurogênicas, embora pouco frequentes, uma vez que o núcleo de Onuf normalmente não sofre degeneração na ELA, foram também reportadas em pacientes portadores da expansão de *C9orf72* (Swinnen & Robberecht, 2014)

1.2 Aspectos Neuropatológicos

1.2.1 Neuroanatomia dos Sistemas Motores

Os neurônios motores inferiores (NMIs) são as células através das quais o SNC regula a contração muscular, em sinapses específicas musculo:neurônio denominadas junções neuromusculares. Anatomicamente, localizam-se nos cornos ventrais da medula espinal e nos núcleos motores do tronco encefálico. A partir desses, os NMIs protraem seus axônios e saem do SNC para conectarem-se às células musculares (Davis-Dusenbery et al., 2014). Eles podem ser subdivididos em neurônios alfa e gama, a depender das fibras musculares que estejam conectados. Enervando as fibras musculares extra-fusais, os neurônios motores inferiores alfa são responsáveis pela força associada à tração muscular. Por outro lado, os subtipos “gama”, por enervarem exclusivamente fibras intra-fusais, estão associados à regulação da distensão muscular (Purves et al, 2010).

Os neurônios motores superiores (NMSs), por sua vez, localizam-se no córtex cerebral e em núcleos do tronco encefálico, como a formação reticular, colículo superior e núcleos vestibulares. A partir dessas estruturas, enviam eferências a neurônios de circuitos locais, através dos quais modulam a atividade dos NMIs. Invariavelmente, tais eferências estão organizadas em tratos mielinizados que são designados conforme suas origens e destinos, e.g. o trato corticoespinal, corticobulbar,

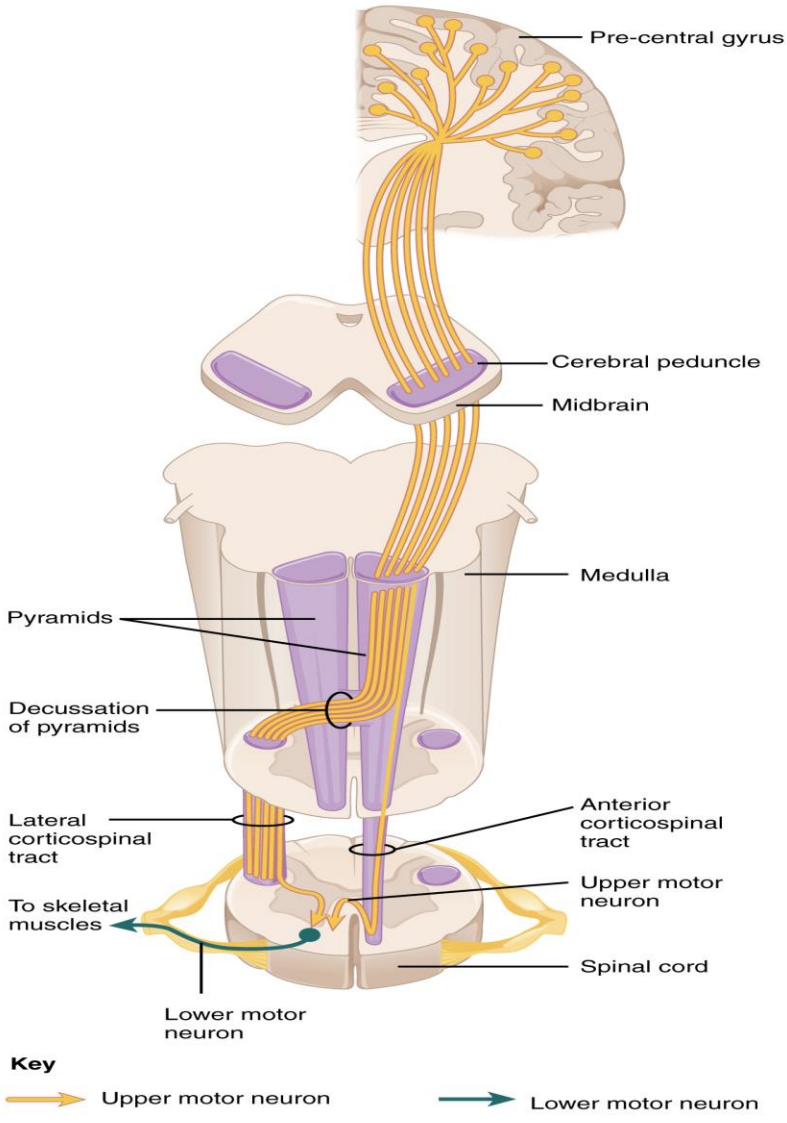
reticulo-espinal (Purves et al., 2010; Martin, 2014). Uma parte significativa desses feixes de axônios possui padrão distinto de degeneração na Esclerose Lateral Amiotrófica, e têm sido especulados como potenciais biomarcadores da doença, através de estudos de neuroimagem (Chió et al., 2014).

O córtex motor primário é a região do encéfalo diretamente ligada ao controle do movimento. Localiza-se na área 4 de Brodmann, e possui conexões com diversas regiões corticais determinantes para o planejamento dos movimentos, como as áreas pré-motoras do lobo frontal (Purves et al., 2010). Também se conectam ao córtex motor primário regiões associadas à resposta motora a estímulos sensoriais e humorais e núcleos subcorticais, e.g. as áreas somatossensoriais parietais e núcleos talâmicos, respectivamente (Martin, 2014).

A coordenação motora por parte do encéfalo é uma novidade evolutiva que surge em *Mammalia*. De fato, em espécies mais antigas como os marsupiais, o controle motor é coordenado eminentemente por circuitos locais localizados na medula espinal. Por outro lado, camundongos (Mus musculus) e chimpanzés (*Pan troglodytes*), possuem regiões corticais destinadas ao controle motor, com uma clara subdivisão entre regiões motoras executivas e planejadoras de movimentos nos primatas (Ebbesen & Brecht, 2017). Outra propriedade do córtex motor primário é a sua regionalização, o que origina grupos de neurônios nessa estrutura enviando eferências para os mesmos segmentos na medula espinal e, por conseguinte, as respectivas áreas onde coordenarão os movimentos. Tal característica permite criar mapas motores a partir de toda extensão dessa região cortical (Kandel et al., 2014).

O cerebelo e os núcleos da base, por fim, atuam de modo a regular a atividade motora indiretamente, via neurônios motores superiores (Purves et al, 2010). Localizado dorsalmente ao tronco encefálico, na região posterior do crânio, o cerebelo é formado por cerca de 50 bilhões de neurônios, cerca de metade do total presente em todo SNC. Através de suas conexões com o córtex motor e núcleos pontinos, essa estrutura está associada à correção do "erro motor", inibindo possíveis alterações entre o movimento pretendido e o executado (Ramnani, 2006). Os núcleos da base compõem um conjunto diverso de estruturas subcorticais dopaminérgicas, entre as quais o estriado, globo pálido e substancia nigra que, através de suas conexões com o tálamo, impedem a execução de movimentos indesejados e preparam as regiões motoras superiores do córtex para os realizarem (Kandel et al., 2014).

Figura 1. Representação do córtex motor conectado às regiões efetoras do movimento, i.e. neurônios motores inferiores e músculos, via trato corticoespinal. Figura obtida em https://open.oregonstate.education/aandp/chapter/14-5-sensory-and-motor-pathways/, no dia 01.07.2020.



1.2.2 Neuropatologia e Neuroimagem

Devido ao comprometimento dos neurônios motores superiores e inferiores, a Esclerose Lateral Amiotrófica tem impacto direto sobre os sistemas associados à atividade motora no SNC. O quadro neuropatológico torna-se mais heterogêneo quando outras subestruturas são afetadas, originando a variabilidade clínica que lhe é característica. Estudos *post-mortem* de tecidos de pacientes com ELA foram fundamentais para a sua caracterização, desde o trabalho pioneiro de Charcot (Goetz, 1999). Atualmente, técnicas mais elaboradas de neuroimagem permitem também avaliar o SNC *in vivo*. Dessa maneira, vários aspectos do processo neurodegenerativo podem ser analisados, e até mesmo acompanhados, conforme a doença progride (Chió et al., 2014; Buchanan et al., 2015). Dentre os parâmetros frequentemente analisados, notabilizam-se: densidade de substância cinzenta, fluxo sanguíneo e de líquido cefalorraquidiano, integridade da substância branca, entre outros (Turner et al., 2012).

Tecidos nervosos de pacientes com ELA possuem em comum a formação de alterações neuropatológicas denominadas Corpos de Bunina (CBs) e inclusões citoplasmáticas TDP43 positivas (Takeda et al, 2019). Devido a essa especificidade, protocolos clínicos como o El Escorial preconizam a avaliação *post-mortem* desses agregados celulares para efeitos de diagnóstico (Brooks et al, 2000).

Os corpos de Bunina são mais frequentemente identificados na medula espinal e em núcleos do tronco encefálico, formando inclusões eosinofílicas (Okamoto et al, 2008). Análises de imunohistoquímica têm mostrado que não reagem com anticorpos contra TDP-43, entretanto, têm sido associados a uma miríade de outras proteínas, como a transferrina, periferina, cistatina C, uma característica que pode ser derivada de sua provável origem no retículo endoplasmático e complexo de Golgi (Mizuno et al, 2006; Mori et al, 2019). As inclusões da TDP-43, por sua vez, são formadas de fragmentos fosforilados dessa proteína em associação com ubiquitina (Neumann et al., 2006; Brettschneider et al., 2013). Ambos os achados patológicos têm sido descritos associados a autofagossomos, o que sugere prováveis mecanismos de resistência à formação dessas inclusões nos citoplasmas de neurônios e células gliais (Mori et al., 2019).

A importância dos achados neuropatológicos como critérios classificatórios têm na Demência Fronto-Temporal um exemplo clássico. Pacientes portadores dessa condição, por exemplo, podem ser agrupados segundo a presença de inclusões TDP-

43, FUS positivas ou marcando para a proteína Tau (Neumann et al., 2006; Elahi & Miller, 2017). Nesses três subtipos há variação quanto à base molecular da doença e manifestações clínicas associadas (Neumann et al., 2009).

Outro aspecto importante do processo degenerativo em ELA e outras condições neurológicas similares é a presença de neurofilamentos no líquor (Delaby et al., 2020). Essas proteínas são componentes do citoesqueleto exclusivamente expressos em neurônios, e são classificados conjuntamente como filamentos intermediários, devido ao seu diâmetro estar entre os filamentos de actina de 10 nm, e os de miosina (15 nm) (Khalil et al., 2018). Em doenças neurodegenerativas, como a ELA, a DFT, Doença de Alzheimer e outras condições, perda axonal é um evento comum. Nessas circunstâncias, ocorre o extravasamento de conteúdos intracelulares, entre eles os neurofilamentos, para a matriz extracelular (Skillback et al, 2014). Evidências experimentais têm demonstrado que alguns componentes dessa classe de proteínas do citoesqueleto podem ser excelentes indicativos da progressão da doença, servindo dessa forma como biomarcadores. Um recente trabalho, envolvendo 715 pacientes com ELA e 87 portadores de DFT, por exemplo, demonstrou que NfLs (Neurofilamentos leves; 78- 86 KDa) acumulam-se em maior quantidade em no líquor dos indivíduos afetados por aquelas doenças que em pessoas normais (Skillback et al, 2017). Trata-se, portanto, de uma área promissora na predição do prognóstico de pacientes com doenças neurodegenerativas (Khalil et al, 2018).

Estudos de neuroimagem têm sido semelhantemente utilizados para avaliação neuropatológica em Esclerose Lateral Amiotrófica. Inicialmente, o emprego de tais técnicas visava apenas excluir entidades correlatas que eventualmente causassem fenótipos similares à ELA (Kiernan et al., 2011). O avanço de técnicas de imageamento, entretanto, possibilitou perscrutar diferentes aspectos do SNC, como a presença de biomarcadores (mio-inositol, N-acetilaspartato, etc), integridade de tratos de substância branca, atividade metabólica e outros parâmetros (Foerster et al., 2013). Hoje, devido a esses avanços, sabe-se que a esclerose lateral amiotrófica apresenta padrões de neurodegeneração sistêmicos, e não apenas circunscritos às áreas motoras do SNC (Trojsi et al, 2020).

Em um amplo estudo envolvendo 292 pacientes e 192 controles, van der Burg et al., (2020) conseguiram observar diferenças no processo neuropatológico, ao comparar pacientes com diferentes manifestações fenotípicas. Os autores observaram,

por exemplo, que indivíduos portadores de expansões do *C9orf72* tendem a apresentar redução de volume cortical em um número muito maior de regiões que os portadores de outras alterações, que se notabilizaram pela perda neuronal no córtex motor, lobos frontal e temporal e estruturas subcorticais dos núcleos da base e hipocampo. Pacientes classificados conforme o início bulbar ou espinal dos sintomas também foram avaliados por esses autores. Apesar de apresentarem padrões similares de neurodegeneração nas regiões motoras primárias e no córtex entorrinal direito, ambos grupos divergiram em relação a outras subestruturas do SNC. Os indivíduos portadores de um início bulbar, por exemplo, caracterizaram-se pela perda cortical em regiões parietais e fronto-temporais (van der Burg et al., 2020).

Esses achados levam a crer, portanto, que estudos de neuroimagem podem servir de biomarcadores para ELA, de maneira similar aos neurofilamentos. Ensaios clínicos, até hoje com resultados conflitantes, podem se beneficiar dessas observações, no sentido de melhor caracterizar fenotipicamente seus pacientes, levando a resultados mais robustos (Foerster et al., 2013).

1.3 Etiologia da Esclerose Lateral Amiotrófica

A grande variabilidade fenotípica na Esclerose Lateral Amiotrófica, como seria de se esperar, implica também em uma grande heterogeneidade de fatores biológicos e/ou ambientais associados à sua etiologia (Mathis et al., 2019). Com o advento de tecnologias de sequenciamento de nova geração, como o *whole genome sequencing* (WGS) e o *whole exome sequencing* (WES), a identificação de fatores de risco genético e de variantes raras, de alta penetrância, tem se acelerado (Johnson et al., 2010). Entretanto, em algumas casuísticas de indivíduos portadores dessa condição, a identificação de um fator etiológico subjacente à ELA chega a 70% em casos familiais e apenas 10% nos esporádicos. Claramente, novos fatores relacionados à ELA aguardam identificação, sobretudo em relação aos casos esporádicos, onde o papel ambiental é amplamente desconhecido (Renton et al., 2014).

Para efeitos de classificação, a Esclerose Lateral Amiotrófica pode também ser subdividida em familial e esporádica (Andersen & Al-Chalabi, 2011). Nesse caso, os tipos familiais caracterizam-se por apresentar recorrência familiar da doença, segregando em genealogias de forma mendeliana. Os casos esporádicos, por outro lado,

correspondem a 90% dos pacientes portadores de ELA identificados em serviços de atendimento a doenças neuromusculares (Perrone & Conforti, 2020).

Estudos comparativos têm demonstrado que o processo neurodegenerativo, em ambos os subtipos ocorrem de modo similar (Ferrante et al., 1997). Com base nisso, os casos familiais têm sido amplamente utilizados como modelos para o estudo dessa patologia (Mitne-Neto et al., 2011; Selvaraj et al., 2018). De fato, variantes genéticas, localizadas nos mesmos genes identificados em casos familiais de ELA, também têm sido descritas nas formas esporádicas, comprovando a relação biológica (Cady et al, 2015).

1.3.1 ELA Familiar

Desde a sua descrição inicial por Charcot e outros no séc XIX, os fatores etiológicos associados à ELA permaneceram desconhecidos por décadas. O advento de técnicas moleculares na década de 90, entretanto, possibilitou mudar essa realidade. Em um trabalho seminal, Rosen et al (1993), identificou valores altos de LOD Score com o marcador D21S223, localizado próximo ao gene da superóxido dismutase (*SOD1*). A análise, por sequenciamento Sanger, do SOD1 em treze famílias diferentes de pacientes com ELA e um padrão autossômico dominante, permitiu a descoberta de onze variantes raras nesse gene que segregavam com a doença. Àquela época, não estava claro de que maneira essas mutações desencadeariam o processo patológico da Esclerose Lateral Amiotrófica, e os autores especularam sobre um possível papel tóxico da acumulação de radicais livres, uma hipótese que atualmente está descartada.

Atualmente, centenas de variantes genéticas, situadas em mais de 30 genes têm sido reportadas em famílias com ELA, um fenômeno que mostra a sua alta heterogeneidade genética (Mathis et al, 2019). Apesar da maioria dos casos manifestarem-se num padrão de herança autossômico dominante, há registros de famílias manifestando a doença de forma ligada ao X e autossômica recessiva (Deng et al., 2011; Helal et al., 2018). (Tabela 1).

Tabela 1. Principais genes associados a diferentes formas de Esclerose Lateral Amiotrófica

Gene	Localização cromossômica	Padrão de herança	OMIM
SOD1 (CuZn-superóxido dismutase)	21q22.11	AD e AR	105400
TARDBP (*TAR-DNA binding protein 43*)	1p13.22	AD	612069
FUS (*Fused in Sarcoma*)	16p11.2	AD e AR	608030
C9orf72 (*chromosome 9 open Reading frame 72*)	9p21.2	AD	105550
VAPB (*Vesicule Trafficking Protein B*)	20q13.32	AD	608627
ALS2 (*Alsin2*)	20q33.1	AR	205100
UBQLN2 (*Ubiquilin 2*)	Xp11.21	L-X	300857
MATR3 (*Matrin-3*)	5q31.2	AD	606070
SQSTM1 (*Sequestosome-1*)	5q35.3	AD	616437
OPTN (*Optneurin*)	10p13	AD e AR	613435
ANG (*Angiogenin*)	14q11.2	AD	611895
ATXN2 (*Ataxin-2*)	12q24.12	AD	183090
PFN1 (*Profilin-1*)	17p13.2	AD	614808
HNRNPA1 (*Heterogeneous Nuclear Ribonucleoprotein A1*)	12q13	AD	615426
ANXA11 (*Annexin A11*)	10q22.3	AD	617839
VCP (*Valosin-Containing Protein*)	9p13.3	AD	613954

Apesar de altamente heterogênea, *screenings* genéticos em pacientes com ELA têm demonstrado que parte significativa de sua etiologia é causada por uma fração pequena de genes (Perrone & Conforti, 2020). Uma meta-análise de 111 estudos de correlação genótipo- fenótipo demonstrou que mutações em *FUS*, *TARDBP*, *SOD1* e *C9orf72* correspondiam a 47,7% dos casos em ELA familial e 5,2% nos casos esporádicos (Zou et al., 2016). Como previsto, populações asiáticas e europeias apresentaram uma discrepância em termos da importância relativa desses genes. Naquelas, *SOD1* correspondia a 30% dos afetados por formas familiais, enquanto o gene *FUS* viria em seguida, com 6,4. Por fim, *C9orf72* e *TARDBP* teriam, respectivamente, 2,3% e 1,5%, de recorrência. Em europeus, diferentemente, a expansão da *C9orf72* foi identificada como a mais frequente (33,7% nas formas familiais). Por outro lado, as mutações em *SOD1*, *TARDBP* e *FUS*, correspondiam a 14,8%, 4,2% e 2,8%, respectivamente (Zou et al., 2016).

1.3.1.1 O gene C9orf72

Estudos de ligação apontando para um loco em 9p21.3-9p21.1 segregando com Esclerose Lateral Amiotrófica e/ou Demência Fronto-Temporal foram reportados na literatura (Vance et al., 2006; Morita et al., 2006). Entretanto, a causa biológica dessas entidades nessa região cromossômica permaneceu um mistério por um longo tempo. Estudos posteriores de GWAS (*genome-wide association studies*) chegaram a resultados similares, não só restringindo a região de ligação, como mostraram a sua elevada prevalência entre populações europeias (Laaksovirta et al., 2010). Por fim, após esforços conjuntos de diferentes grupos, identificou-se nesses pacientes uma expansão de hexanucleotídeos GGGGCC (G_4C_2) no gene C9orf72 (De Jesus-Hernandez et al., 2011; Renton et al., 2011). Dada a sua ampla distribuição entre portadores tanto de ELA quanto DFT, a expansão do C9orf72 foi considerada a prova definitiva da origem biológica comum entre ambas as entidades, embora já houvesse descrição de casos com mutações em *FUS*, *TARDBP*, *MAPT* e *VCP* (Gijselinck et al, 2018).

Como em outras doenças de expansão de nucleotídeos, logo se especulou sobre a variabilidade clínica associada ao número de repetições G_4C_2. De fato, as repetições

de C9orf72 variam entre 2 e mais de 4000 repetições, onde o número de repetições tidas como "normais" corresponderiam entre 2 e 20, a faixa limítrofe entre 20 e 30, e indivíduos com repetições acima de 30 seriam os portadores de ELA e/ou DFT (De Jesus-Hernandez, et al, 2011; Renton et al., 2011; Nuytemans et al., 2013; van Blitterswijk et al., 2013). Nessas genealogias, a antecipação do início dos sintomas ao longo de gerações também é um fenômeno frequente (Hsiung et al., 2012; Stewart et al., 2012). Curiosamente, modificadores genéticos em portadores da DFT causada pela expansão G_4C_2 foram descritos: a variante rs1990622 do gene *TMEM106B* tem sido associada a uma maior sobrevida nesses indivíduos, enquanto expansões intermediárias de *ATXN2* aumentam o risco de desenvolvimento da ELA (Gallagher et al., 2014; van Blitterswijk et al., 2014).

Estudos de hidridização fluorescente *in situ* (FISH) demonstraram que as expansões do C9orf72 eram transcritas e acumulavam-se no interior do núcleo dos neurônios em forma de "foci" de RNA (Xi et al., 2015). Esses achados levaram à hipótese de uma suposta toxicidade dessas moléculas, como já havia sido sugerido para outras doenças neurodegenerativas, como a FXTAS (Asamitsu et al., 2020). Trabalhos posteriores demonstraram também a presença de dipeptídeos aberrantes no interior dos neurônios, produto de um mecanismo não-convencional de tradução daqueles mesmos RNAs da expansão G_4C_2 do gene *C9orf72* (Cheng et al., 2018) Essas espécies peptídicas foram observadas interferindo em diversos aspectos da fisiologia celular, como o tráfego nucleocitoplasmático e metabolismo mitocondrial (Jovicic et al., 2015; Allen et al., 2019). Atualmente, não está clara a importância relativa desses dois aspectos patológicos da *C9orf72* para o desenvolvimento da ELA/DFT e, embora não sejam mutuamente exclusivos, são alvos de intensos debates.

1.3.1.2 O gene SOD1

Atualmente, mais de 180 mutações patogênicas no gene da superóxido dismutase (*SOD1*) foram descritas em pacientes portadores da Esclerose Lateral Amiotrófica (Mathis et al., 2019). Dentre essas variantes genéticas também se observa uma grande diversidade de evolução clínica. Pacientes com a mutação em heterozigose A4V, por exemplo, desenvolvem rapidamente uma forma agressiva de ELA, levando a óbito em cerca de um ano (Cudkowicz et al, 1997). Outras variantes, entretanto,

aparentam manifestações fenotípicas mais brandas, como a mutação em homozigose D90A. Esta, causadora de uma forma autossômica recessiva de ELA, evolui numa progressão mais lenta, que pode chegar até uma década (Andersen et al., 1996).

A atividade de dismutase da SOD1 depende da formação de homodímeros associados a íons Cu^{+} e Zn^{2+}. Estudos funcionais com proteínas portadoras de alterações patogênicas, como C6S, N90A, A89V demonstraram que sua atividade enzimática não é perdida em alguns modelos mutantes. Isso sugere, portanto, que a perda de função proteica não desempenha um papel preponderante no processo neurodegenerativo do *SOD1* em ELA (Andersen & Al-Chalabi, 2011). De fato, atualmente acredita-se que essas mutações ocasionam um ganho de função tóxica na proteína. O seu acúmulo progressivo no citosol, interferiria em vários processos celulares, como tráfego de vesículas e atividade mitocondrial, levando à morte celular (Tafuri et al., 2015; Burk & Jeroen Pasterkump, 2019).

1.3.1.3 Os genes *TARDBP* e *FUS*

Fragmentos altamente fosforilados da proteína TDP-43, codificada pelo gene *TARDBP,* constituem em um dos achados patológicos clássicos em preparações histológicas do SNC de pacientes com ELA (Brooks et al., 2000; Neumann et al., 2006). A sua presença em subtipos diferentes da Esclerose Lateral Amiotrófica/ Demência Fronto-Temporal é uma das evidências mais fortes para a unificação desses fenótipos em um espectro fenotípico. Devido a esse caráter, atualmente, tem sido proposto um modelo de progressão patológica para a ELA, onde essa proteína desempenha um papel similar ao da alfa-sinucleína na Doença de Parkinson ou da proteína tau em Alzheimer (Braak et al., 2013). Sob essa perspectiva, a TDP-43 desempenharia um papel "*prion-like*", se espalhando a partir de regiões motoras do cortex (áreas 4 e 6 de Brodmann) via neurônios de projeção para outras regiões corticais e subcorticais do SNC. O proposto modelo, denominado "corticofugal", é subdividido em quatro etapas, onde a progressão seguiria dessas regiões motoras fundamentais e de porções da substância cinzenta da medula espinal para outros alvos, seguindo transporte axonal (Braak et al., 2013).

Um aspecto relevante do papel fisiológico da TDP-43 é a sua atuação no metabolismo de RNAs. De fato, diferentes trabalhos têm atribuído à essa proteína a

regulação de passos importantes do splicing alternativo, por meio do qual regularia a expressão de genes importantes para o desenvolvimento e manutenção do CNS (Xue et al, 2020). A subsequente descoberta de mutações no gene *FUS* reforçou a importância do metabolismo de RNA na patologia da ELA, uma vez que o produto proteico desse gene tem sido associado mecanismos como biogênese de microRNAs, *splicing* alternativo, etc (Vance et al., 2009; Qiu et al., 2014). Atualmente, mutações patogênicas vários outros genes que sintetizam proteínas de ligação a RNA estão descritas para ELA, como *hnRNPA1*, *hnRNPA2B1*, *ATXN2* e *TIA1* (Xue et al., 2020).

Tanto a TDP-43 quanto a FUS são proteínas nucleares, e como tal, possuem um peptídeo sinal de endereçamento a essa organela (Kapeli et al., 2017). No citoplasma, essas proteínas também desempenham papel importante. Um deles é a capacidade de formar grânulos de estresse. Essas "organelas não membranosas", são constituídos por aglomerados proteicos com afinidade por moléculas de RNA, que ao ficarem presas a tais estruturas, são impedidas de se associarem à maquinaria de tradução (Hofweber & Dormann, 2018; Ivanov et al., 2019). Dessa forma, a célula consegue regular a expressão gênica para se adaptar a mudanças fisiológicas. Passadas as condições de estresse, tais aglomerados conseguem naturalmente se desfazer no citosol, liberando os RNAs ligados às suas proteínas. A propriedade dinâmica desses grânulos se deve a domínios proteicos chamados de LCDs (*low complexity domains*). Estes, ao associarem-se, modificam as propriedades físico-químicas dos grânulos, separando-os do coloide circundante (Cornicella et al, 2016).

Frequentemente, mutações do FUS estão localizadas em seu domínio de endereçamento nuclear (Kapeli et al, 2017). Nessas ocasiões, a sua própria retenção citoplasmática faz com que essa proteína se acumule no citosol a níveis intoleráveis. A habilidade natural de formar "agregados" proteicos fisiológicos, necessária para regulação da expressão gênica, torna-se, a longo prazo, um fardo para estabilidade do SNC (Cao et al, 2020).

1.3.1.4 O gene VAPB

Mutações no gene *VAPB* têm sido majoritariamente associadas a um subtipo familial autossômico dominante de Esclerose Lateral Amiotrófica, a ELA8 (Nishimura et al. 2004[a]). Os pacientes portadores dessa condição apresentaram, na sua descrição

inicial, duas manifestações distintas de ELA, classificadas como ELA "típica" e "atípica", que se diferenciavam pela presença de tremor apenas na forma atípica. Além disso, foi observado entre alguns indivíduos da genealogia portadores de mutação no gene *VAPB*, a manifestação de atrofia muscular espinhal (Nishimura et al. 2004[b]). Tal fenômeno reforça a grande variabilidade fenotípica já descrita nos fenótipos associados à ELA, e sugere que a *VAPB* desempenha um papel fundamental em circuitos eminentemente espinais.

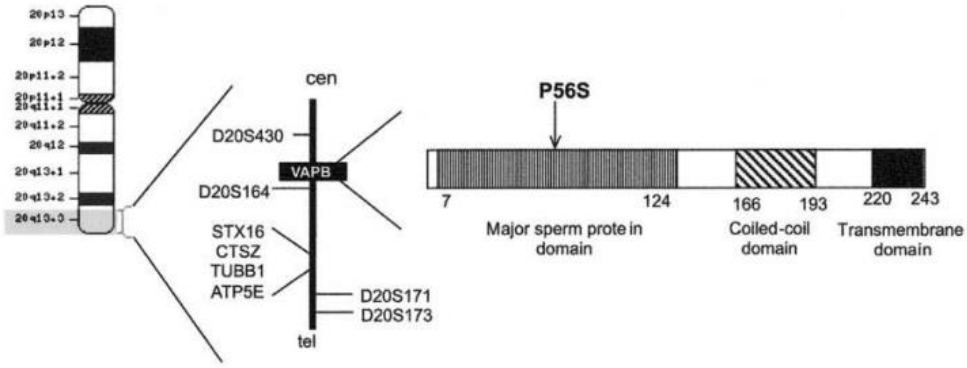
Até o momento, a maioria dos indivíduos afetados pela ELA8 são de nacionalidade brasileira, caucasoide e portadores de uma mesma mutação no exon 2 do gene VAPB (c.166C>T; p.P56S VAPB). Um efeito fundador foi então postulado para essa mutação no Brasil, que muito provavelmente chegou ao país via colonização portuguesa (Nishimura et al. 2005). Posteriormente, outros indivíduos com ancestralidade europeia, mas não portadores do mesmo haplótipo da *VAPB*, foram descritos na Alemanha (Funke et al, 201), sugerindo que a mutação P56S também surgiu de forma independente. Outros pacientes na China, Reino Unido e Estados Unidos também foram reportados com a variante P56S, e mais recentemente, outras duas mutações nesse gene, T46I e P56H, também foram associadas à ELA8 (Sun et al., 2017).

O gene *VAPB* (*vesicule trafficking protein B*) codifica para uma proteína localizada na superfície externa do reticulo endoplasmático (RE). Esta é composta de dois domínios coiled-coil, um domínio MSP (*Major Sperm Domain*) e um domínio transmembrana, cuja função é a sua ancoragem na membrana do RE (Mitne-Neto et al., 2011). Curiosamente, a proteína VAPB tem sido descrita localizada em sítios de contato entre o RE e outras organelas, especialmente mitocôndrias, endossomos e vesículas lipídicas (Phillips & Voeltz, 2015). É através dessas intersecções que diferentes processos celulares têm lugar, como autofagia, regulação do fluxo de cálcio, maturação de endossomos, *unfolded protein response* e síntese proteica (Burgoyne et al 2017; Lee et al., 2020; Nakatogawa et al., 2020).

Curiosamente, o domínio MSP da proteína VAPB foi observado, em drosófilas e no *C. elegans,* atuando como uma molécula secretória capaz de ligar-se a receptores de efrinas (EPh) (Tsuda et al, 2008). Estes compõem uma família de tirosina quinases que, em humanos, notabilizam-se por regular diversos aspectos do desenvolvimento e fisiologia neuronais, como extensão de axônios, formação de espinhos dendríticos,

nucleação de receptores de NMDA, etc (Goldsmith et al, 2006; Yang et al, 2018). Através da interrupção da sinalização autócrina MSP - Ephs, mutações na VAPB poderiam, portanto, interferir num aspecto central da sinapse dos neurônios motores, que é a formação dos receptores de NMDA. Esse desequilíbrio, por conseguinte, ocasionaria a excitotoxicidade, um fenômeno capaz de gerar apoptose devido a estimulação excessiva dos neurônios (Tsuda et al, 2008). Embora sugestiva, essa hipótese ainda necessita de avaliação em modelos experimentais humanos, mantendo-se até hoje como uma especulação.

Figura 2. Representação esquemática da localização cromossômica e domínios proteicos da *VAPB*. Adaptado de Nishimura et al., 2004[b]



Em outros trabalhos, curiosamente, verificou-se que a diminuição da expressão da VAPB não é característica apenas da ELA8. Teuling et al. (2007) observaram o mesmo fenômeno em camundongos portadores da mutação G93A do *SOD1*. Anagnostou et al (2010), por outro lado, constataram a diminuição dos níveis de *VAPB* em lisados de medula espinal de pacientes com ELA esporádica. Esses achados levam a sugerir um papel central da VAPB no processo degenerativo da Esclerose Lateral Amiotrófica. A associação entre a VAPB e os sítios de contato RE e outras organelas pode ser, em parte, a chave para esse fenômeno. Uma prova disso é o crescente interesse que essas estruturas têm recebido da comunidade científica (Paillusson et al. 2016). Recentemente, por exemplo, foi demonstrado que a α-sinucleína portadora da mutação A53T, amplamente ligada à Doença de Parkinson, interferia em sítios RE- mitocôndria. Essa alteração, causada pela disrupção da interação entre VAPB e a proteína mitocondrial PTPIP51, teria implicações fisiológicas nas mitocôndrias e no metabolismo de cálcio (Paillusson et al., 2017).

1.3.2 Esclerose Lateral Amiotrófica Esporádica

Conceitualmente, esclerose lateral amiotrófica esporádica é assim classificada devido à ausência de recorrência familiar num padrão mendeliano. Trata-se da forma mais frequente de ELA, correspondendo a 90% dos casos em registros epidemiológicos (Schymick et al., 2007). Estudos de herdabilidade têm demonstrado que cerca de 40 – 60% dos casos esporádicos têm causa genética, sugerindo que fatores de susceptibilidade à ELA esporádica ainda aguardam identificação (Wingo et al., 2011; Ryan et al., 2019).

Alguns mecanismos genéticos têm sido propostos como os mais associados à ELA esporádica. Dentre eles, pode-se citar mecanismos poligênicos, oligogênicos ou até mesmo mutações *de novo* (McCann et al., 2020). Estudos de GWAS foram amplamente utilizados no início para identificar fatores de risco genético para a ELA. Entretanto, uma parte significativa desses achados não têm sido replicados (Schymick et al., 2007; Ajroud-Driss & Siddique, 2015). As próprias limitações técnicas dos estudos de GWAS levaram a esses resultados, visto que a identificação de variantes nesses casos depende do desequilíbrio de ligação com SNPs marcadores polimórficos (Nicolae, 2016). A popularização do sequenciamento de genomas completos (*Whole-Genome Sequencing*; WGS) e o desenvolvimento de ferramentas metodológicas para a o estudo de variantes raras têm se mostrado promissoras. Esses podem contribuir para o aumento da compreensão da arquitetura genética na ELA esporádica, embora esbarrem na necessidade do recrutamento de um número cada vez maior de indivíduos (Lee et al., 2014). Apesar disso, resultados mais robustos têm sido publicados, como a recente identificação de mutações no gene *KIF5A* (Nicolas et al,, 2018).

O primeiro trabalho visando estudar mecanismos *de novo* em pacientes com ELA analisou 47 trios, previamente excluídos para expansões em *C9orf72*. O sequenciamento dos 141 exomas identificou uma mutação introduzindo um códon de parada prematuro no gene *CREST* (p.Q388stop; *CREST*) (Chesi et al., 2013). Desde então, outras iniciativas têm sido empreendidas para identificar tais variantes. Um achado frequente nesses trabalhos é a associação de variantes em genes comumente reportados para casos familiais. *Screenings* genéticos em ELA esporádica em

populações europeias têm demonstrado que as expansões de *C9orf72* correspondem a 5,1% dos casos, enquanto variantes no *SOD1*, 1,2%. *TARDBP* e *FUS*, por outro lado, seriam responsáveis por 0,8% e 0,3% dos casos, respectivamente (Zou et al., 2017). Esses dados reforçam a base genética comum que os casos familiais e esporádicos possuem, e têm sido utilizados como argumento por críticos do sistema de classificação da ELA baseado em agregação familiar. Numa iniciativa de construir um modelo único para a Esclerose Lateral Amiotrófica, alguns autores têm proposto um modelo sequencial para a patogênese dessa doença (*multistep model*) (Al-Chalabi et al., 2014; Chió et al, 2018). Nessa proposta, "seis passos" ou seis fatores de risco deveriam incidir na história de vida de um paciente para a manifestação do fenótipo. Predisposição genética, fatores de risco ambiental e outras alterações epigenéticas seriam alguns desses "passos", que poderiam variar de importância relativa de acordo com o grau de penetrância das variantes causais.

Em uma análise de mais de 612 casos de ELA esporádica na Austrália, McCann et al (2020) identificaram 42 indivíduos portadores de mais de uma variante já associada a essa doença. Desses, 38 carregavam duas mutações, e 4 tinham 3 mutações. Um modelo oligogênico foi então proposto para esses indivíduos, como o mais provável mecanismo genético subjacente à ELA. Críticos desse modelo frequentemente apontam a possibilidade de mutações raras, como as identificadas em tais coortes, apresentarem-se cumulativamente em um mesmo indivíduo. Entretanto, para esses casos, o mais provável é que mutações de baixa penetrância e aspectos genético-populacionais sejam os fatores mais prováveis na construção desse genótipo atípico. A mesma avaliação na ilha de Sardenha, por exemplo, mostrou que 25% dos casos de ELA esporádica têm ou a expansão patogênica de *C9orf72,* ou a mutação A328T em *TARDBP* (Chio et al 2012). Curiosamente, a análise haplotípica em portadores dessas variantes demonstrou que ambas seriam resultado de um efeito fundador, durante o povoamento dessa região.

Casos onde há claro efeito epistático têm sido também reportados na literatura. Uma variante no gene *UNC13A* (rs12608932 C) foi identificada como fator modulador da progressão da ELA, em uma meta-análise que associou dados genômicos de diversos estudos de GWAS, e curvas de sobrevida dos pacientes neles envolvidos. Concluiu-se, através desse estudo, que portadores dessa variante tinham um prognóstico pior da doença, levando-os ao óbito mais rapidamente. (Diekstra et al., 2012). Análises

posteriores não só confirmaram esses achados, como especularam sobre o papel da variante genética no processo neurodegenerativo da ELA. Esses trabalhos têm demonstrado que UNC13 está associado ao recrutamento do complexo SNARE, a partir do qual membranas se fusionariam (Yang et al. 2015). Dessa maneira, uma disrupção nesse sistema provavelmente comprometeria o funcionamento de sinapses, e implicaria no agravamento da doença (Reddy-Alla et al, 2017).

Outro efeito epistático bem caracterizado é a de mutações de perda de função no gene do receptor A4 da efrina (*EPHA4*). *Screenings* genéticos em *Danio rerio* (Peixe-zebra), portadores de mutações em SOD1 (A4V, G93A, G37R), mostraram que mutações hipomórficas naquele gene resgatavam o fenótipo normal (Van Hoecke et al., 2012). Esses achados foram replicados em camundongos e em coortes de pacientes com ELA esporádica. Nesse último caso, observou-se uma progressão mais lenta da doença quando os pacientes possuíam mutações de perda de função em *EPHA4* com a diminuição da sua expressão (Van Hoecke et al., 2012). Devido à sua importância no SNC, vários estudos têm sido feitos sobre o gene receptor da efrina. Esses trabalhos têm atribuído ao produto proteico desse gene várias funções fisiológicas, tais como regulação de formação de dendritos e extensão de axônios durante o desenvolvimento (Klein, 2004; Yang et al., 2018). Por outro lado, *EPHA4* tem sido associado à regulação da atividade do recepto ionotópico de glutamato (receptores AMPA), a partir dos quais modularia a atividade sináptica (Fu et al., 2010).

Não está claro ainda como mutações de perda de função no *EPHA4* protegeriam seus portadores de uma forma mais grave de ELA. Entretanto, a identificação de fatores modificadores epistáticos é altamente informativa para a Esclerose Lateral Amiotrófica, uma doença cujas manifestações clínicas se apresentam de forma tão variável (Swinnen & Robberecht, 2014).

1.3.3 Fatores ambientais associados à ELA

Devido à complexidade, tanto em termos de recorrência em populações quanto em manifestações clínicas, tem havido um grande interesse na identificação dos fatores ambientais associados à ELA. Entretanto, a maioria das tentativas de estabelecer uma correlação entre esses e a patogênese dessa doença tem sido alvo de questionamentos metodológicos (Bozzoni et al., 2016). Isso, provavelmente, se deve à dificuldade de

isolar tais agentes ambientais num contexto onde o genótipo dos indivíduos observados seja homogêneo. Outros fatores complicadores nesses estudos seriam a variabilidade quanto ao grau de exposição ambiental, e a dificuldade da observação de uma grande coorte por um longo período de tempo. Até o momento, vários candidatos têm sido apontados como fatores de risco ambiental para a ELA, como exposição a metais pesados, pesticidas, atividade física intensa e cianotoxinas (Al-Chalabi & Hardiman, 2013).

A Esclerose Lateral Amiotrófica do subtipo "Guam" é um caso clássico onde fatores ambientais parecem apresentar um papel preponderante. Esse tipo de ELA notabiliza-se também pela manifestação de sinais clínicos típicos de Doença de Parkinson e Alzheimer (Steele & McGeer, 2008). Sua recorrência apresenta-se particularmente alta na ilha de Guam, ilhas Marianas, na Nova Guiné e em regiões do Japão, chegando a ser até cem vezes maior que em outros locais (Takeda et al., 2019). Análises nessas populações locais identificaram níveis altos de uma cianotoxina (BMAA, beta-metil-amino-L-alanina) no SNC dos pacientes afetados. Esse composto foi observado também particularmente alto em morcegos que se alimentavam de frutos de *Cyca* sp., um vegetal que vive em simbiose com cianobactérias produtoras de BMAA. O hábito de se alimentar desses animais foi então sugerido como o mecanismo mais provável de magnificação trófica da toxina em humanos (Al-Chalabi &Hardiman, 2013).

Estudos posteriores demonstraram que o beta-metil-amino-L-alanina é capaz de realizar profundas alterações proteômicas em modelos celulares, ativando vias biológicas ligadas a processos neurodegenerativos, como UPR (*unfolded protein response*), disfunção mitocondrial e vias de ubiquitinação (Beri et al., 2017). Modelos murinos tratados com BMAA também desenvolveram fenótipos consistentes com o processo neuropatológico da ELA, como alterações mitocondriais, astrogliose e expressão de citocinas pró-inflamatorias (Tian et al., 2015).

1.4 Objetivos

Os objetivos do presente trabalhos foram:

- Desenvolver modelos de célula-tronco pluripotente induzida (iPSC) para pacientes portadores de Esclerose Lateral Amiotrófica tipo 8 com diferentes manifestações fenotípicas;
- Identificar, através de estudos funcionais, técnicas de sequenciamento de nova geração e de análise de expressão gênica, potenciais fatores modificadores da progressão clínica na ELA;
- Estudar uma grande família consanguínea com um padrão autossômico recessivo de Esclerose Lateral Amiotrófica.

Capítulo 2

Estudo de pacientes portadores da ELA8 com diferentes perfis clínicos

Different gene expression profiles in iPSC-derived motor neurons from ALS8 patients with variable clinical courses suggest mitigating pathways for neurodegeneration

Human Molecular Genetics 29 (2020) 1465 – 1475.

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Abstract

Amyotrophic lateral sclerosis type 8 (ALS8) is an autosomal dominant form of ALS, which is caused by pathogenic variants in the *VAPB* gene. Here we investigated five ALS8 patients, classified as 'severe' and 'mild' from a gigantic Brazilian kindred, carrying the same *VAPB* mutation but displaying different clinical courses. Copy number variation and whole exome sequencing analyses in such individuals ruled out previously described genetic modifiers of pathogenicity. After deriving induced pluripotent stem cells (iPSCs) for each patient (N=5) and controls (N=3), motor neurons were differentiated, and high-throughput RNA-Seq gene expression measurements were performed. Functional cell death and oxidative metabolism assays were also carried out in patients' iPSC-derived motor neurons. The degree of cell death and mitochondrial oxidative metabolism were similar in iPSC-derived motor neurons from mild patients and controls and were distinct from those of severe patients. Similar findings were obtained when RNA-Seq from such cells was performed. Overall, 43 genes were upregulated and 66 downregulated in the two mild ALS8 patients when compared with severe ALS8 individuals and controls. Interestingly, significantly enriched pathways found among differentially expressed genes, such as protein translation and protein targeting to the endoplasmic reticulum (ER), are known to be associated with neurodegenerative processes. Taken together, the mitigating mechanisms here presented appear to maintain motor neuron survival by keeping translational activity and protein targeting to the ER in such cells. As ALS8 physiopathology has been associated with proteostasis mechanisms in ER–mitochondria contact sites, such differentially expressed genes appear to relate to the bypass of VAPB deficiency.

Resumo

Esclerose Lateral Amiotrófica tipo 8 (ELA8) é uma forma autossômica dominante de ELA, que é causada por mutações patogênicas no gene *VAPB*. Neste trabalho, investigamos cinco pacientes com ELA8, classificados como “severos” ou “brandos”, de uma mesma família brasileira gigante, portando a mesma mutação no gene VAPB, mas apresentando diferentes cursos clínicos. A análise do número de cópias (CNVs) e sequenciamento do exoma nesses indivíduos excluiu modificadores genéticos previamente descritos. Depois de derivar células tronco pluritpotentes induzidas para cada paciente (N=5) e controles (N=3), neurônios motores foram diferenciados, e experimentos de expressão gênica através do *RNA Sequencing* foram executados. Ensaios funcionais de morte celular e metabolismo oxidativo foram também realizados nos neurônios derivados de iPSCs dos pacientes. O grau de morte celular e metabolismo oxidativo mitocondrial foram similares nos neurônios motores de pacientes brandos e controles, e diferentes dos pacientes com quadros severos. Resultados similares foram obtidos quando o *RNA Sequencing* dessas células foi realizado. No total, 43 genes estavam superexpressos e 66 diminuídos em ambos os pacientes brandos, quando comparados aos indivíduos ELA8 severos e os controles. Interessantemente, vias biológicas significantemente enriquecidas identificadas entre os genes diferencialmente expressos, tais como tradução proteica e endereçamento proteico para o retículo endoplasmático (RE), estão sabidamente associadas a processos neurodegenerativos. No geral, os mecanismos mitigadores aqui apresentados aparentemente mantêm sobrevivência dos neurônios motores pela manutenção da tradução e o endereçamento de proteínas para o RE nessas células. Como a patofisiologia da ELA8 tem sido associada com mecanismos de proteostase em sítios de contato RE- mitocôndria, tais genes diferencialmente expressos parecem estar relacionados à resiliência à deficiência da VAPB.

Human Molecular Genetics, 2020, Vol. 29, No. 9 1465–1475

doi: 10.1093/hmg/ddaa069
Advance Access Publication Date: 13 April 2020
General Article

GENERAL ARTICLE

Different gene expression profiles in iPSC-derived motor neurons from ALS8 patients with variable clinical courses suggest mitigating pathways for neurodegeneration

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Abstract

Amyotrophic lateral sclerosis type 8 (ALS8) is an autosomal dominant form of ALS, which is caused by pathogenic variants in the *VAPB* gene. Here we investigated five ALS8 patients, classified as 'severe' and 'mild' from a gigantic Brazilian kindred, carrying the same *VAPB* mutation but displaying different clinical courses. Copy number variation and whole exome sequencing analyses in such individuals ruled out previously described genetic modifiers of pathogenicity. After deriving induced pluripotent stem cells (iPSCs) for each patient ($N = 5$) and controls ($N = 3$), motor neurons were differentiated, and high-throughput RNA-Seq gene expression measurements were performed. Functional cell death and oxidative metabolism assays were also carried out in patients' iPSC-derived motor neurons. The degree of cell death and mitochondrial oxidative metabolism were similar in iPSC-derived motor neurons from mild patients and controls and were distinct from those of severe patients. Similar findings were obtained when RNA-Seq from such cells was performed. Overall, 43 genes were

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Received: November 8, 2019. **Revised:** April 4, 2020. **Accepted:** April 9, 2020

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by the selective death of upper and lower motor neurons (MNs) in the spinal cord, brainstem and motor cortex (1). Affected individuals typically present fasciculations and progressive weakness, which is followed by skeletal muscle atrophy, dysphagia and dysarthria. Death commonly occurs within 3 years from the disease onset, mainly due to respiratory failure (2). However, ALS patients may show variable disease onset and progression, as well as comorbidities (3). Amyotrophic lateral sclerosis type 8 (ALS8) is a familial autosomal dominant type of ALS described by our group, which is caused by pathogenic mutations in *VAPB* gene (4). Most ALS8 patients carry the same mutation (c.C166T; p.P56S), although other variants have been described in China and elsewhere (5).

VAPB is a highly conserved gene which encodes a transmembrane protein localized to the endoplasmic reticulum (ER). It is composed of three main functional domains, namely, a central coiled coil transmembrane region, a C-terminal intra-ER domain and a N-terminal major sperm protein (MSP) domain located at the ER's outer surface (6). The MSP domain, where the p.P56S variant is located, has been suggested to play an important role in the interaction of *VAPB* with other organellar membrane-associated proteins, thus mediating ER organelle contacts (7,8). A growing evidence has shown that the contacts that ER forms with other membranous organelles, such as endosomes and mitochondria, play a variety of functions important for cell homeostasis, such as regulation of endosome maturation and traffic (7), as well as mitochondria calcium metabolism and fission/fusion dynamics (7,9).

Similar to other types of ALS, phenotypic manifestations in ALS8 are highly variable, ranging from early- to late-onset ALS, or late-onset spinal muscular atrophy. In addition, clinical progression varies from a typical course of 5–10 years up to 30 years (4). More recent studies expanded the plethora of signals and symptoms associated with ALS8 by describing patients with tremor and chronic pain (10). The mechanisms underlying phenotypic variability in ALS8 as well as in different forms of ALS are poorly understood. Currently, only a few genetic modifiers have been proposed to modulate ALS clinical presentation, but the mechanisms of action are still poorly understood for most of them (11–13). Investigating the molecular pathways underlying ALS clinical variability might provide novel therapeutic targets, paving the way for more effective treatments.

Herein, by using different genetic and cellular approaches, we investigated the mechanisms of phenotypic variability in ALS8 Brazilian patients belonging to the same family, harboring the p.P56S mutation and presenting different clinical profiles. The patients were classified as presenting either 'highly severe' (S1 and S3) and 'severe' (S2) symptoms or 'mild' symptoms (M1 and M2) based on the clinical course and neurological examination (Table 1).

Results

ALS8 presents phenotypic variability, which is not due to other ALS-associated variants or copy number variations

The patients enrolled to this study were evaluated by a single neurologist, and their clinical data are summarized in Table 1.

Patient S1. A 59-year-old male started showing symptoms around 38 years of age, which included tremor, cramps and weakness in the legs. He also presented enlarged abdomen, fasciculation and absence of deep tendon reflexes. He developed progressive muscle weakness with impaired ambulation after 8 years of diagnosis, becoming wheelchair-bound at the age of 54 and requiring respiratory aid (BiPAP) at the age of 56. At the age of 57, he was submitted to gastrostomy and enteral nutrition, and, currently, he exhibits frequent choking signs as well as voice changes. His proximal muscular strength grade is 0 in the upper and lower limbs and grade 3 in distal arms with atrophy.

Patient S2. She is a 57-year-old female, and her symptoms started 7 years ago, with tremor in the hands and weakness in the legs. Apart from that, she also presents cramps, and 2 years ago she started walking with a cane (1 support). She has been treated with riluzole for 5 years and does not have choking or respiratory symptoms. She has an enlarged abdomen and distal tremor in the hands. She shows grade 3 proximal muscular strength in the legs and degree 4 distal in the legs and grade 3+ proximal in the arms and grade 4 distal in the arms. Also, she shows absent deep tendon reflexes in the legs and hypoactive deep tendon reflexes in the arms. There's no fasciculations.

Patient S3. A 54-year-old male started showing weakness in the legs, cramps and tremors at the age of 36. His symptoms worsened progressively, and at the age of 49 he stopped walking. He has been dependent on respiratory assistance since the age of 54, and, currently, he presents sporadic choking. At examination, he also presented an enlarged abdomen, absent deep tendon reflexes and presence of fasciculations in the arms and tongue. His proximal muscular strength is grade 0 in the upper and lower limbs, grade 3 in the right arm and grade 2 in the left arm distally and degree 2 in the legs distally.

Patient M1. She is a 73-year-old female, who referred first symptoms at the age of 67 years, after feeling difficulty for climbing stairs. When first seen at our center at age 70, she could walk independently. Her symptoms worsened and now she refers little tremor, with sporadic choking. She shows good function in the arms without weakness or respiratory complaints. She presents tremor in the hands, and her deep

Table 1. Clinical variability of ALS8 patients

ID	S1	S2	S3	M1	M2
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Age	59	57	54	73	75
Gender	Male	Female	Male	Female	Male
Age to onset	38	50	36	67	30
Deambulation	No	Yes	No	Yes	Yes
Support	No	1 Support	No	1 Support	2 Support
Wheelchair	Yes	No	Yes	No	No
BiPAP	Yes	No	Yes	No	No
Gastrostomy	Yes	No	No	No	No
Weakness	0 Proximal 2 Distal	3 Proximal 4 Distal	0 Proximal 2 Distal	4 Proximal 4 Distal	3 Proximal 4 Distal
Cramps	No	Yes	No	Yes	No
Tremor	Yes	Yes	Yes	Yes	Yes
Fasciculation	Yes	No	Yes	Yes	Yes
Dilated Abdomen	Yes	Yes	Yes	No	No
Age at sample collection	54	52	49	68	70
Riluzole	Yes	Yes	Yes	No	No
Myotatic reflexes	Absent	Absent in the legs; present in the arms	Absent	Present	Absent

Phenotypic diversity of ALS8 patients enrolled to this study. Patients S1 and S3 were considered highly severe and S2, severely affected. On the other hand, M1 and M2 were classified as mildly affected.

tendon reflexes are present in the arms and legs. Hoffman's reflex is present bilaterally. She exhibits grade 4 proximal and distal muscular strength in the legs and arms, the left a little weaker than the right. Rare fasciculations are present in the right arm.

Patient M2. He is a 75-year-old male. He refers onset of his symptoms at age 35 with weakness in the legs, with a slow and progressive worsening. Five years ago, he started walking with a cane (1 support), and 3 years ago with bilateral support. He presents tremor and sporadic choking. He does not present cramps or respiratory symptoms. His muscle strength is grade 4 proximal in the arms and 4+ distal in the arms, grade 3+ proximal in the legs and 4 distal in the legs. He also shows rare fasciculations in the arms and tongue.

Interestingly, although the two mild patients are only slightly affected in their seventies, their clinical progression was quite different. Patient M1 was completely asymptomatic until the age of 67, and she probably would not be ascertained if she did not have many affected relatives, including her father from whom she inherited the mutated allele. On the other hand, M2 refers to disease onset at the age of 35 but with a very slow progression during the next 40 years.

As copy number variants have been largely shown to be associated with milder clinical courses in other neuromuscular disorders, such as spinal muscular atrophy, we performed an array comparative genome hybridization (aCGH) for all patients enrolled to this study. However, we only identified polymorphic alterations, unlikely to play a determinant role in the phenotypic variability here observed. We also performed exome sequencing for such individuals and considered different patterns of inheritance when analyzing genetic data. Although we cannot rule out either oligogenic or multifactorial inheritance for the phenotypic variability here observed, we did not observe potentially pathogenic variants in ALS genes other than *VAPB*.

ALS8 iPSC-derived MNs present similar reduced *VAPB* expression, different cell death rates and increased mTOR signaling activity in the mild patients

As VAPB haploinsufficiency has been pointed as a pathological mechanism for ALS8, we hypothesized that different expression levels of this gene could be related to phenotypic variability. However, RT-qPCR and western blotting analyses showed that both the *VAPB* mRNA and the protein expression were significantly reduced in all patient induced pluripotent stem cell (iPSC)-derived MNs compared with control cells, independently of the clinical status (Fig. 1B and E).

We then decided to evaluate the degree of cell death in the three groups of iPSC-derived MNs using propidium iodide (PI) staining (Fig. 1C). This analysis showed that the cell death levels were indistinguishable among MNs derived from the highly severe and severe patients; therefore, we placed these three individuals in a single group, namely, 'severe ALS'. Interestingly, MNs from mildly affected patients presented significantly less cell death than from severe ALS patients, and the degree of cell death did not differ between mild ALS patients and the control group (Fig. 1C).

Besides, as VAPB has been implicated in regulating protein synthesis, and mTOR signaling pathway plays a critical role in regulating translation in mammalian central nervous system, we sought to evaluate key members of this molecular cascade in our experimental groups. In order to do that, we subjected the MNs to caloric restriction by cultivating them with DMEM/F12 for 48 h and performed western blotting for pmTOR, RPS6 (Ser 235/236) and 4EBP1 (Fig. 2A). Curiously, we observed an increased protein expression of pmTOR in ALS8 patients, which was more pronounced in the mild ones (Fig. 2B and E). On the other hand, when compared together, 4EBP1 did not differ among the groups (Fig. 2C and F), and RPS6 only appeared to be increased in the ALS8 severe patients when compared to controls (Fig. 2D). When individually compared, however, 4EBP1 and RPS6 appeared to be highly expressed in M1 and M2, respectively (Fig. 2F and G).

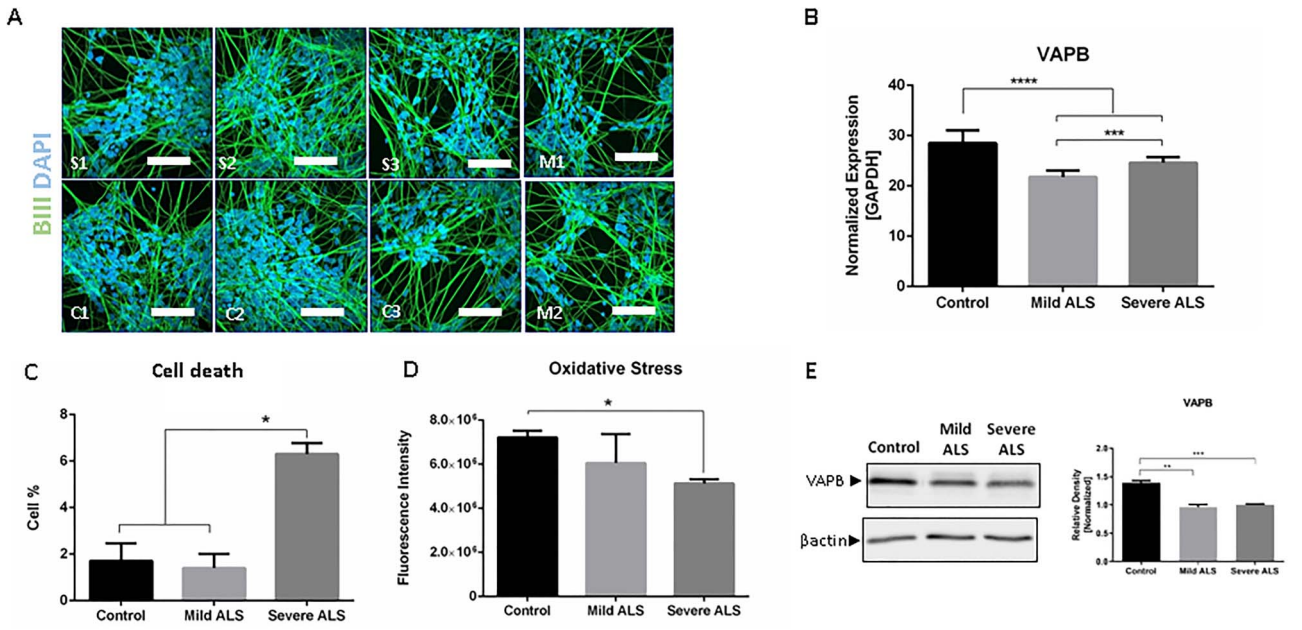

Figure 1. Functional studies in iPSC-derived ALS8 MNs. The graphs represent the mean ± SD for three experimental replicates, represented as fold change compared with controls $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (**A**) Confocal imaging of differentiated MNs stained for β-3 tubulin (green) and DAPI (blue). (**B**) RT-qPCR of *VAPB* levels in the experimental groups. Control individuals (C1, C2 and C3), mild individuals (M1 and M2) and highly severe plus severe patients (S1, S2 and S3) were compared against each other. (**C**) Cell death assay showing higher cell death rates in the severe ALS group when compared with controls and mild ALS patients. (**D**) DCFDA assay showing ROS levels in severe ALS group was lower than in controls and in mild ALS. (**E**) Western blotting for VAPB indicating ALS8 patients show decreased levels of this protein, regardless of the degree of severity.

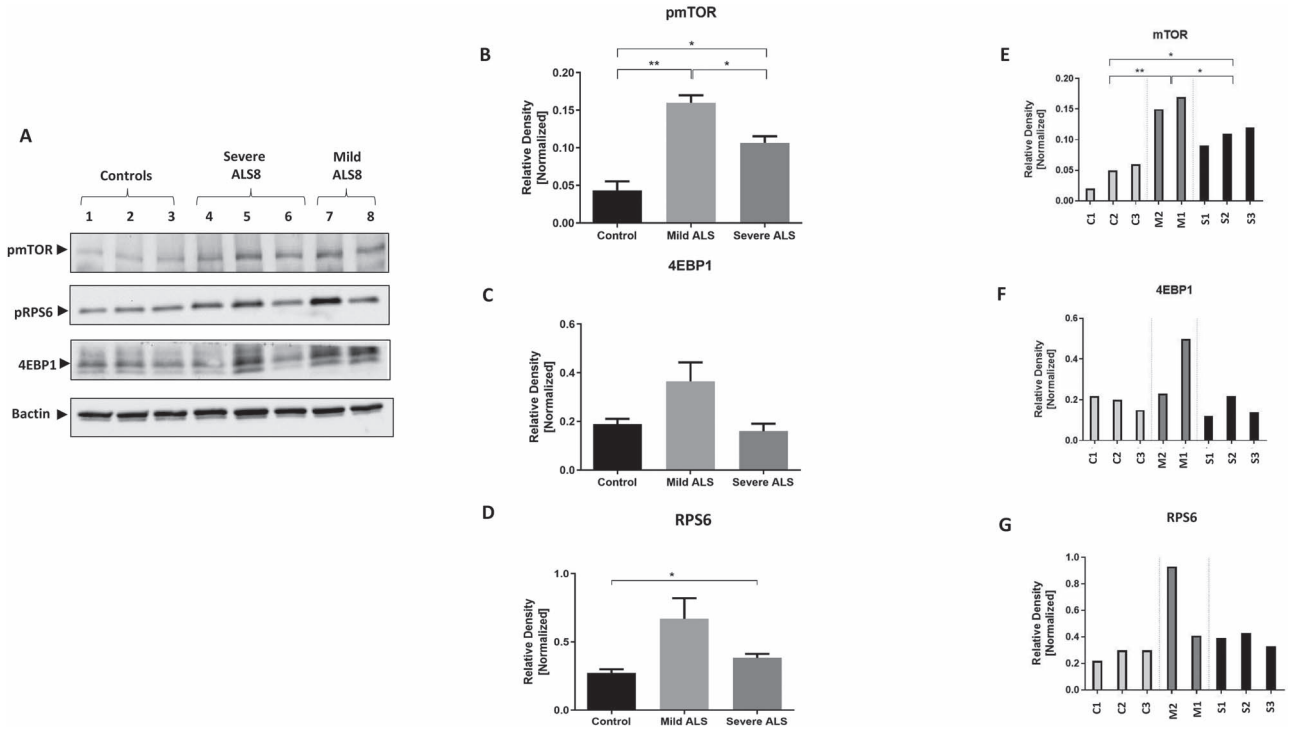

Figure 2. Western blotting assays for evaluating mTOR signaling pathway activity. (**A**) Electrophoresis gel representation of pmTOR, RPS6 and 4EBP1 from MN lysates after 48 h of starvation. (**B**) pmTOR protein levels in controls, severe ALS and mild ALS patients, suggesting increased levels of mTOR signaling pathway in mild ALS8 individuals, when compared to the other experimental groups. Data are shown as mean ± SEM and SD of controls, severe ALS and mild ALS. *P*-value is represented as $^{*}0.05$ and $^{**}0.01$. (**C**) 4EBP1 protein levels in controls, severe ALS and mild ALS patients when compared in group. (**D**) RPS6 protein expression in controls, severe ALS and mild ALS when compared in group. (**E**) pmTOR protein levels represented as in individual band densitometry for MN lysates of each individual employed in this study. (**F**) 4EBP1 band densitometry for each patient, pointing out to a high expression of this protein in M1. (**G**) RPS6 band densitometry for each patient, also suggesting its high protein expression in M2.

ALS8 iPSC-derived MNs have impaired mitochondrial activity, which is partially rescued in mild patients

In the iPSC-derived MNs from severe ALS patients, all estimated oxygen consumption rate (OCR) parameters—basal, ATP-linked, proton leak-linked and maximal OCR—were decreased when compared with controls (Supplementary Material, Fig. S3A–D), while in MNs from mildly affected patients only the ATP-linked and maximal OCR were significantly reduced (Supplementary Material, Fig. S3B and D). These data indicate a lower mitochondrial oxidative activity. Because mitochondria produce a major part of reactive oxygen species (ROS) in the cells, we then performed a 2′,7′- dichlorofluorescein diacetate (DCFDA) assay in order to quantify the ROS levels in MNs from ALS8 patients and controls. We observed that ROS levels were lower in cells derived from the severe ALS patients compared with cells from controls (Fig. 1D), while ROS levels in MNs from mildly affected patients were not significantly different from controls (Fig. 1D). Although we cannot associate ROS production exclusively with mitochondrial metabolism, such findings suggest an energetic depletion in MNs from severe ALS patients.

RNA sequencing shows differentially expressed genes in pathways highly associated with VAPB deficiency and points out to mitigating mechanisms

First, we compared MNs from severely affected patients and controls. A total of 979 genes were differentially expressed, of which 552 were upregulated and 427 downregulated in the MNs from patients (Fig. 3A; Supplementary Material, Table S1). The top 100 most significant differentially expressed (DE) genes are shown in Fig. 3B and in Supplementary Material, Fig. S11B. The Gene Ontology (GO) enriched terms for upregulated genes in the severely affected patients were trans-synaptic signaling, chemical synapse transmission and regulation of synapse organization (Fig. 3C; Supplementary Material, Table S2, Supplementary Material, Fig. S11C). In addition, GO-enriched terms for the downregulated genes in the severely affected patients were mostly associated with protein translation, protein targeting to the ER and rRNA biogenesis and metabolism (Fig. 3D; Supplementary Material, Table S3, Supplementary Material, Fig. S11D).

Because the global gene expression patterns of MNs were different between the two mildly affected individuals (M1 and M2) and the severe patients (Supplementary Material, Fig. S4), we separately compared each one with the severely affected patients' group. Analyzing M1 gene expression data, we observed 1352 upregulated and 1492 downregulated genes, when compared with the severe ALS8 group (Supplementary Material, Fig. S5A; Supplementary Material, Table S4). The top 100 most significant DE genes are shown in Supplementary Material, Fig. S5B. In addition, when compared with the control group, 1136 genes were found to be upregulated, and 1203 downregulated (Supplementary Material, Fig. S6A; Supplementary Material, Table S5). Again, the top 100 most significant DE genes are shown in Supplementary Material, Fig. S6B. In both analyses, the most prominent enriched GO terms identified among all the upregulated genes were associated with modulation of synaptic transmission and neurotransmitter secretion (Supplementary Material, Figs S5C and S 6C; Supplementary Material, Tables S6 and S7). Similarly, the GOs enriched among all the downregulated genes were associated with cell matrix and cellular adhesion (Supplementary Material, Figs S5D and S6D; Supplementary Material, Tables S8 and S9). The M2 patient was compared using the same parameters: when compared with the severe group, 2517 genes were found to be upregulated and 2887 were downregulated (Supplementary Material, Fig. S7A; Supplementary Material, Table S10). The top 100 most significant DE genes are shown in Supplementary Material, Fig. S7B. The enriched GO terms among all the upregulated genes were associated with nucleotide and lipid biosynthetic pathways, cytoskeleton organization and vesicle transport (Supplementary Material, Fig. S7C; Supplementary Material, Table S11). There were no enriched GO terms for the downregulated genes in M2. In addition, the comparison of M2 patient with the control group showed that 3167 genes were upregulated and 3056 were downregulated (Supplementary Material, Fig. S8A; Supplementary Material, Table S12, Fig. S11A). The top 100 most significant DE genes are also represented in Supplementary Material, Fig. S8B. The enriched GO terms among all the upregulated genes were associated with cilium organization, Golgi vesicle transport, nucleotide biosynthetic process, glycoprotein biosynthetic process and peroxisome organization (Supplementary Material, Fig. S8C; Supplementary Material, Table S13). No enriched GO terms were found among M2 downregulated gene sets.

Next, we identified the genes altered in common between the two mild ALS8 patients. For this, we first identified six different sets of genes (Fig. 4A, lower left bars; Supplementary Material, Fig. S12A) with significant differential expression among three comparisons: (1) severe versus control patients (genes up- or downregulated in the severe patients); (2) M1 patient versus severe group (genes up- or downregulated in the M1 patient); (3) M2 patient versus severe group (genes up- or downregulated in the M2 patient). Approximately 500–2500 genes were identified in each of the six different sets (Fig. 4A, lower left bars; Supplementary Material, Fig. S12A). Among the genes altered in common between the two mild ALS8 patients, we identified 43 genes that were downregulated when the severe group was compared with the controls, and in contrast were upregulated in both mildly affected M1 and M2 individuals when compared with the severe group (Fig. 4A; Supplementary Material, Fig. S12A yellow bar; Fig. 4B; Supplementary Material, Fig. S12B; Supplementary Material, Table S14). Given the upregulation of these 43 genes in M1 and M2, the expression pattern in the MNs of the mildly affected individuals was similar to the controls (Fig. 4B and Supplementary Material, Fig. S12B). Most of the genes here identified are related to biological functions associated with VAPB, such as protein localization to ER, translation initiation and SRP-dependent cotranslational targeting to membrane (Fig. 4C and D; Supplementary Material, Fig. S12C and D; Supplementary Material, Table S15).

We also identified 66 genes that were upregulated when the severe group was compared with the controls, and in contrast were downregulated in both mildly affected M1 and M2 individuals when compared with the severe group (Fig. 4A and Supplementary Material, Fig. S12A, blue bar; Fig. 4E and Supplementary Material, Fig. S12E). Again, given the downregulation of these 66 genes in M1 and M2, the expression pattern in the MNs of the mildly affected individuals was similar to the controls (Fig. 4E; Supplementary Material, Fig. S12E; Supplementary Material, Table S14, Supplementary Material, Fig. S12). No GO categories enrichment was found for these 66 genes.

Interestingly, we found that between 7 and 14% of the DE genes in our experiments of Figures 3 and 4 are long non-coding RNAs (lncRNAs), mostly lincRNAs (long intervening non-coding RNAs).

We also sought to evaluate, by RT-qPCR, the expression profile among the three experimental groups (severe ALS8, mild ALS8 and control) of a selected set of genes. We chose candidate

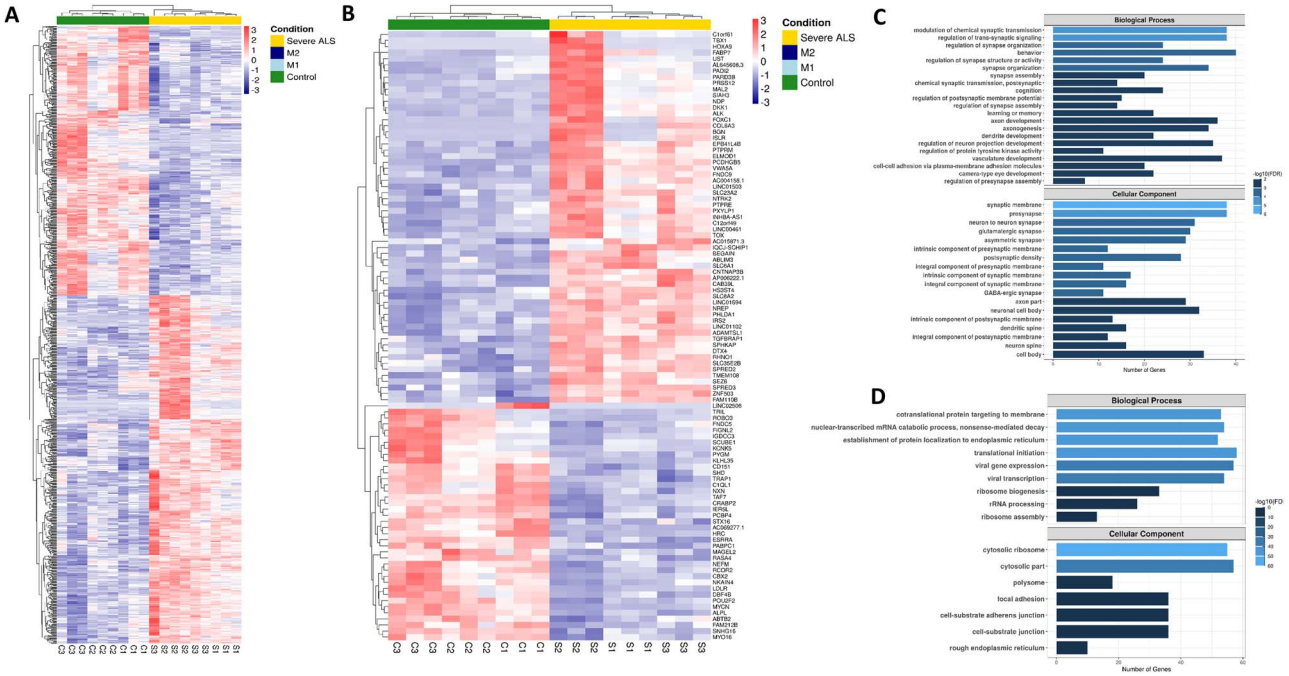

Figure 3. Gene expression analysis of iPSC-derived MNs from severe ALS and control individuals. (**A**) Unsupervised heatmap of all significant DE genes (corrected *P*-value <0.01) showing that S1, S2 and S3 patients have a similar gene expression profile of upregulated (red) and downregulated (blue) genes when compared with controls (C1, C2 and C3). The z-scores' (−3 to +3) color scale is shown at the right. (**B**) Unsupervised heatmap with the top 100 most significant DE genes in the comparison between the severe and control groups. The z-scores' (−3 to +3) color scale is shown at the right. (**C**) GO terms for Biological Processes (upper panel) and Cellular Components (lower panel) significantly enriched among all the downregulated genes in ALS8 pathology compared with controls. Colors indicate the −log10(FDR) as shown in the scale at the right. (**D**) GO terms for Biological Processes (upper panel) and Cellular Components (lower panel) significantly enriched among all the upregulated genes in ALS8 pathology compared with controls. Colors indicate the −log10(FDR) as shown in the scale at the right. See Supplementary Material, Fig. S11 for colour image.

genes based on their relationship with the most frequent GO terms associated with a potential protecting mechanism (protein translation, translation initiation and synaptic function). Interestingly, for those whose fold change value appeared to be higher, we observed a similar pattern to what was identified in our RNA-Seq analysis (Supplementary Material, Fig. S9).

Using transcriptomic data, we also analyzed the expression levels of two already described modifiers for ALS, *EPHA4* and *UNC13A* (11,13). *UNC13A* was not found to be DE, among the different groups (Supplementary Material, Fig. S10). However, *EPHA4* was significantly downregulated in the controls and in the mildly affected M1 patient relative to the severely affected group (Supplementary Material, Fig. S6). Such gene expression information rules out *UNC13A* as a genetic modifier for ALS8 in these patients. As we identified a downregulation of *EPHA4*, we sought for rare, loss-of-function, variants in this gene in M1 using whole exome sequencing (WES) data. However, in this patient we did not find suggestive mutations which could explain such finding. Therefore, although we cannot completely exclude *EPHA4* as a modifier in M1, this gene downregulation is most likely due to secondary effects of other biological factors (Supplementary Material, Fig. S10).

Discussion

Taken together, our findings suggest mitigating mechanisms underlying ALS8 clinical heterogeneity and present, for the first time, the most relevant biological pathways involved in this process in a patient-based model. It is also noteworthy that, even though the severely affected patient (S2) was not as affected as the highly severe ones (S1 and S3), all of them looked similar in the functional assays and in the gene expression signatures. Such information further indicates that the clinical heterogeneity in ALS8 might be a phenotypic continuum with a so far poorly understood environmental influence. The mild phenotype is most likely due to protecting variants of small effect which we were not able to identify due to our limited sample size.

Interestingly, our analyses converge into suggesting potential mechanisms of resilience to neurodegeneration. Based on the GO terms associated with protein translation, we assessed the mTOR signaling pathway downstream key members, which are employed in the literature to evaluate its level of activity. pmTOR is a prominent member of this molecular cascade and phosphorylates different targets, such as 4EBP1 and S6K1, whereby it regulates protein, nucleotide and other macromolecules' biosynthesis (14). Besides, it has been a widely known target for modulating autophagy in the lifespan extension experiments in animal models such as *Caenorhabditis elegans* (15). RPS6 is a component of the ribosomal machinery, being directly associated with regulation of translation initiation through its phosphorylation in multiple sites (16); it is also acknowledged as a measure of mTOR pathway output (17). 4EBP1, however, is a translation inhibitor which, when phosphorylated, releases initiation factor eIF4E to bind 40S ribosomal subunit to mRNA in order to start protein synthesis (18). In our assay, performed upon caloric restriction in MNs, we observed that pMTOR was highly expressed in the mildly affected ALS8, when compared either to controls or severe ALS8. However, both RPS6 and 4EBP1 protein levels did not reach statistically significant differences when both mild patients were compared together. On the other hand,

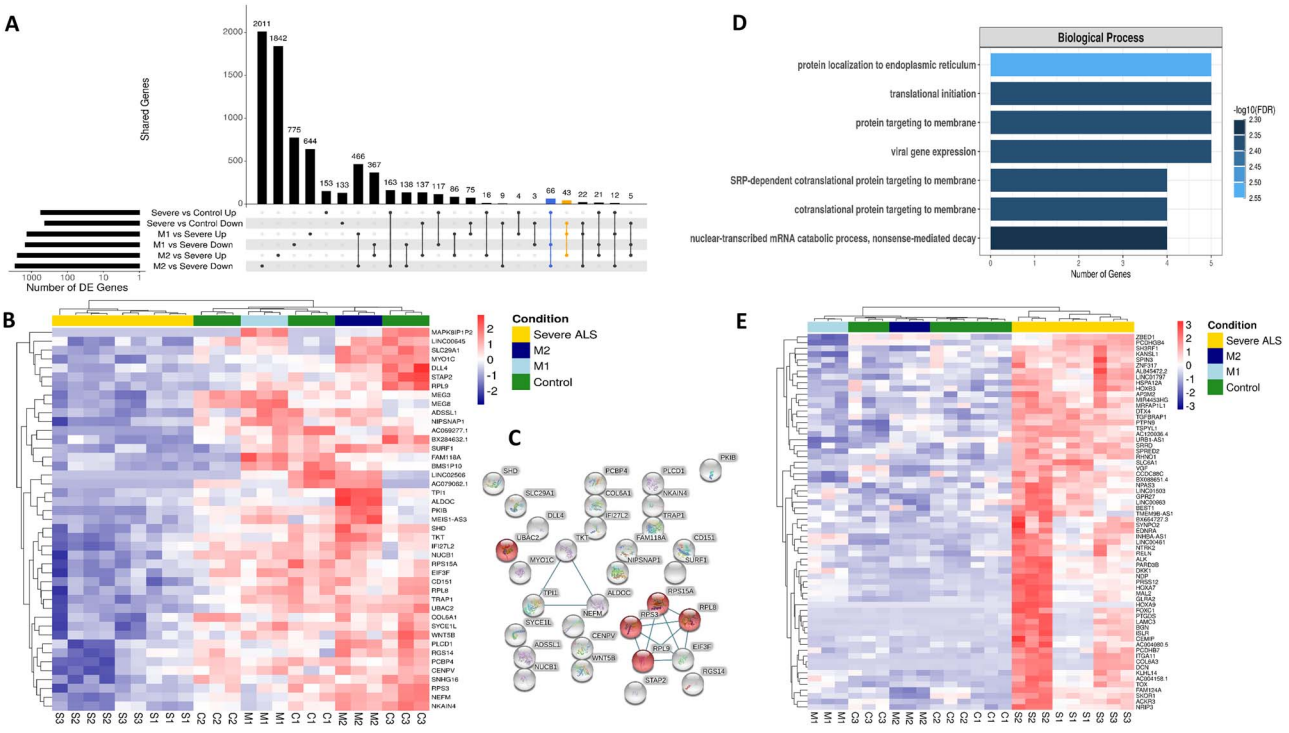

Figure 4. Identification of DE genes shared by mild ALS8 patients, suggesting modulatory roles in neurodegenerative process. (**A**) The UpSet intersection diagram shows the number of genes (*y*-axis) that have been detected in each of the intersection sets, indicated by the connected points in the lower part of the plot. The total number of DE genes comprising each set is indicated by black horizontal bars. The intersection sets in yellow and blue correspond to the genes shown in panels B and E, respectively. (**B**) Unsupervised heatmap of genes that were downregulated in the 'severe ALS' group (S1, S2 and S3 patients) when compared with controls, which were identified to be upregulated in common in M1 and M2 patients. The z-scores' (−2 to +2) color scale is shown at the right. (**C**) String enriched pathways for upregulated genes shared by M1 and M2 evidencing ribosome-associated mitigating mechanisms. (**D**) Enriched GO Biological Process terms associated with the genes in panel B. (**E**) Unsupervised heatmap of genes that were upregulated in the 'severe ALS' group (S1, S2 and S3 patients) when compared with controls, which were identified to be downregulated in common in M1 and M2 patients. The z-scores' (−3 to +3) color scale is shown at the right. See Supplementary Material, Fig. S12 for colour image.

interestingly, when compared isolatedly, there was a significant difference between 4EBP1 in M1 and the severe ALS8 and control samples and RPS6 in M2 and severe ALS8 and control samples. Such qualitative information corroborates the hypothesis indicated by pmTOR western blot, showing increased translation in the mild patients (Fig. 2). The heterogeneity in the final steps of this central cascade must be due to epistatic influence of other genes regulating different points of the pathway (19,20). Particularly, the RT-qPCR assay of genes following RNA-Seq expected patterns of expression further demonstrated the differential expression of them. Most of such genes are key regulators of pathways associated with mTOR, calcium efflux and cell cycle progression.

There is a large amount of evidence for the direct association between synaptic plasticity and protein synthesis in neurons. In fact, molecular mechanisms underlying synaptic plasticity, releasing of neurotransmitters at synaptic cleft and synapse formation require precisely regulated protein synthesis, in a direct association with calcium (Ca^{2+}) efflux regulation (21,22). *VAPB*, as an ER–mitochondria interface protein, might work most likely as a bridge between Ca^{2+} buffering in mitochondria and the Ca^{2+}-induced changes in protein synthesis and synaptic activity. Interestingly, *VAPB* downregulation was also found to occur in *SOD1* G93A transgenic mice, and in the spinal cord of sporadic ALS patients (23,24), strongly suggesting common mechanisms of disease.

It is also known that lincRNAs are important gene expression regulators in the brain and that DE lincRNAs are found up- and downstream of driver genes in neuroblastoma (25). Also, a recent review has pointed to a few lncRNAs dysregulated in ALS (26). Here, among the lincRNAs upregulated in common in the two mild ALS8 patients, we found *HOXD-AS2* and the imprinted maternally expressed lincRNA genes *MEG8* and *MEG3*. Recently, it has been found that *MEG3* is highly expressed in mouse embryonic stem cell-derived MNs and that the loss of *MEG3* leads to aberrant expression of progenitor and caudal *Hox* genes in postmitotic MNs (27). The DE lincRNAs found here in iPSC-derived MNs from different ALS8 patients should be an interesting avenue to be further explored.

Although we did not identify a single genetic factor associated with the clinical discrepancy in the *VAPB* p.P56S patients, the gene expression signatures here presented suggest that the clinical variability might be due to complex genetic interactions of modifiers regulating protein translation, cell survival and synaptic function. A multifactorial mechanism is therefore the most suitable model to explain the phenotypic diversity observed in the ALS8. In this report we present evidence for mitigating mechanisms for ALS8, which involve pathways related to the neurodegenerative process (28,29). Such information might be of fundamental importance for broadening the understanding of MN functioning both in pathological and physiologic states. Additionally, as control of proteostasis has been an active research field for neurological disorders, a fuller grasp of the mechanisms underlying its regulation might shed light on ALS and other neurodegenerative diseases' pathology (30).

Materials and methods

Cellular reprogramming and MN differentiation

After informed consent, primary blood mononuclear cells (PBMCs) were isolated from all affected individuals and controls ($N = 8$, 5 affected and 3 controls) and were used for reprogramming. iPSCs were then obtained for such individuals (Supplementary Material, Fig. S1A). Expression of pluripotency markers *SSEA4* and *OCT4* was checked through an immunofluorescence assay and for *OCT4*, *Nanog* and *SOX2* by RT-qPCR (Supplementary Material, Fig. S1B–D). The reprogramming procedure followed well-established protocols for obtaining iPSCs from PBMCs and using episomal vectors (31). Differentiation into MNs was performed by using a previously published protocol able to generate up to 95% of such cells (32). Briefly, the iPSCs were grown in Essential 8 medium (Thermo) until they reach 80% of confluency. Then, they were cultivated in NB medium containing DMEM/F12, Neurobasal medium, N2, B27 and GlutaMAX (all from Thermo) and subjected to a two-step protocol of neural induction/caudalization and ventralization for obtaining motor neuron progenitors (MNPs). The first phase was achieved after cultivating iPSCs for 6 days in NB containing dorsomorphin (2 μM), SB431542 (2 μM), CHIR99021 (3 μM) and ascorbic acid (0.1 mM). The following step, which also lasted 6 days, consisted of cultivating the cells in NB medium and dorsomorphin (2 μM), SB431542 (2 μM), CHIR99021 (1 μM), retinoic acid (0.1 μM), ascorbic acid (0.1 mM) and purmorphamine (0.5 μM). After obtaining the MNPs, the cells were seeded in 60 mm^2 plates containing Matrigel (all from Corning) and subjected to MN differentiation by cultivating them for 6 days in NB medium containing retinoic acid (0.5 μM), purmorphamine (0.1 μM) and ascorbic acid (0.1 mM). A further step of neural maturation was also carried out by adding compound E (0.1 μM) to the same medium used for MN differentiation. Although this last phase can last up to 10 days, for the experiments here reported, the MNs were differentiated until the third day, when the expression of cell markers was already evident. The presence of β-tubulin, a pan-neuronal marker, was evidenced in all samples through immunofluorescence (Fig. 1A). In addition, *MAP2*, a pan-neuronal marker, and *ISL1*, a MN specific maker, were measured by RT-qPCR (Supplementary Material, Fig. S1E and F), and *ISL1*, *ChAT*, *MNX1* and *TUBB3* expression levels were retrieved from the RNA-Seq data (Supplementary Material, Fig. S1G).

Multiplex ligation probe amplification assay

Genomic DNA was extracted from cultured iPSCs using the NucleoSpin kit (Macherey-Nagel, Düren, Germany), following manufacturer instructions. Multiplex ligation probe amplification assay was performed using subtelomeric probes (MRC-Holland, Amsterdam, The Netherlands), in order to test for the presence of chromosomal aneuploidies (Supplementary Material, Fig. S2).

WES and aCGH

aCGH, with a Human CGH 180 K platform (Agilent Technologies, Santa Clara, CA, USA), was performed using genomic DNA extracted from peripheral blood, collected after informed consent. The scanned images were analyzed using the SLC Genomic Workbench tool (Agilent Technologies), and the detected CNVs were compared with the following databanks: DECIPHER (https://decipher.sanger.ac.uk/) and Database of Genomic Variants (http://projects.tcag.ca/variation/). WES libraries were prepared using SureSelect kit (Agilent), and sequencing was performed in a HiSeq2500 (Illumina, San Diego, CA, USA). For alignment to the human reference genome GRCh37, Burrows-Wheeler Aligner (33) and an established pipeline to evaluate the pair-end reads were used. Variant annotation was carried out using ABraOM database (http://abraom.ib.usp.br/), ANNOVAR (34), 1000 Genomes Project National Institute of Health (http://www.1000genomes.org/) and 6500 Exome Sequencing Project Washington University (https://evs.gs.washington.edu/EVS/). Genetic variants were filtered according to their allelic frequency (MAF < 0.01), also taking into consideration expression patterns of the corresponding genes and their previous association with ALS.

Real-time quantitative PCR

RNA was obtained from MNs following standard protocols. Three biological replicates representing three differentiations of the same iPSC from each patient were assayed. After quantifying and evaluating its quality, 1 μg of RNA was employed for cDNA synthesis, with SuperScript III First-Strand Synthesis System (Thermo Fischer Scientific, Waltham, MA, USA) and oligo-dT primers, according to manufacturer's instructions. Real-time qPCR assays were performed in technical triplicates using SYBR Green PCR Master Mix (Thermo Fischer Scientific, Waltham, MA, USA) (Supplementary methods for primers' sequences). For *VAPB* expression, TaqMan probes were used, following manufacturer protocols. Probes used for the expression assays are as follows: *VAPB* forward (Hs00191003_m1) and reverse (Hs00427749_m1).

For the RT-qPCR validation assays, RNA was extracted from iPSC-derived MNs using RNeasy Micro Kit (Qiagen, 70 004, Hilden, Germany) and treated with TURBO DNase (Ambion, AM2238) for 1 h at 37°C. For each sample, the reverse transcription (RT) reaction was performed with 800 ng total RNA using the SuperScript IV First-Strand Synthesis System (Life Technologies, cat. #18091050) and random hexamer primers in a 20-μl final volume. The obtained cDNAs were diluted 10 times in water, and quantitative PCR was performed using 2.5 μl of each diluted cDNA in a total volume of 10 μl containing 1× LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics, cat. #04707516001, Rotkreuz, Switzerland) and 800 nM of each primer in a LightCycler 480 System (Roche Diagnostics). RT-qPCR was run in three biological replicates representing three independent differentiations of the same iPSC from each patient, with three technical replicates each. Primers are shown in Supplementary methods. To find adequate normalizer genes for RT-qPCR, we looked at the RNA-Seq data for the genes with the lowest variation in expression across all samples; four from these genes were tested in RT-qPCR and the three most stable genes were used: *ZFAND3* (AN1-type zinc finger protein 3), *TRPC4AP* (short transient receptor potential channel 4-associated protein) and *BOD1* (biorientation of chromosomes in cell division protein 1). In all cases, the geometric mean expression of these three genes was used as normalizer for calculating the expression levels of genes of interest. One-way analysis of variance with Tukey's *post hoc* analysis was used for comparisons. GraphPad Prism software was used to perform statistical analysis (version 8.0). Quantification of data is represented as mean ± SEM, and *P*-value threshold was *0.05, **0.01, ***0.001 and ****0.0001.

Western blotting

Protein extracts from MNs were obtained using RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich,

St Louis, MO, USA), and BCA assay (Thermo Fischer Scientific) was employed for its quantification. Western blotting was performed for VAPB using a specific antibody (Sigma, HPA013144) and β-actin as a normalizing factor (Sigma, A1978). Besides, the following antibodies were used for evaluating mTOR pathway activity and protein translation levels: pmTOR (Sigma, 2971), pRPS6 (Sigma, 5364) and 4EBP1 (Sigma, 9452).

Seahorse assay

For mitochondrial assay, 2x 10^4 MNs were plated in XF 24-well cell culture microplates (Agilent Technologies), in technical quadruplicates. Assays were performed by adding oligomycin (1 µM), carbonyl cyanide chlorophenylhydrazone (CCCP 5 µM) and antimycin + rotenone (2 µM). After performing the experiment, protein was extracted from each well and used for normalizing the OCR results.

DCFDA assay

After plating 2×10^4 MNs in a 24-well plate (Corning, Corning, NY, USA), cells were harvested and incubated with 2′,7′-dichlorofluorescein diacetate (DCFDA) for 30 min at room temperature, as indicated in the manufacturer instructions. Fluorescence signal was read in a Flow Cytometer BD Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence

For cell imaging, 2×10^5 neurons were plated in a two-well glass chamber slide and, after restoring cellular morphology, were fixed with 4% PFA, permeabilized with 0.01% Triton X-100 and blocked with 5% bovine serum albumin for 1 h. Then, the MNs were incubated overnight with primary antibody for β-tubulin (Merck, TU-20, Darmstadt, Germany), at 4°C. On the following day, the cell preparations were treated with secondary antibody (Thermo, A-11001) (1 h) at room temperature. Finally, DAPI (4′-6-diamidino-2-phenylindole) staining was performed for 2 min at room temperature. Confocal imaging was performed in a Zeiss LSM 800.

Cell death assay

For PI assay, MNs previously plated in 24-well plates (Corning) were washed in PBS 1X, harvested and then incubated with 2 µl of a solution containing PI (1 mg/ml), fluorescein diacetate (1.5 mg/ml), Hoechst33342 (1 mg/ml) and PBS. Then, the cells were placed in glass slides, and the number of apoptotic cells was counted in a fluorescence microscope (Zeiss Axiovert 200).

RNA sequencing and analyses

RNA-Seq was done in triplicate independently differentiated iPSC-derived MN culture samples, from a single iPSC clone from each individual. Cells were collected at the third day of the last differentiation phase (see above). RNA-Seq raw data was deposited in the SRA database at NCBI (Accession number SRP223674). RNA was extracted from iPSC-derived MNs using RNeasy Micro Kit (Qiagen, 70004) and treated with TURBO DNase (Ambion, AM2238) for 1 h at 37°C. The three biological replicate samples were obtained in parallel for iPSC-derived MNs from each individual. After that, the samples underwent a new step of purification with RNeasy Micro Kit and were quantified with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Q32852). For assessing RNA purity, a NanoDrop-1000 spectrophotometer (NanoDrop technologies) was used, and RNA integrity was evaluated with Agilent RNA 6000 Pico Kit (Agilent Technologies, 5067–1513) in the 2100 Bioanalyzer Instrument (Agilent Technologies). Samples were sent to Duke University Center for Genomic and Computational Biology sequencing facility for preparing stranded tagged cDNA libraries and cluster generation, using KAPA Stranded mRNA-Seq Kit (Illumina, KK8421) and an Illumina HiSeq 4000 PE cluster Kit (Illumina-PE-410-1001), respectively. After running the tagged and pooled libraries in an Illumina HiSeq 4000, using a HiSeq 4000 SBS Kit (Illumina FC-410-1003), the paired-end 150-bp-long reads were de-multiplexed and processed using an Illumina pipeline to generate a FASTQ sequence file for each sample. On average, 18.4 million paired-end reads were produced for each sample.

Files were retrieved from the sequencing facility, and paired-end adapters and low-quality reads were removed by fastp (35) version 0.20.0 using the quality-filtering parameters –l 20–5 3 –3 3. Filtered paired-end reads were mapped with STAR (36) (2.5.3a), mapping on average 17.5 million pair-ended reads per sample, which were quantified at gene level by the RSEM pipeline (37), using an index generated from the GRCh38.p12 GencodeV.28 annotation (https://www.gencodegenes.org/human/release_28.html). To call DE genes using the Bioconductor package edgeR (38), quasi-likelihood tests were performed on fitted generalized linear models where the biological replicates and gender were added as blocking factors, then *P*-values were adjusted using FDR and all genes with an FDR < 0.05 were considered DE genes, unless otherwise indicated. To identify upregulated or downregulated genes, the logCPM data from edgeR were used. Unsupervised heatmaps were plotted using the R package pheatmap (39). Enriched GO analysis was performed using the Bioconductor package clusterProfiler (40) and org.Hs.eg.db (41). Graphs were plotted with the R package ggplot2 (42) and plot3D (43). The intersection diagram was plotted using the UpSetR tool v 1.3.3 (44).

Supplementary material

Supplementary Material is available at *HMG* online.

Acknowledgements

We would like to thank the patients and their collaborative support throughout this study. We would also like to thank FAPESP for the following grants: 2013/08028-1, 2014/50931-3 and 465355/2014-5.

Funding

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP: 2015/14821-1, 2017/16283-2); Instituto Nacional de Ciência e Tecnologia (INCT: 465355/2014-5). D.A.M.-V. received a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code 001. S.V.-A. is the recipient of an established investigator fellowship award from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Brazil.

Conflict of interests. The authors declare no conflict of interest.

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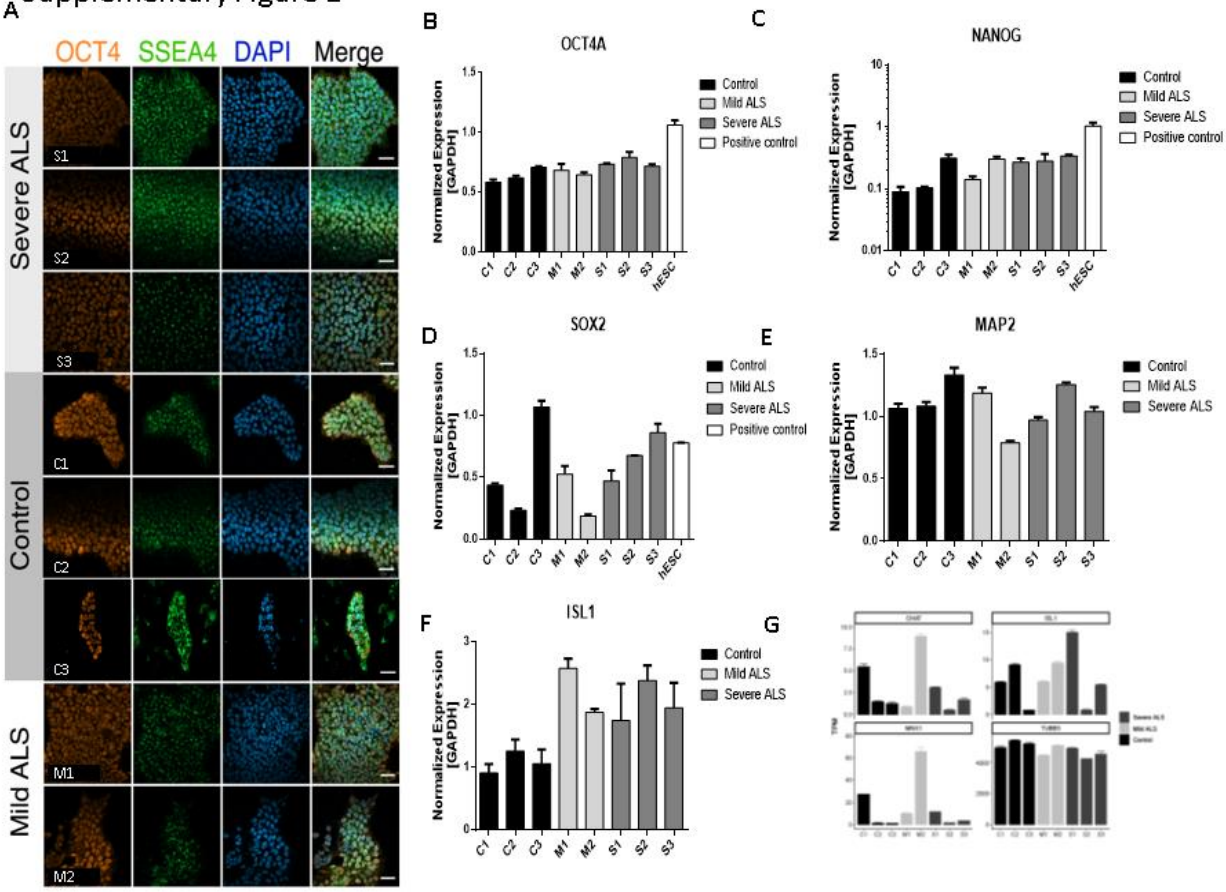
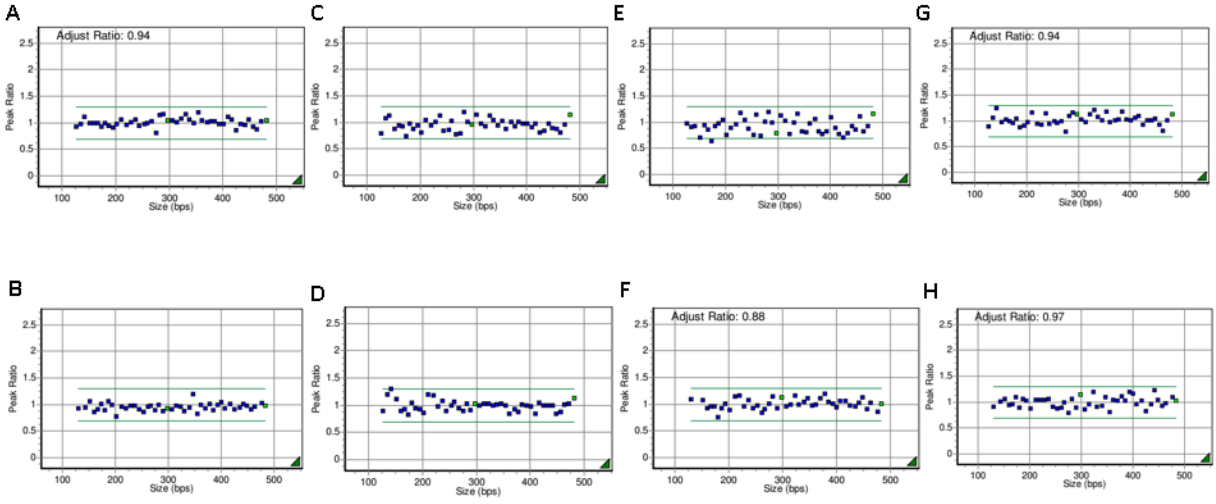
Supplementary Figure 1



Supplementary Figure 2



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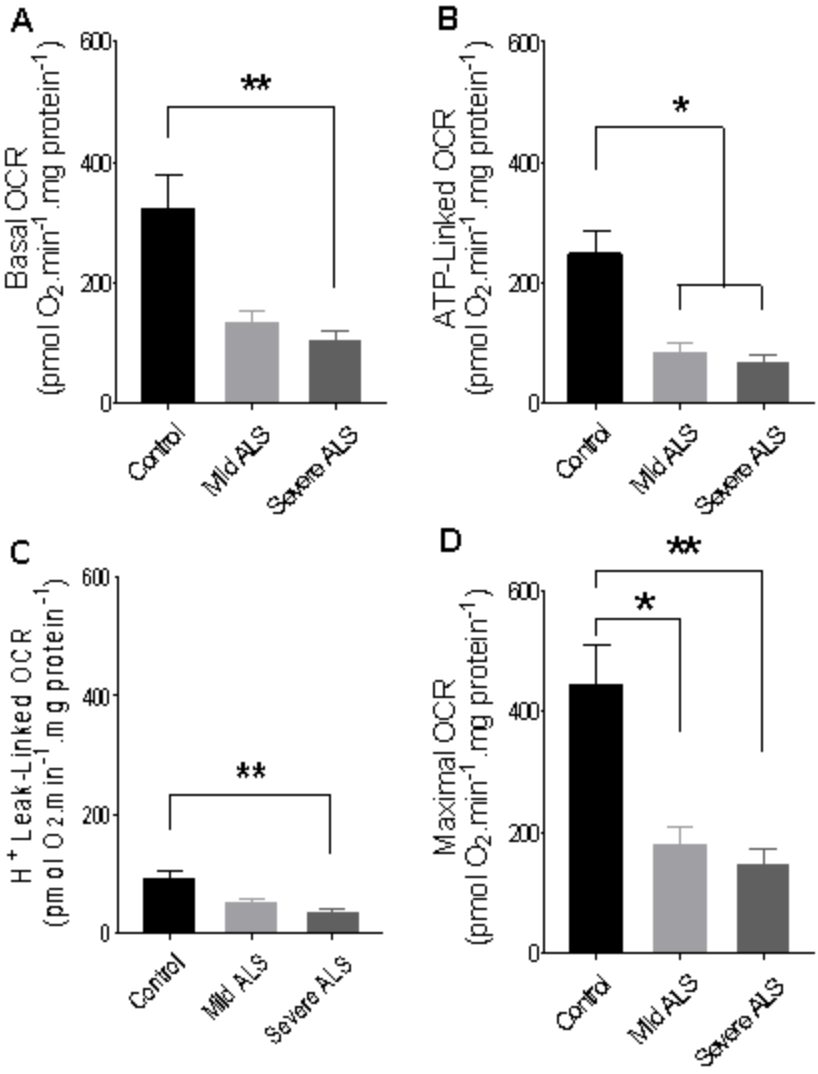
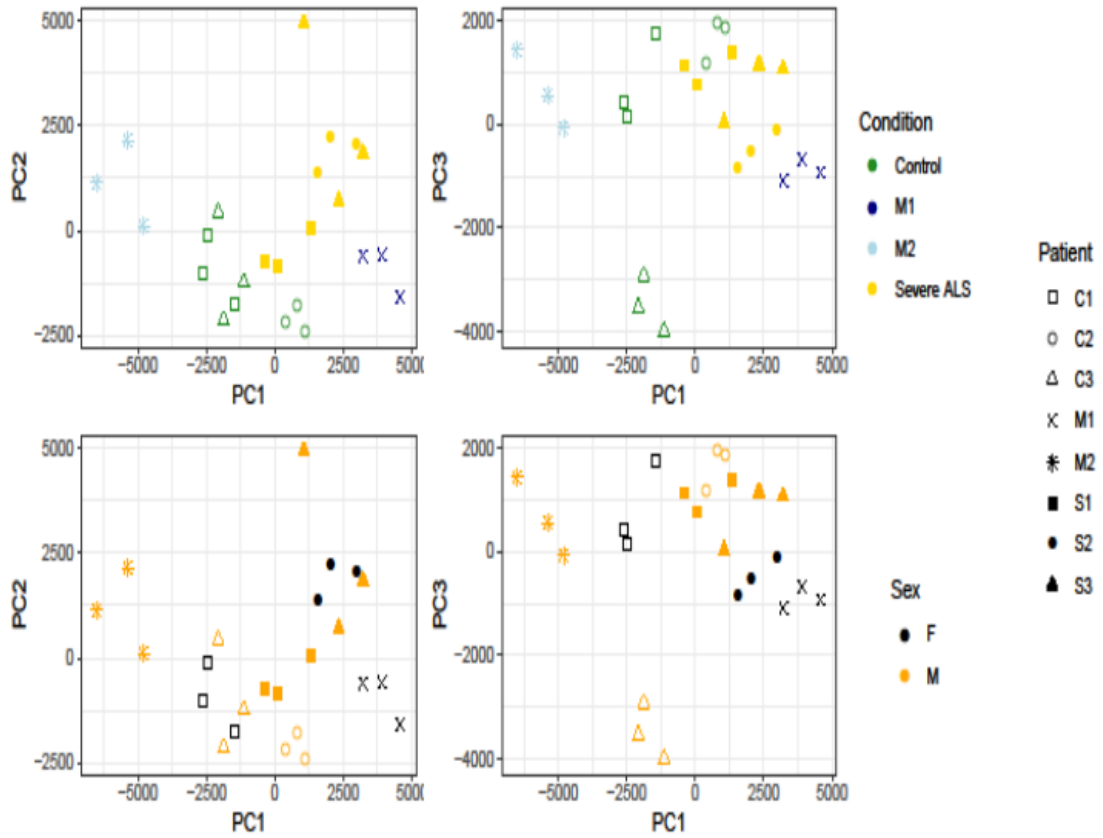
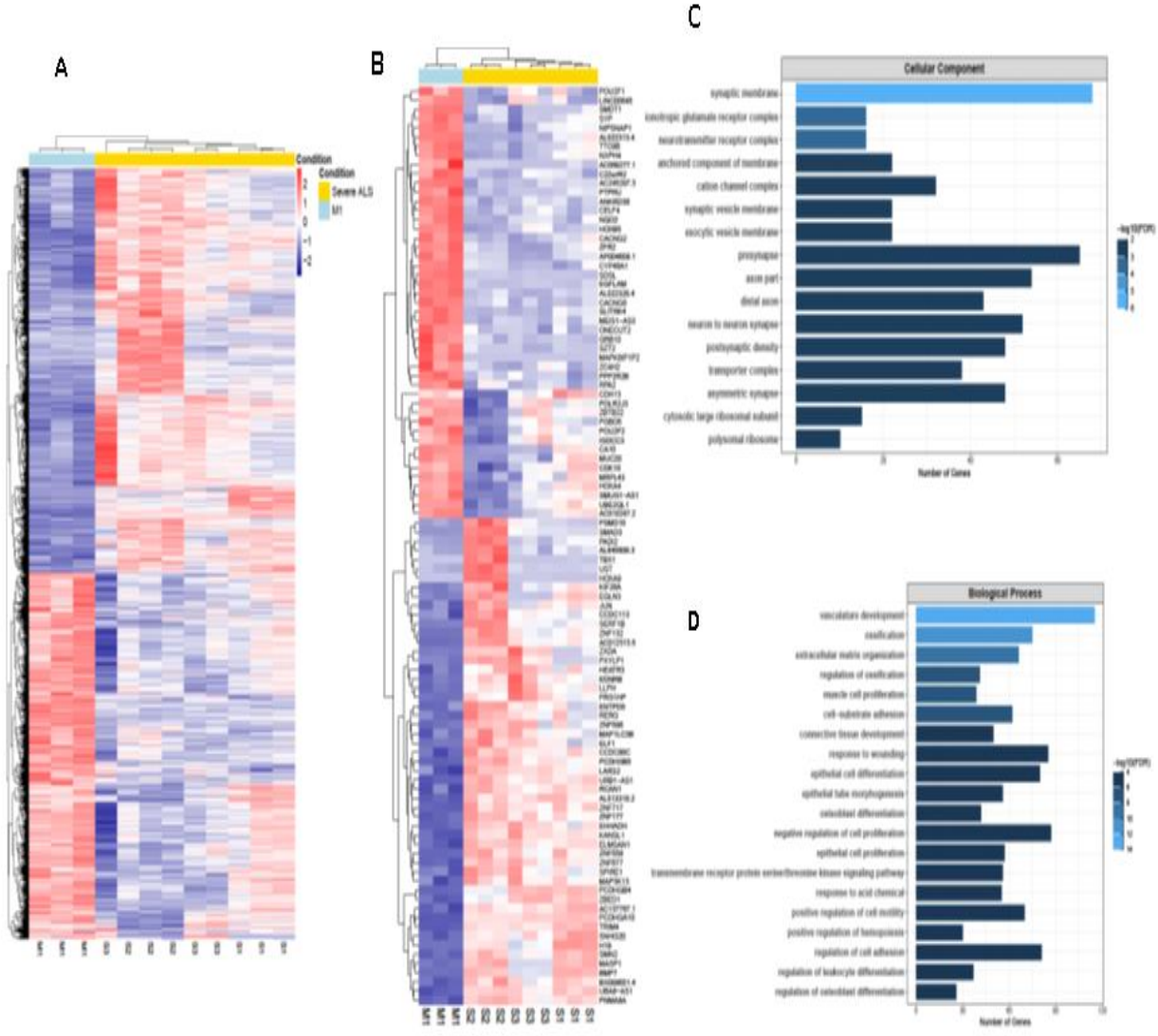


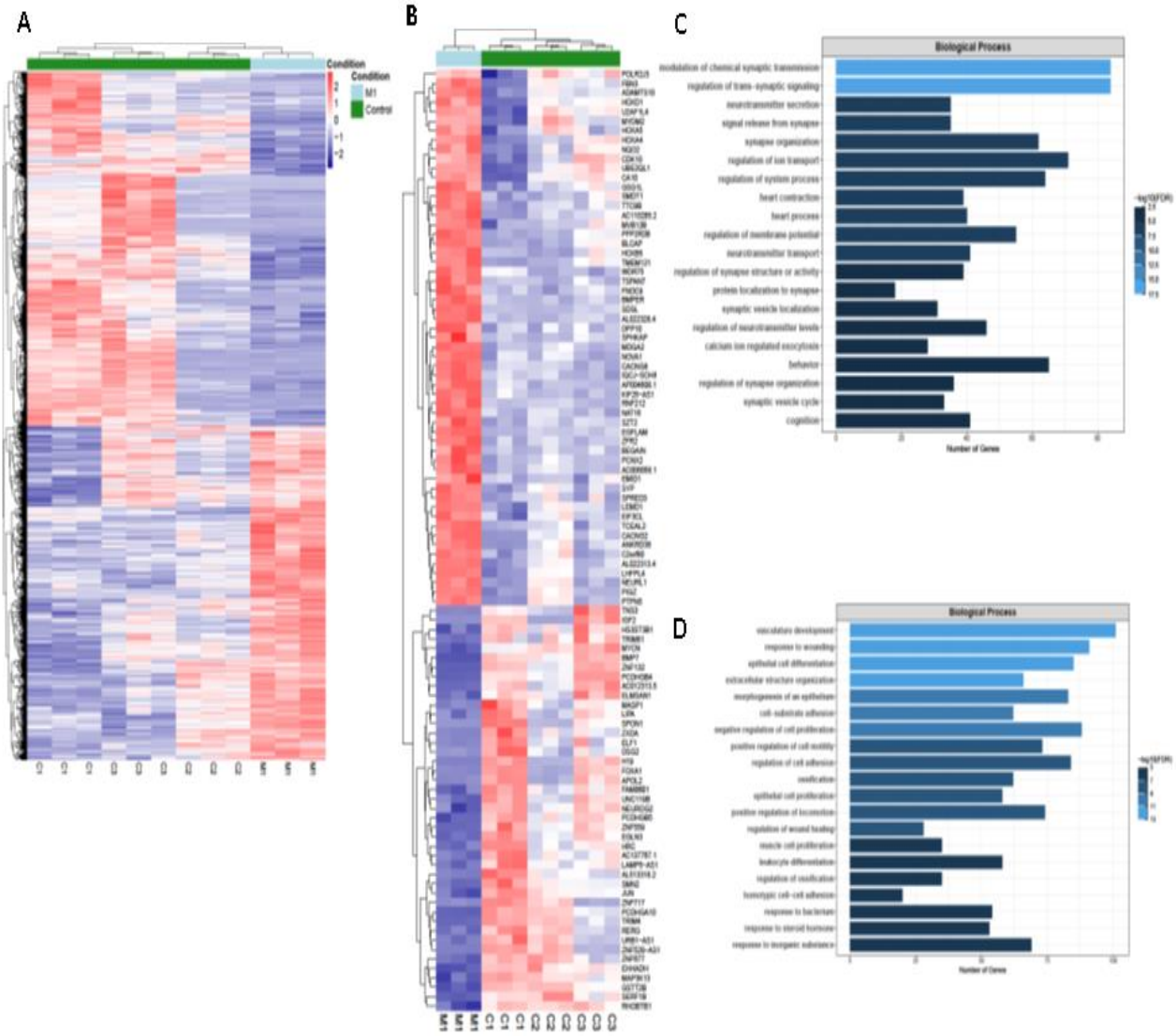
Supplementary Figure 4



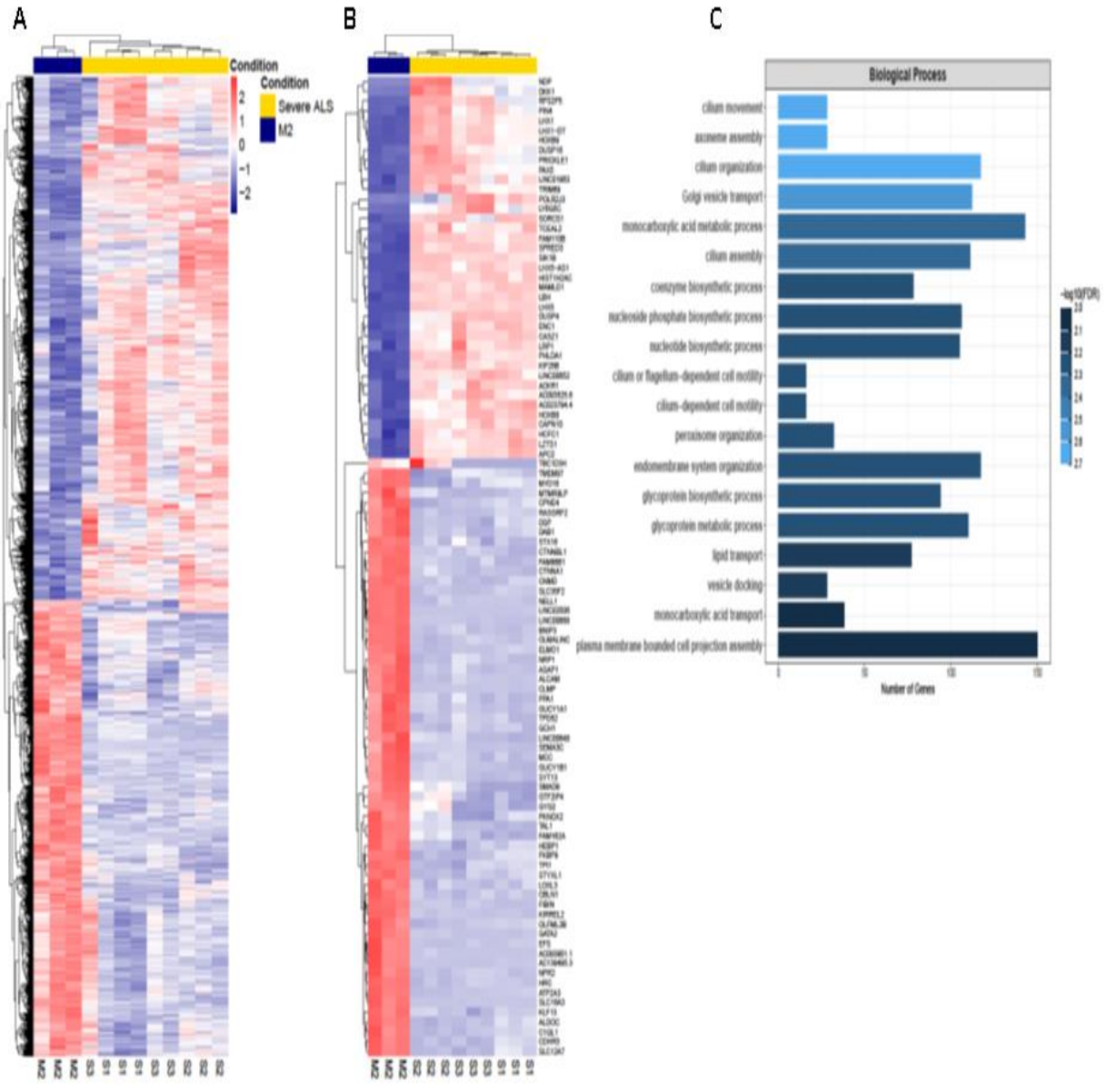
Supplementary figure 5



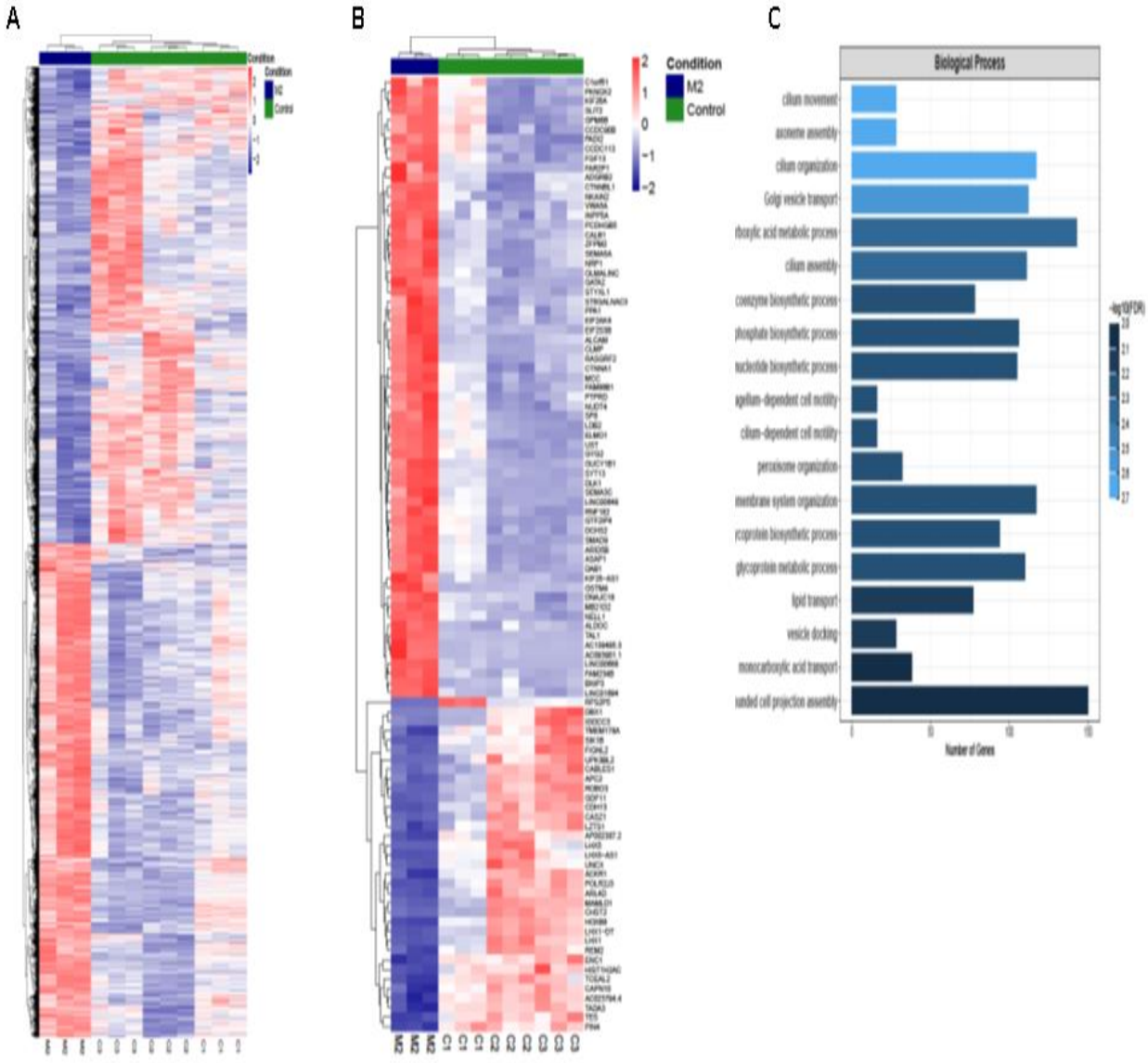
Supplementary figure 6



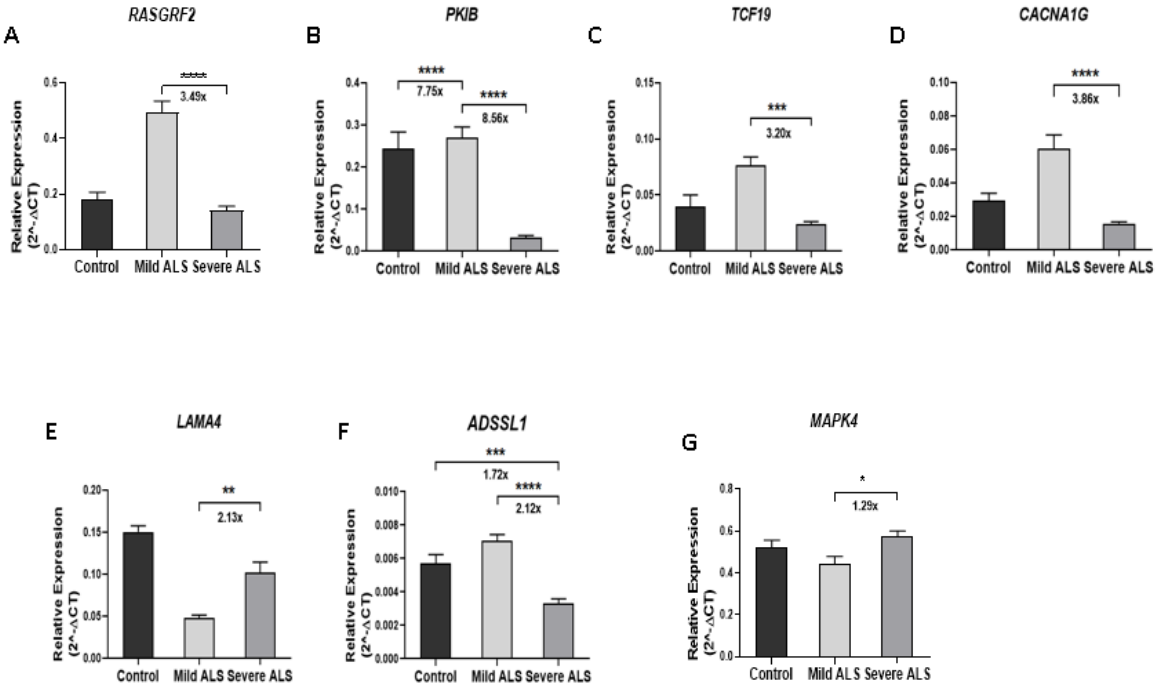
Supplementary figure 7



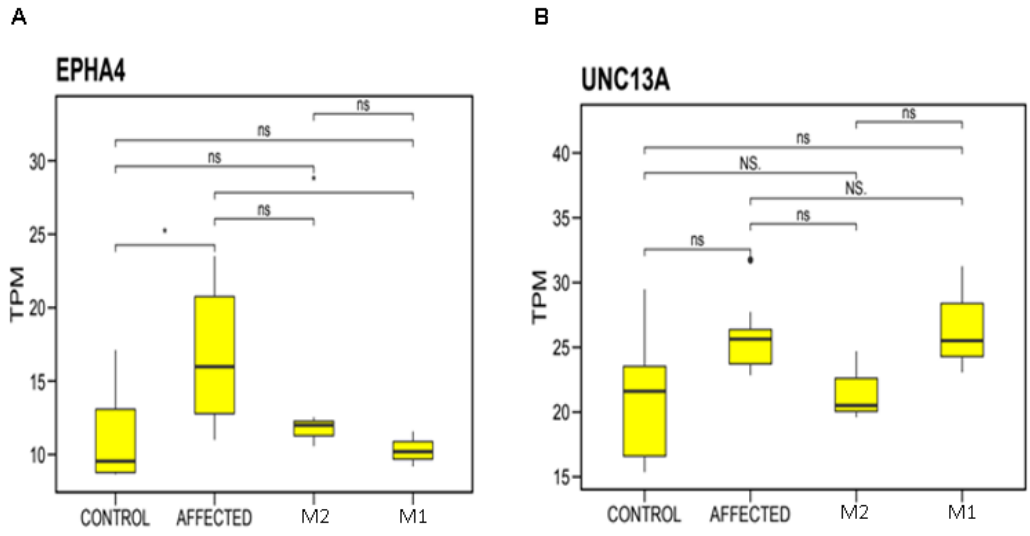
Supplementary figure 8



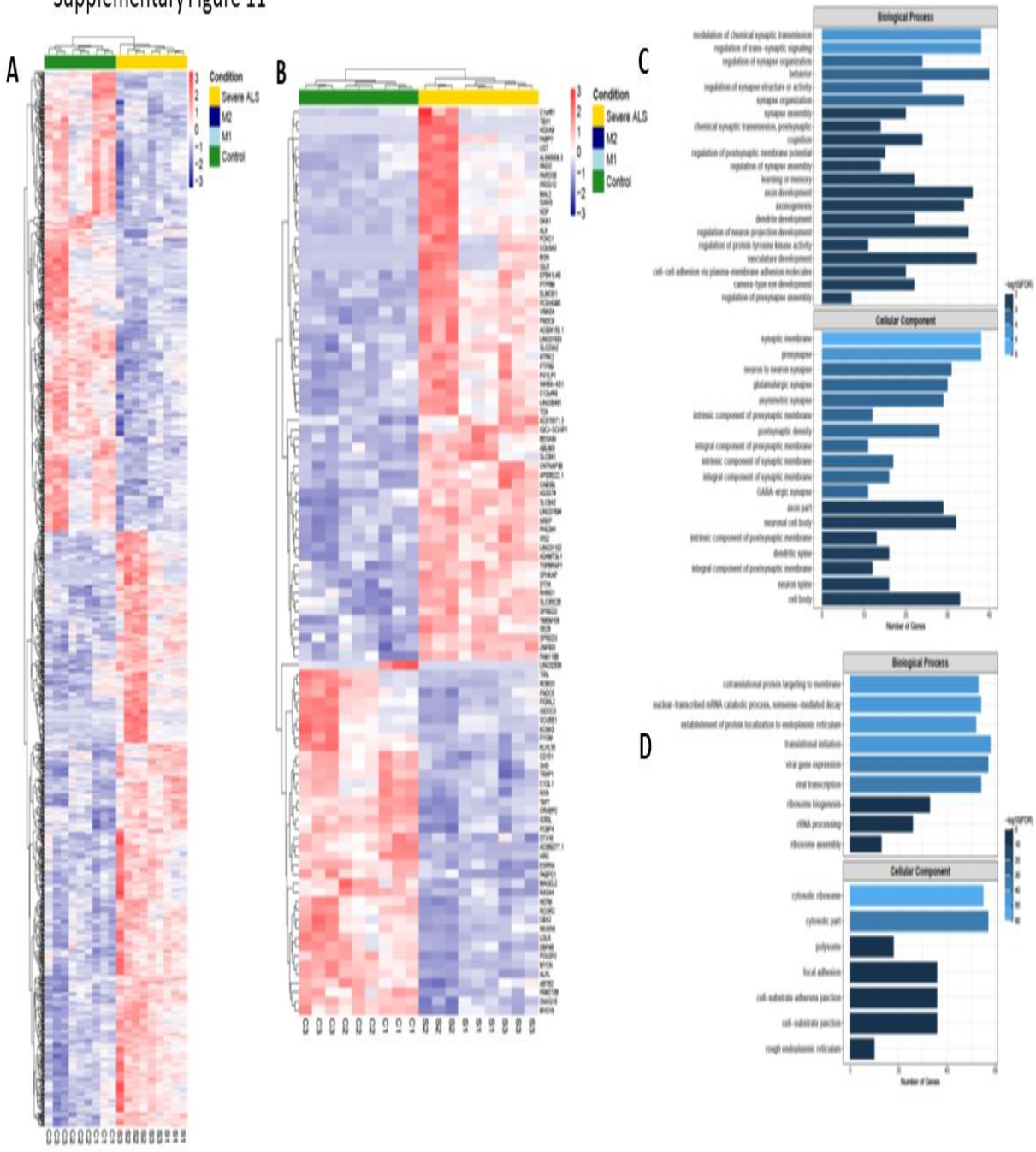
Supplementary Figure 9



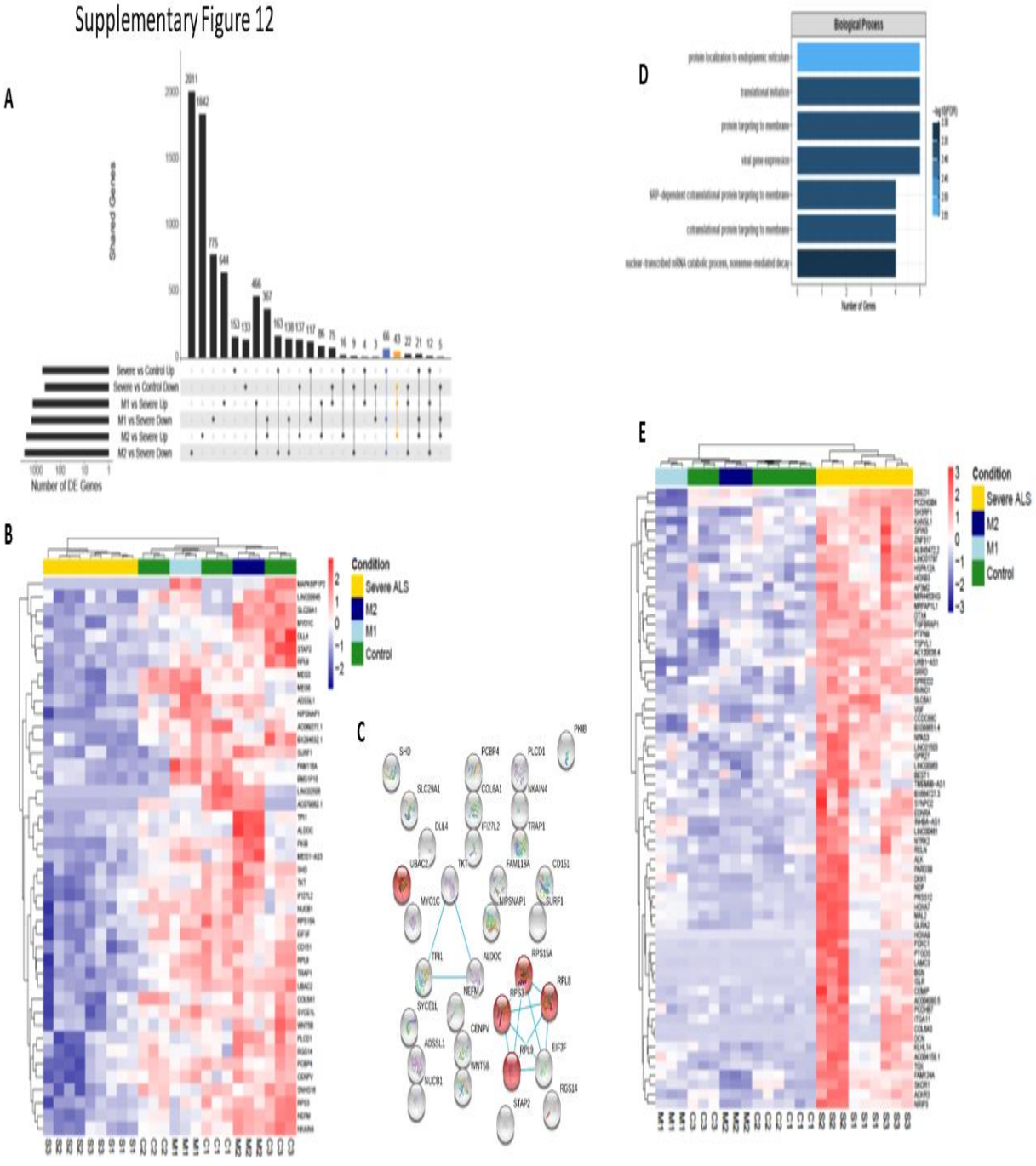
Supplementary Figure 10



Supplementary Figure 11



Supplementary Figure 12



Supplementary methods: Primers' sequencies used in gene expression assays

Oligonucleotide	Sequence
TRPC4AP-F	ACAAGGACAAGGACAGCAC
TRPC4AP-R	CTAACGAGAGCAGAGGGTG
BOD1-F	GCTCATCGCTCTCATCGTGG
BOD1-R	TGCTTGTCCAGATGTGTTGAC
GAPDH-F	ACAACTTTGGTATCGTGGAAGG
GAPDH – R	GCCATCACGCCACAGTTTC
OCT4 - F	CCTGAAGCAGAAGAGGATCACC
OCT4 - R	AAAGCGGCAGATGGTCGTTTGG
NANOG - F	CCTGAAGAAAACTATCCATCC
NANOG- R	CCTTGTCTTCCTTTTTTGCGA
SOX2- F	GCTACAGCATGATGCAGGACCA
SOX2 – R	TCTGCGAGCTGGTCATGGAGTT
ISL1 –F	AAACAGGAGCTCCAGCAAAA
ISL1- R	GCTACAGGACAGGCCAAGAG
MAP2-F	CTCCTTCTCCACCCCCATCA
MAP2- R	TGGAACTCCATCTTCGAGGC
ADSSL1-F	CCAGAACTACATCCGCTTTG
ADSSL1-R	TTTACACAAGTCCAGACGC
PKIB-F	ACAGACGGAACCTCAGATTTG
PKIB-R	AGCACTCTTGATAGATTATGAGCC
RASGRF2-F	ACCACAGAACTTTCACCTTG
RASGRF2-R	ACTCCCATGTCCTGCTG
CACNA1G-F	TGCCCAATGACAGCTACATG
CACNA1G-R	GGGAAGCTGCAGGATGTAG
LAMA4-F	GTGGTTCAGTTGGATGTGG
LAMA4-R	CTGTGAAGGGTTTGCTGG
MAPK4-F	CCCATACTCGTGCCCTG
MAPK4-R	ACAGGCTCACAGGGTAC
TCF19-F	TGTTGGCGGAACTGGAT
TCF19-R	GCTCACTGGGTACTTCCG
ZFAND3-F	AGGAAACCAGTCGATCTAAACAG
ZFAND3-R	GGGAGGCGATGTAACATACA

Capítulo 3

A Heterogeneidade fenotípica da esclerose lateral amiotrófica

Artigo submetido (a convite) à revista *Neural Regeneration Research,* onde uma perspectiva é dada sobre os resultados recentemente publicados.

Phenotypic heterogeneity in Amyotrophic Lateral Sclerosis and modifying mechanisms of neurodegeneration

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In his accounts "On longevity and shortness of life", Aristotle considered how diseased states could be interchangeably associated with long and short lifespans. He believed that the presence of opposite elements and the environment were the sole determinants of this variability. Nowadays, also struck by the same phenomenon observed by the Greek philosopher, human geneticists are still trying to define the etiological basis of the phenotypic plasticity in neurodegenerative disorders. Among such diseases, Amyotrophic Lateral Sclerosis (ALS) stands out as a highly heterogeneous condition. Patients affected by ALS commonly start manifesting symptoms such as weakness in

the upper or lower limbs, difficulty in climbing stairs, fasciculations and loss of muscular mass. As the disease progresses, patients become wheelchair-bound and bulbar signs such as dysarthria and dysphagia become more pronounced. Death occurs on average after 3 years of onset, mainly due to respiratory failure (Renton et al., 2014). However, this canonical course is frequently variable, with a myriad of phenotypic alterations (Swinnen & Robberecht, 2014). Here we describe different aspects of ALS8 clinical variability, both in terms of clinical manifestations and in rate of disease progression. Then, we outline our recent work on ALS8 patients (Oliveira et al., 2020), in which we tried to address the molecular underpinnings of clinical progression variation in the patients we studied. We were able to rule out well-described genetic modifiers, such as *EPHA4* and *UNC13A*, and potential copy number variation alterations. Interestingly, both cell death rates and energetic metabolism appeared to be different among the severe ALS8, mild ALS8 and controls, suggesting an attenuation of pathological process in the less affected patients. Whole transcriptomic analysis of iPSC-derived motor neurons pointed that both "mild patients" presented 43 upregulated and 66 downregulated genes, when compared to controls and the "severe" group. Interestingly, most of the identified genes were associated with protein synthesis and protein targeting to endoplasmic reticulum. Expression of protein translation markers' pMTOR, 4EBP1 and RPS6 were found to be high in the mild ALS8 individuals, when compared to both controls and the severe group. To sum up, our data point that mitigating factors are most likely preventing neurodegeneration in ALS8 through maintenance of protein synthesis. Further studies, assessing the relationship among these potential genetic modifiers and the pathophysiology in ALS8, are fundamental. They might shed light on venues for treatment of this devastating disease.

Amyotrophic lateral sclerosis 8 (ALS8)

First reported in Brazil, Amyotrophic Lateral Sclerosis type 8 (ALS8) is an autosomal dominant form of ALS that mainly presents phenotypes of spinal onset (Nishimura et al., 2004a), where patients commonly present with weakness, fasciculation, muscular atrophy and tremor. In its initial description, in a seminal paper reporting seven related families, a remarkable clinical variability was observed. There were patients classified

as “typical ALS8” or “atypical ALS8”, showing a rapid progression, and differing only by the presence of tremor in the “atypical group”. Additionally, other affected patients from this same cohort were classified as carrying late-onset spinal muscular atrophy (SMA) (Nishimura et al., 2004b).

Linkage analysis, followed by Sanger sequencing of candidate genes, identified in 20q13 a single mutation (C>T at exon 2) of *Vesicle Associated Protein B* (*VAPB;* c.166 C>T). This mutation was found to change proline to serine at amino acid 56 of *VAPB* gene product, which segregated with the three main phenotypic groups (Nishimura et al., 2004b). Interestingly, since then *VAPB* has become the focus of extensive research in ALS physiopathology, once it has been found to regulate autophagy, calcium metabolism and Ephrin pathway. As its overexpression was also found to decrease neuropathology in *SOD1* mice, common pathways for neurodegeneration between *VAPB* and other ALS associated genes were hypothesized as the underlying mechanisms (Mitne-Neto et al., 2011; Kim et al., 2016).

ALS8 patients present a great variability in age of onset. In some individuals, the first symptoms are observed in their 30’s, while others remain asymptomatic until their late 60’s. Progression of clinical manifestations also varies greatly, with some patients surviving with only mild alterations for as long as 40 years, and others having a more typical ALS clinical course of < 5 years (Nishimura et al., 2004b). This finding suggests that mitigating factors might be playing a role in ALS8 progression. The search for genetic modifiers underlying ALS severity is of great interest since it might pave the way for novel therapies. In this scenario, the investigation of familial cases may be more informative, once patients carrying the same pathogenic mutation share more similar environmental influences and genetic background.

Searching for modifying mechanisms in ALS8

In order to address this issue, we recently undertook the search for modifying genetic variants in five ALS8 patients from the same Brazilian family (Oliveira et al., 2020). Although these individuals carry the same genetic mutation (VAPB P56S), they displayed different disease progression rates. We then hypothesized that genetic factors could be playing a role in such phenotypic heterogeneity (Oliveira et al., 2020).

Three ALS8 patients classified as “severe” started the disease symptoms around their 50’s. When clinical data and sample collections were taken, they were wheelchair bound or walking with the help of a cane. On the other hand, the two patients classified as “mild”, a man and a woman were in their seventies but differed from each other in terms of clinical progression. While the woman was asymptomatic until her late sixties and started to present symptoms at the age of 70, the man reported onset of symptoms in his thirties, but with a very mild disease progression for 40 years. At physical evaluation, both presented tremor and fasciculations but good physical strength in upper and lower limbs. The small size of the sample did not allow us to perform linkage analysis. However, copy number variation and whole exome sequencing analyses enabled us to rule out the well-established genetic modifiers for ALS progression, namely, *EPHA4* and *UNC13A* genes (Diekstra et al., 2012; Van-Hoecke et al., 2012). Such data suggested that unknown genetic modifiers might be playing a role in disease progression in these ALS8 patients (Oliveira et al., 2020).

Aiming to further evaluate the mechanism underlying their discordant clinical progressions, we reprogrammed erythroblasts from ALS8 severe and mild patients into induced pluripotent stem cells (iPSCs), differentiated them into motor neurons, and compared them to similar samples from three controls (Oliveira et al., 2020). Considering that ALS8 is probably caused by *VAPB* haploinsufficiency (Mitne-Neto et al., 2011) we first sought to investigate whether a higher expression of this gene in the iPSC-derived motor neurons from milder patients could explain the phenotypic attenuation. Nonetheless, VAPB was found to be equally downregulated in both affected ALS8 groups, regardless of their clinical status. Surprisingly, however, we observed that severe ALS8 patients presented higher degrees of cell death and lower oxidative metabolism than the controls and mildly affected individuals (Oliveira et al., 2020).

As mTOR signaling pathway is a key regulator of several aspects of neuron physiology, such as autophagy and protein synthesis, we set out to investigate its key components (pmTOR, 4EBP1 and RPS6). Interestingly, upon caloric restriction for 48 hours, we found increased levels of pmTOR in the mild ALS8 compared to severe ALS8 and

controls. On the other hand, surprisingly, we identified a higher expression of 4EBP1 in one mild patient and RPS6 in the other, when compared to controls and the "severe" group (Oliveira et al., 2020). Such data suggested, therefore, that the mild ALS8 individuals present a high protein translation activity. The difference between 4EBP1 and RPS6 protein levels is most likely due to epistatic effects.

A whole transcriptomic analysis of the iPSC-derived motor neurons showed that, indeed, the three experimental groups differed among them with respect to their gene expression patterns. Most interestingly, when comparing the two mild patients with severe ALS8 individuals and controls, we were able to identify 43 upregulated and 66 downregulated genes. Most of them, such as *RPL9*, *RPS3*, *RPS15A*, *RPL8* among others were associated with pathways regulating protein translation and targeting to endoplasmic reticulum (ER) (Oliveira et al., 2020). The main pathways found to be affected supported previous reports using other animal and cellular models, which demonstrated the role played by these genes in synaptic physiology.

Therefore, taken together, our data suggest a direct association between the differentially expressed protein translation genes, identified in our whole transcriptomic analysis, and our western blots for protein translation activity. Indeed, both experiments indicate that the motor neurons from mild ALS8 patients are able to keep translation at higher levels than those from the severe ALS8 and the controls. The differential patterns of energetic metabolism and cell death, additionally, are most likely a reflex of this sustained protein synthesis in mild ALS8 individuals, in spite of *VAPB* haploinsufficiency. Several lines of evidence support this idea, as a direct relationship between mitochondrial activity and protein translation has been reported largely in the literature. We hypothesize that VAPB, as a protein located at ER contact sites with other organelles, among them mitochondria, could work as a bridge between these biochemical systems. Similar findings for *VAPB*-induced neurodegeneration were also identified in drosophila, strengthening the idea of *VAPB* as a regulator of protein synthesis in motor neurons (Deivasigamani et al., 2014).

Proteostasis has also been a central aspect of investigation of Amyotrophic Lateral Sclerosis physiopathology. Spinal motor neurons, apart from their decreased capacity

of triggering heat shock protein response, possess a highly concentrated colloid, due to protein concentrations above their solubility levels (Yerbury et al., 2020). Because of such characteristics, these cells appear to be more susceptible to protein homeostasis imbalances than other neural cell subtypes. Our recent work (Oliveira et al., 2020) goes in line with such observations, as it suggests that differentially expressed genes associated with translation are bypassing *VAPB* deficiency to maintain protein synthesis. This would result in greater cell viability and mitochondrial activity in mild ALS8 patients.

Although much progress has been made in the past decade, Amyotrophic Lateral Sclerosis still poses a challenge for biomedical research. The biological underpinnings of its highly heterogeneous phenotypic manifestations, for instance, are only beginning to be addressed. Such information might be extremely useful, as it can shed light on molecular mechanisms of resilience in the Central Nervous System. Potential sites amenable for pharmacological intervention could then be established, giving rise to effective treatments.

Acknowledgements:

The work in the laboratories of the authors was supported by FAPESP (grant numbers: 2013/08028-1, 2015/14821-1, 2017/16283-2 to MZ and 2018/23414-9 to SVA), INCT (465355/2014-5 to MZ) and CAPES. We would like to express our gratitude to the patients who collaborated with us in the study. Without their support, the work in the laboratories of the authors would not have been possible.

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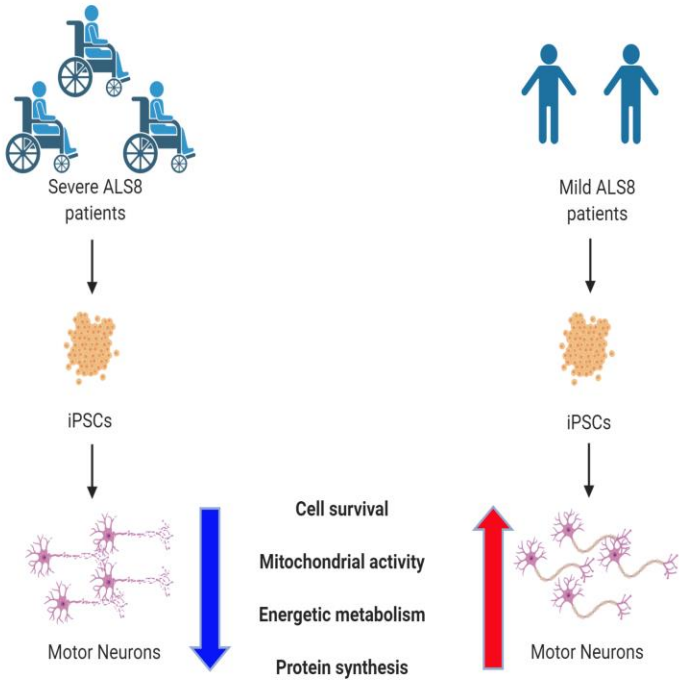

Legend for figure:

PBMCs from three severe and two mild ALS8 patients were collected, and then reprogrammed into induced pluripotent stem cells (iPSCs). IPSCs were differentiated into motor neurons, which were found to be different in terms of cell death, energetic metabolism and protein synthesis, suggesting mitigating mechanisms of neurodegeneration.

Capítulo 4

Estudo genético e clínico de uma família com Esclerose Lateral Amiotrófica de herança autossômica recessiva

Artigo a ser submetido à publicação reportando uma família brasileira consanguínea com uma mutação em homozigose no gene VRK1, e portadora Esclerose Lateral Amiotrófica.

Autosomal recessive amyotrophic lateral sclerosis caused by *VRK1* mutations.

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Abstract

Amyotrophic Lateral Sclerosis is a severe motor neuron disease, which so far has no treatment. In spite of having already been associated to hundreds of pathogenic mutations, located at more than 30 genes, its genetic etiology is far from being fully understood. Vaccinia-related kinase 1 (*VRK1*) is a gene located at 14q32.2 which has been implicated in the pathological process of a broad range of neurodevelopmental disorders as well as neuropathies, including microcephaly, spinal muscular atrophy with cerebellar hypoplasia (SMA-PCH) and Amyotrophic Lateral Sclerosis (ALS). Here we report a large, endogamic, family presenting ALS in an autosomal recessive mode of inheritance, segregating with a homozygous missense mutation located at VRK1 gene (p.Arg321Cys). The proband's age of onset was at 38 years, after referring weakness in legs, which progressed to the upper limbs. After two years, the patient was wheelchair confined. Patellar, radial and tricipital brisk reflexes were described, suggesting upper motor neuron impairment. Similar findings were also observed in the other two affected ALS siblings. To our knowledge it is the first Brazilian family reported with Amyotrophic Lateral Sclerosis caused by a mutation at VRK1.

Resumo

Esclerose Lateral Amiotrófica é uma doença de neurônio motor severa, que até o momento não possui tratamento. A despeito de já ter sido associada a centenas de variantes patogênicas, localizadas em mais de trinta genes, sua etiologia genética está longe de ser completamente compreendida. *Vaccinia-Related Kinase 1* (*VRK1*) é um gene localizado em 14q32.2, que tem sido implicado no processo patológico de uma ampla variedade de alterações do neurodesenvolvimento, bem como neuropatias, incluindo microcefalia, atrofia muscular espinal com hipoplasia de cerebelo (SMA-PCH) e esclerose lateral amiotrófica (ELA). Aqui reportamos uma ampla e endogâmica família, manifestando ELA num padrão de herança autossômico recessivo, que segregava com uma mutação em homozigose localizada no gene VRK1 (p.Arg321Cys). A idade de início dos sintomas no propósito foi de 38 anos, depois de referir fraqueza nas pernas, que progrediu em seguida para os membros superiores. Depois de dois anos de progressão, a paciente tornou-se dependente de cadeira de rodas. Reflexos patelares, radiais e tricipitais rápidos foram descritos, sugerindo dano em neurônios motores superiores. Achados similares foram também observados nos

outros dois afetados da irmandade. Até onde nos consta, trata-se da primeira família brasileira deportada com Esclerose Lateral Amiotrófica causada por mutações no gene VRK1.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron disorder, affecting around 3:100 000 individuals worldwide [1]. It is pathologically characterized by the selective death of lower and upper motor neurons in motor cortex, brainstem and spinal cord [2]. Albeit having a variable clinical course, ALS patients commonly start referring weakness, loss of muscular mass, fasciculations and difficulty of swallowing. After a relentless progress, which takes on average 3 years, affected patients become wheelchair-bound. Death is usually due to respiratory failure [3].

So far, more than 850 mutations, localized at 31 genes, have been associated with ALS [4]. In spite of this enormous genetic heterogeneity, this list is still expected to grow, particularly for sporadic ALS, where large fractions of patients remain without an association to any known causative genetic factor [1]. Amongst the familial cases, most of them present an autosomal dominant pattern of inheritance (AD) [5]. Interestingly, however, there have been cases in which genes presenting mostly AD mutations also manifest ALS with autosomal recessive inheritance (AR)[6,7]. The best-described examples for such events are *FUS* and *SOD1*. Other genes, however, cause ALS only in an AR mode of inheritance, this is the case of the genes *ALS2*, *SPG11* and *VRK1* [5,8]

VRK1 is a serine-threonine kinase which has been reported as playing a role in cell cycle regulation and genomic integrity [9]. So far, apart from AR Amyotrophic Lateral Sclerosis, a broad spectrum of neurological diseases have been associated to it, such as Spinal Muscular Atrophy with Pontocerebellar Hypoplasia (SMA-PCH) and distal Spinal Muscular Atrophy/ distal hereditary motor neuron neuropathy [10].

Herein, we report a large consanguineous family from the Brazilian state of Bahia, in which the three affected patients with Amyotrophic Lateral Sclerosis presented homozygous *VRK1* mutations. To our knowledge, it is the first report of ALS individuals from Brazil carrying pathogenic mutations at this gene.

Methods

Clinical data collection

Familial history supporting an autosomal recessive inheritance, and clinical data were obtained upon evaluation from an experienced neurologist, both at São Paulo and at the city of Juazeiro. Electroneuromyography (EMG) was performed in the three affected individuals . The clinical hypothesis of Amyotrophic Lateral Sclerosis was then proposed for all of them. In order to identify a possible causal genetic variant associated with the phenotype, peripheral blood was collected for the ALS patients and the other normal seven siblings

Whole exome sequencing (WES) and segregation analysis

Genomic DNA from the three affected individuals and a non-affected sibling (individuals IV-2, IV-4, IV-7 and IV-9) were extracted, following standard procedures. The sequencing libraries were prepared by using the SureSelect Kit (Agilent). The captured libraries were sequenced in a HiSeq2500 (Illumina, San Diego, California, USA). After that, paired-ends were submitted to a stablished pipeline, for evaluation using Burrows-Wheeler Aligner for alignment to the reference genome GRCh37 [11]. Finally, variant annotation was performed using ANNOVAR, 1000 Genomes Project National Institute of Health (https://www.internationalgenome.org/), the 6500 Exome Sequencing Project Washington University (https://evs.gs.washington.edu/EVS/), and database of 609 healthy aged controls from Brazilian population (ABraOM, http://abraom.ib.usp.br/) [12,13].

Following a candidate variant identification at VRK1 gene, PCR assays and Sanger sequencing of amplified fragments were performed. The primers sequences employed in the experiments were as follows - Forward: 5'GCCAAATACATGGAAACAGTGA3', and Reverse: 5'TCAAACCTCCATTCTCCACA3'.

Results

Clinical data

Three affected siblings were clinically and neurologically evaluated. The proband, a 48-year-old female (Figure 1A), had no symptoms until 10 years ago. At

age 38, she referred distal weakness in both legs which progressed in approximately one year to weakness in both hands. At age of 40, she was confined to a wheelchair. Since then the weakness is slowly worsening with episodes of cramps in the legs and abdomen but no bulbar or sensitive sphincters involvement. At clinical examination, she presented pronounced distal and symmetric muscular atrophy and weakness in the lower and upper limbs, affecting more the lower limbs distally than proximally. The patellar, radial and tricipital reflexes were brisk and abolished in the achilles. No signs of sensitive or ataxia involvement were observed. Electroneuromyography (EMG) revealed the presence of diffuse impairment of the lower motor neurons.

The other two affected siblings (a 51 years-old sister and 53-years old brother; Figure 1A) presented similar clinical manifestations. The sister had slowly progressive weakness for 20 years, and currently she presents distal and symmetrical atrophy with muscular weakness of distal predominance and deep hyperactive (brisk) reflexes. However, her progression is milder and she is still walking without support. The brother reports onset of symptoms at age 35. Currently he has distal weakness, more evident in the lower limbs, also with deep brisk reflexes and depends on crutches to walk. EMG revealed chronic lesion of lower motor neurons in both.

Mutation identification

Whole exome sequencing (WES) was performed in the three patients and one non-affected sister (IV-2, IV-4, IV-7 and IV-9, respectively; Figure 1). Data was filtered for homozygous variants, as suggested for autosomal recessive inheritance due to consanguinity and pedigree structure. We took into consideration filters of frequency (MAF <0.01) in population databases from Brazil (ABraOM) and abroad (ExAC and 1000 genomes) and pathogenicity prediction websites. Homozygous variants shared by all three affected and absent in the non-affected individual were identified in *AK7*, *DRD4*, *OR51A4*, *OSBPL5*, *RHOBTB3*, *SLC22A18*, *TSPAN32* and *VRK1* as potential candidates. Based on database information, we observed that the *VRK1* mutation (c.961C>T; p.Arg321Cys), had been previously associated with ALS in a sporadic patient, as reported by Nguyen et al (2015).

Segregation analysis showed that the single nucleotide variant here reported was present in homozigosity in the three affected individuals and in none of the normal

siblings. The variant was also found to be highly conserved across different species (Figure 1B and C).

Discussion

A nonsense mutation (c.1072C>T; p.Arg358*) in Vaccinia-Related Kinase gene (*VRK1*) has been first reported in a consanguineous family of Askenazi Jewish ancestry presenting spinal muscular atrophy with pontocerebellar hypoplasia [14]. Other pathogenic variants (p.Arg133Cys, p.Val236Met, p.Arg89Gln, p.Pro358*) were described in patients with SMA-PCH, microcephaly, neuropathy in sensory neurons and other brain dysmorphic findings [15]. Two unrelated individuals presenting early-onset amyotrophic lateral sclerosis (ALS) and compound heterozygous mutations in *VRK1* (p.His119Arg and p.Arg321Cys; p.Gly135Arg and p.Leu195Val) have also been described [8,16]

More recently, two other individuals of Jewish Moroccan ancestry presented distal motor neuropathy and VRK1 mutations (p.Arg387His). Both were also members of consanguineous families and, differently from the other early-onset cases, started referring clinical symptoms around age 40 [17]. Such data further expanded the plethora of VRK1-associated clinical entities, and increased the number of pathogenic variants associated with this gene, which are so far 16. Here we describe a consanguineous Brazilian family of Caucasian ancestry, presenting ALS in an autosomal recessive mode of inheritance. Whole-exome sequencing (WES) and segregation analysis identified a homozygous mutation in *VRK1* (p.Arg321Cys) as the underlying pathogenic factor in the ALS individuals.

Interestingly, the patients here described present brisk tendon deep reflexes, which are clinical signs suggesting a higher involvement of the upper motor neurons. Such finding is in agreement with previous reports of other motor neuron disease patients carrying *VRK1* mutations, and highlights the clinical and genetic heterogeneity in amyotrophic lateral sclerosis [3]

VRK1 is a 396 amino acids protein, which has been observed mainly located at cell nuclei. There, it phosphorylates several targets, such as VRK1, p53, c-jun, ATF2, and CREB, whereby it may regulate different aspects of transcription and cell proliferation [18]. Its role in coordinating DNA repair responses to double strand breaks is believed to be due to its kinase activity on H2AX, NBS1 and 53BP1 [19].

Interestingly, *VRK1* has also been associated with nuclear membrane physiology, as it plays a role in its assembly after cell division [20]. Molecular mechanisms of ageing, coincidentally, overlap with the ones reported to be damaged by *VRK1* mutations, as alterations in repair machinery have been associated with premature senescence [21]. Its multiple roles in DNA integrity, transcription, mitosis and nuclear integrity appear to be central for maintenance of cell viability. Therefore, studies focusing on understanding VRK1-induced neurodegenerative processes might also shed light on broader cell mechanisms, such as brain ageing itself.

Conclusion

We described a large consanguineous family from Brazil presenting autosomal recessive Amyotrophic Lateral Sclerosis. Genetic analysis enabled us to identify the underlying pathogenic mutation segregating in all three affected siblings, and absent in the seven non-affected (*VRK1*; p.Arg321Cys). Patients' clinical assessment revealed mainly upper motor neuron impairment, confirming previous findings. Therefore, *VRK1* should be considered for genetic screening, in cases of motor neuron disease with autosomal recessive inheritance. Further molecular studies, aiming to understand the role of this gene in neurodegenerative processes are needed.

Acknowledgements:

We would like to thank the patients reported in this study. This work was supported by Fundação de Amparo à Pesquisa do Estado de Sao Paulo (FAPESP-CEPID), INCT, CAPES and CNPq.

Compliance with ethical standars

Conflicts of interest: The authors declare no conflict of interest

Ethical standards: The study was approved by ethics Committee from the Department of Genetics and Evolutionary Biology at the Institute of Biosciences, University of São Paulo, Brazil.

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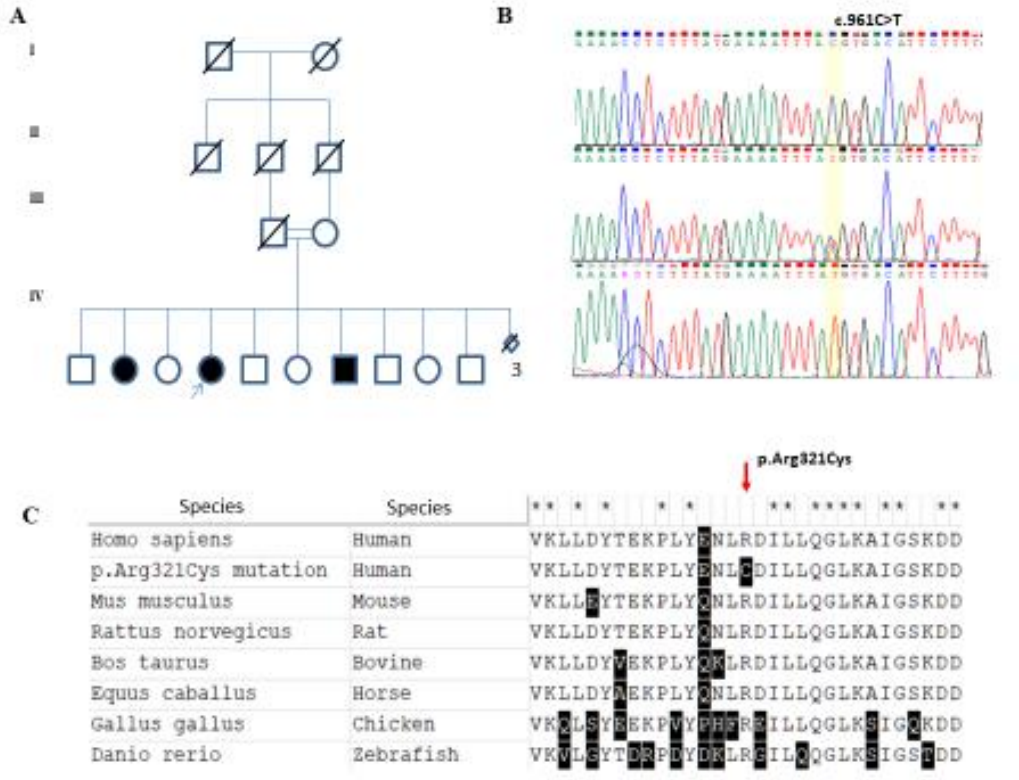

Legend for figure:

A large endogamic family presenting Amyotrophic Lateral Sclerosis in a autosomal recessive pattern of inheritance (A) Heredogram representation, depicting the propositus and the other two affected individuals. (B) Sanger sequencing of the detected variant, showing this is repsent in all affected patients. (C) Bioinformatic alignment of VRK1 sequence in wild type and mutated individuals, and comparison with orthologs in different animal species.

Capítulo 5

Discussão Geral e Conclusões

A Esclerose Lateral Amiotrófica é a doença de neurônio motor mais comum. Ao longo do seu curso clínico, compromete fisicamente o indivíduo de forma severa, inabilitando-o a realizar os movimentos mais simples. Como visto no capítulo 1, toda musculatura esquelética é comprometida, restando ao paciente, quando muito, o controle esfincteriano nos sistemas gastrointestinal, urinário e movimentos sacádicos dos olhos. Ambos são dependentes de estruturas anatômicas comumente preservadas do processo neurodegenerativo da ELA, a saber, núcleo de Onuf e colículos superiores (Mesencéfalo) (Sharma et al., 2011; Swinnen & Robberecht, 2014). O portador da esclerose lateral amiotrófica torna-se, portanto, prisioneiro de si mesmo e dos seus pensamentos.

Devido à grave morbidade causada por essa doença, esforços objetivando a elucidação de sua base molecular, e a identificação de uma terapia ocorrem há mais de duas décadas. Atualmente, duas drogas aprovadas pelo FDA (*Food and Drug Admnistration*) edaravone e riluzole foram aprovadas para uso em pacientes com ELA. Apesar disso, ambas têm pouco efeito e o prognóstico continua pobre (Barp et al., 2020).

O primeiro medicamento aprovado para uso em pacientes com ELA, o riluzole foi reportado, em alguns estudos clínicos, aumentando a sobrevida de casos graves dessa doença em até 35% (Bensimon et al., 1994). Poucos efeitos colaterais foram descritos, tais como tonturas e alterações gastrointestinais. Entretanto, a maioria dos trabalhos subsequentes avaliando o potencial terapêutico do riluzole têm demonstrado pouco efeito sobre a progressão da doença (Bedlack et al., 2007). A edaravone, de modo similar, foi aprovada mais recentemente para uso em pacientes, baseado na sua capacidade de reduzir estresse oxidativo (Yoshino & Kimura, 2006). Apesar da discrepância entre os resultados dos estudos clínicos, seu uso ainda hoje é mantido (Abe et al., 2014).

Devido à ausência, portanto, de estratégias terapêuticas eficazes para a ELA, vários estudos têm se dedicado a testar fármacos novos. Nesse sentido, eritropoietina,

prepampanel, Inosina, L-serina e mais uma gama de outras drogas tem sido avaliada, sem que haja um consenso ou resultado definitivo em relação à melhora do prognóstico (Barp et al., 2020). Claramente, tratamentos capazes de trazer a cura ou melhorar a sobrevida de portadores da ELA são inexistentes, até o momento.

Portanto, ainda se faz necessário o investimento de recursos, tanto para a compreensão dos mecanismos de fisiopatologia da ELA quanto para estudos clínicos de potenciais terapias. Estima-se que a sua incidência média, em populações ocidentais, que é de cerca de 3:100 000 indivíduos, sofrerá nas próximas décadas um aumento significativo (Arthur et al., 2016). Estudos recentes, visando modelar a prevalência da Esclerose Lateral Amiotrófica até 2040 preveem um aumento de até 69% dos casos globalmente. Entre os fatores contribuindo para esse incremento substancial, encontram-se o envelhecimento das populações e a expansão de programas públicos de saúde, principalmente em países em desenvolvimento (Arthur et al., 2016).

A Esclerose lateral Amiotrófica tipo 8 (ELA8), tem sido foco de investigações extensivas por nosso grupo, desde sua descrição inicial. Fomos capazes de esclarecer sua etiologia molecular – uma mutação de sentido trocado no gene VAPB (p.P56S), e demonstrar, em modelos celulares, os principais aspectos do seu processo patológico (Nishimura et al., 2004[b] ; Mitne-Neto et al., 2011). Entre as características observadas está a diminuição da expressão da VAPB em indivíduos com ELA8, na contramão do seu aumento ao longo da maturação dos neurônios motores, em condições fisiologicamente normais.

Outro aspecto patológico da ELA8 previamente caracterizado por nosso grupo é a total ausência de agregados proteicos nos neurônios derivados de células iPSCs (*induced pluripotent stem cells*) (Mitne-Neto et al., 2011). Esse achado vai de encontro ao reportado em modelos de superexpressão da VAPB P56S em outros sistemas biológicos, células HeLa e culturas primarias de neurônios (Teuling et al., 2007). Algumas hipóteses têm sido aventadas para explicar a discordância entre esses achados, como o próprio perfil de expressão embrionário dos neurônios, impedindo a observação de fenótipos típicos de um ambiente celular envelhecido (Miller et al., 2013). Por outro lado, entretanto, é importante ressaltar que a superexpressão da *VAPB* naqueles modelos também produz níveis aberrantes dessa proteína. Portanto, a formação desses aglomerados tóxicos pode ser um artefato que não corresponde à realidade.

Simular processos neurodegenerativos em modelos de *iPSC* é de fato um desafio. Linhagens derivadas a partir de pacientes com diferentes mutações demonstraram uma capacidade diferencial de formar agregados proteicos, característica típica de sua patogênese. Algumas estratégias têm sido desenvolvidas para contornar essas limitações, como o estresse artificial dos neurônios. Nesse caso, tratamentos com puromicina e arsenito, por exemplo, têm sido eficientes na produção desses fenótipos (Martinez et al., 2016). Com base nesses trabalhos, testamos a inibição da via de proteassomas, pela adição de MG132, mas não houve diferença em relação à distribuição da *VAPB* em relação ao grupo controle. Considerando as limitações dos modelos derivados de iPSCs, a hipótese mais aceita para a patogênese da *VAPB* continua sendo, portanto, a da haploinsuficiência (Mitne-Neto et al., 2011). Futuros estudos anatomopatológicos, em tecidos do SNC dos pacientes ELA8, poderão esclarecer esse ponto importante de sua fisiopatologia.

Nesse trabalho, fomos capazes de demonstrar pela primeira vez as vias biológicas relevantes para a patogênese da ELA8, em modelos celulares humanos. Como mencionado no capítulo 2, identificamos 979 genes diferencialmente expressos, dos quais 552 estavam superexpressos e 427 hipoexpressos. Curiosamente, os genes cuja expressão estava aumentada agrupavam para vias biológicas associadas à formação e manutenção de sinapses, sinalização sináptica, entre outros. Esses achados corroboram indícios já descritos na literatura, sugerindo um papel regulador da *VAPB* nesse importante processo fisiológico. Gomez-Suaga et al, (2019) usando neurônios hipocampais murinos, verificaram que tanto VAPB quanto o seu ligante PTPIP51 localizavam-se em sinapses, e o knockdown de um ou o outro causava alterações morfológicas em dendritos e na atividade sináptica. Não está claro, entretanto, de que maneira a interação VAPB – PTPIP51, ocorrendo em sítios de contato RE-mitocôndrias, está relacionada a esses processos.

Outro aspecto importante observado nos nossos achados, é que, dentre os genes hipoexpressos, identificamos vias biológicas relacionadas à síntese proteica, biogênese de rRNA e endereçamento proteico ao RE. Embora já tendo sido avaliada como reguladora de processos autofágicos, e indutora de *unfolded protein response* (UPR) em modelos celulares diferentes, a *VAPB* nunca tinha sido descrita anteriormente como ligada a mecanismos de tradução.

Similarmente ao que está descrito na literatura em outros sistemas biológicos, a mutação da *VAPB* induz alterações no metabolismo energético dos neurônios. Pudemos evidenciar esse fenótipo molecular através de dois ensaios funcionais (DCFDA e *SeaHorse*).

Nesse trabalho, fomos também capazes de descrever alterações funcionais e de expressão gênica correlacionadas com a variabilidade clínica da ELA8. Observamos que os possíveis fatores mitigadores do seu processo patológico não estavam associados diferenças na expressão da *VAPB*, nem devido ao acúmulo de mutações já associadas à ELA nos indivíduos mais graves. Por outro lado, os níveis de morte celular e metabolismo energético apresentavam um padrão distinto: indivíduos ELA8 "severos" apresentaram mais morte celular e menos atividade mitocondrial que os "leves" e os "controles".

Em nosso ensaio de transcriptômica, identificamos 43 genes superexpressos e 66 hipoexpressos em ambos os indivíduos ELA8 "leves", quando comparados aos "severos" e "controles". Surpreendentemente, a maioria desses genes estava associada a processos de tradução proteica e endereçamento de proteínas ao RE. Visando validar esse achado, analisamos com técnica de *western blottings* as proteínas marcadoras de tradução pMTOR, RPS6 e 4EBP1. Observamos que, em condições de estresse calórico, os três marcadores mostraram-se altamente expressos nos indivíduos "leves", em relação aos "controles" e "severos". Dessa maneira, nossos achados mostram que mecanismos de síntese proteica estão diminuídos na presença da mutação da *VAPB* P56S. Isso sugere que processos biológicos capazes de os reestimular poderiam, provavelmente, retardar a progressão da doença.

Como mencionado no capítulo 3, a proteostase é um mecanismo central em neurônios motores. O papel desempenhado pela síntese proteica em aspectos fundamentais da sinapse, como plasticidade, síntese de neurotransmissores, etc, sugere que os genes candidatos por nós identificados no capítulo 2, possam de fato, mitigar a neurodegeneração na ELA8. Devido ao pouco sucesso das terapias atuais para Esclerose Lateral Amiotrófica, vias moleculares identificadas a partir de pacientes com fenótipo brando são de extrema relevância. Estudos visando identificar a correlação entre aqueles genes e os fenótipos moleculares por nós descritos podem apontar para sítios de intervenção farmacológica.

Nesse trabalho, também fomos capazes de identificar a causa genética de uma forma autossômica recessiva de Esclerose Lateral Amiotrófica. Como descrito no capítulo 4, todos os indivíduos afetados dessa grande irmandade apresentavam uma mutação em homozigose no gene *Vaccinia-Related Kinase 1* (*VRK1*). Clinicamente, os pacientes foram observados com sinais típicos de perda de neurônios motores superiores, pela manifestação de hiperreflexia. Esse achado é oposto ao apresentado pelos pacientes com a mutação P56S no gene *VAPB*, descritos por nós nos capítulos 1 e 2. A ELA8 caracteriza-se, principalmente, pela presença de fraqueza muscular e fasciculações, alterações tipicamente derivadas de morte dos neurônios motores inferiores.

Mutações em *VRK1* estão associadas a um amplo espectro fenotípico. Inicialmente descritas em indivíduos portadores de atrofia muscular espinal com hipoplasia pontocerebelar (SMA-PCH), foram observadas posteriormente em pacientes manifestando neuropatias distais de início juvenil, formas de microcefalia e esclerose lateral amiotrófica (Sedghi et al, 2019). Trata-se, portanto, de um gene com preponderante papel na neurogênese e na manutenção do SNC adulto (Vinograd-Byk et al, 2018).

Estudos funcionais em modelos animais e celulares sobre o gene *VRK1* têm evidenciado diversos aspectos do seu papel biológico (Salzano et al, 2015). Atualmente, sabe-se que esse gene atua na regulação de mecanismos de reparo de fita dupla (*DSB repair*), replicação do DNA e reagrupamento da membrana nuclear após a divisão celular. São também constituintes dos corpos de Cajal, complexos ribonucleoproteicos encontrados no interior do núcleo celular, onde atuam no metabolismo de RNAs (El-Bazzal et al, 2018).

Ainda não está claro o papel desempenhado pelo VRK1 na patogênese da ELA, nem a sua correlação ou não com outras proteínas ligadas à processos fisiológicos semelhantes, como TDP-43 e FUS. Devido à sua atividade reguladora de mecanismos de reparo, é provável que os neurônios motores portadores dos pacientes apresentem instabilidade na sua integridade genômica, levando a maiores níveis de apoptose. Nesse cenário, variantes causais no *VRK1* poderiam ser consideradas mutações progeróides, uma vez que defeitos no reparo de DNA são reconhecidos indutores do envelhecimento celular. Estudos visando corrigir seus efeitos patológicos poderão, portanto, ser de

grande valia para não só o estudo da Esclerose Lateral Amiotrófica, mas também do envelhecimento cerebral.

Resumo

A Esclerose Lateral Amiotrófica tipo 8, causada por mutações no gene VAPB, notabiliza-se pela grande variabilidade clínica e de início dos sintomas. Nesse trabalho, avaliamos possíveis fatores biológicos subjacentes a esse fenômeno. Identificamos dois pacientes que apresentavam uma manifestação branda dessa doença, aos quais denominamos ELA8 "leves", que foram comparados com outros três, com um curso clínico típico, classificados como ELA8 "graves". As análises genéticas através de a-CGH e exoma, não identificaram a presença de modificadores clássicos para essa doença.

Células iPSCs foram derivadas para os três grupos experimentais (ELA8 "graves", "leves" e "controles". A partir desse modelo celular, neurônios motores foram então obtidos, e vários parâmetros funcionais e de expressão gênica puderam ser avaliados. Identificamos que os neurônios motores dos ELA8 "leves" possuíam níveis de metabolismo energético similares aos controles, bem como taxas de morte celular inferiores aos ELA8 "severos". Por outro lado, fomos também capazes de identificar 43 genes superexpressos e 66 hipoexpressos em comum entre os ELA8 "leves", quando comparados aos "controles" e "severos". A maioria desses estão relacionados a proteostase. A análise de *Western blottings* para proteínas marcadoras dessa via biológica (pMTOR, RPS6 e 4EBP1) confirmou esse achado, sugerindo a sua preponderância na mitigação da neurodegeneração em ELA8.

Identificamos também uma grande família consanguínea portadora de um tipo autossômico recessivo de Esclerose Lateral Amiotrófica. A avaliação clínica dos três afetados evidenciou a presença de alterações típicas de dano em neurônios motores superiores (hiperreflexia). O estudo do exoma dos afetados levou à identificação de uma mutação em homozigose no gene *Vaccinia Related Kinase 1* (*VRK1* p.R321C). A avaliação da segregação dessa variante na família confirmou sua presença apenas nos pacientes. Estudos funcionais serão, portanto, necessários para avaliar o papel dessa mutação no processo neurodegenerativo da ELA.

Abstract

Amyotrophic Lateral Sclerosis type 8, caused by mutations at VAPB gene, characterizes by its great clinical variability and onset of symptoms. In the present work, we evaluated the possible biological factors underlying this phenomenon. We identified two patients with a very mild manifestation of this disease, which we named ALS8 "mild", who were compared with three others, with a typical clinical course, classified as ALS8 "severe". Genetic analyses through a-CGH and exome sequencing did not identify the presence of classical modifiers for this disease.

iPSC cells were derived for the three experimental groups (ALS8 "severe", "mild" and "controls"). By using this cellular model, motor neurons were obtained, and several functional parameters and gene expression were evaluated. We identified that the motor neurons from the "mild" ALS8 presented levels of energetic metabolism similar to controls, as well as less cell death rates, when compared to severe ALS8 individuals. We were also able to identify 43 overexpressed genes, and 66 underexpressed, in common between the "mild"ALS when compared to "controls" and "severe" patients. Most of them, were related to proteostasis. *Western blotting* for marker proteins of this biological pathway (pMTOR, RPS6 and 4EBP1) confirmed this finding, suggesting its importance for mitigating ALS8 neurodegeneration.

We also identified a large consanguineous family presenting an autosomal recessive type of Amyotrophic Lateral Sclerosis. Clinical evaluation of the three affected individuals evidenced the presence of typical alterations of upper motor neurons (hyperreflexia). Exome sequencing for the three patients led to the identification of a homozygous mutation in the gene Vaccinia Related Kinase 1 (*VRK1* p.R321C). Segregation analyses of this variant in the family confirmed its presence only in the affected individuals. Functional studies will be necessary for evaluating the role of this genetic alteration in the ALS neurodegenerative process.

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Anexos

Anexo 1: co – autoria de artigo publicado na revista Nature Communications

ARTICLE

Corrected: Publisher correction

DOI: 10.1038/s41467-017-02790-9 OPEN

Discordant congenital Zika syndrome twins show differential in vitro viral susceptibility of neural progenitor cells

Luiz Carlos Caires-Júnior *et al.*#

Congenital Zika syndrome (CZS) causes early brain development impairment by affecting neural progenitor cells (NPCs). Here, we analyze NPCs from three pairs of dizygotic twins discordant for CZS. We compare by RNA-Seq the NPCs derived from CZS-affected and CZS-unaffected twins. Prior to Zika virus (ZIKV) infection the NPCs from CZS babies show a significantly different gene expression signature of mTOR and Wnt pathway regulators, key to a neurodevelopmental program. Following ZIKV in vitro infection, cells from affected individuals have significantly higher ZIKV replication and reduced cell growth. Whole-exome analysis in 18 affected CZS babies as compared to 5 unaffected twins and 609 controls excludes a monogenic model to explain resistance or increased susceptibility to CZS development. Overall, our results indicate that CZS is not a stochastic event and depends on NPC intrinsic susceptibility, possibly related to oligogenic and/or epigenetic mechanisms.

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Anexo 2: autoria de artigo publicado na revista Journal of Medical Genetics

SHORT REPORT

10q23.31 microduplication encompassing *PTEN* decreases mTOR signalling activity and is associated with autosomal dominant primary microcephaly

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Received 4 May 2018
Revised 7 August 2018
Accepted 29 August 2018
Published Online First 9 October 2018

ABSTRACT

Background Hereditary primary microcephaly (MCPH) is mainly characterised by decreased occipitofrontal circumference and variable degree of intellectual disability. MCPH with a dominant pattern of inheritance is a rare condition, so far causally linked to pathogenic variants in the *ALFY*, *DPP6*, *KIF11* and *DYRK1A* genes.

Objective This study aimed at identifying the causative variant of the autosomal dominant form of MCPH in a Brazilian family with three affected members.

Methods Following clinical evaluation of two sibs and their mother presenting with autosomal dominant MCPH, array comparative genome hybridisation was performed using genomic DNA from peripheral blood of the family members. Gene and protein expression studies were carried out in cultured skin fibroblasts.

Results A 382 kb microduplication at 10q23.31 was detected, encompassing the entire *PTEN*, *KLLN* and *ATAD1* genes. *PTEN* haploinsufficiency has been causally associated with macrocephaly and autism spectrum disorder and, therefore, was considered the most likely candidate gene to be involved in this autosomal dominant form of MCPH. In the patients' fibroblasts, *PTEN* mRNA and protein were found to be overexpressed, and the phosphorylation patterns of upstream and downstream components of the mammalian target of rapamycin (mTOR) signalling pathway were dysregulated.

Conclusions Taken together, our results demonstrate that the identified submicroscopic 10q23.31 duplication in a family with MCPH leads to markedly increased expression of *PTEN* and reduced activity of the mTOR signalling pathway. These results suggest that the most probable pathomechanism underlying the microcephaly phenotype in this family involves downregulation of the mTOR pathway through overexpression of *PTEN*.

To cite: Oliveira D, Leal GF, Sertié AL, *et al*. *J Med Genet* 2019;**56**:543–547.

INTRODUCTION

Hereditary primary microcephaly (MCPH), characterised by occipitofrontal circumference (OFC) two or three SD below the population mean,[1 2] is a genetically heterogeneous disorder whose overall incidence varies across different populations, from 1.3 to 150 per 100 000 newborns. The population prevalence of this condition is directly related to the rates of endogamy, since most cases of MCPH have an autosomal recessive mode of inheritance.[1 3] Nevertheless, families have been reported, in which MCPH has either autosomal dominant or X linked inheritance.[1 4]

Patients with MCPH often display varying degrees of intellectual disability (ID), which is correlated to the level of brain hypoplasia. Despite being structurally smaller, the patients' brains usually exhibit normal cytoarchitecture,[2] suggesting that the genes implicated in MCPH have an important role in determining human brain size. For instance, the *ASPM* gene, associated with autosomal recessive MCPH, has been reported as undergoing positive selection for cortical size expansion during recent primate evolution.[5]

The main processes through which MCPH-linked genes regulate brain growth involve those associated with neural progenitor cells (NPCs) divisions. During neocortical development, these cells, particularly concentrated at ventricular zone (VZ) and subventricular zone (SVZ) of the developing telencephalon, undergo both symmetric and asymmetric divisions, which maintain the stem cell pool and generate newborn neurons that, through radial migration, originate the cortical plate.[6] Accurate positioning of centrosomes and spindles is essential for proper NPCs division and cortical development, since vertical cleavage planes perpendicular to the VZ and SVZ usually result in symmetric divisions, while horizontal cleavages lead to asymmetric divisions.[6] Most of the known pathogenic variants responsible for autosomal recessive MCPH are in genes encoding centrosomal proteins or proteins required for spindle formation and function, such as *ASPM*, *CEP152*, *CEP235*, *CENPJ* and *CDK5RAP2*. In addition, disruption of chromatin dynamics and condensation, caused by pathogenic variants in the *PCH1* and *MCPH1* genes, has also been implicated in the pathogenesis of autosomal recessive MCPH.[7]

Patients with autosomal dominant MCPH[4] usually show moderate-to-mild ID or no cognitive impairment. The term 'silent microcephaly' has been proposed to refer to this entity without any neurological or dysmorphic manifestations.[4] Recently, linkage studies and exome sequencing identified a pathogenic variant in the *ALFY* gene, which encodes an autophagy scaffold protein, segregating along with microcephaly through three generations of a family.[8] Functional studies using animal models showed that the mutated protein

Anexo 3: co – autoria de artigo publicado na revista Stem Cell Research & Therapy

Goulart *et al. Stem Cell Research & Therapy* (2019) 10:258
https://doi.org/10.1186/s13287-019-1367-x

Stem Cell Research & Therapy

RESEARCH Open Access

Adult and iPS-derived non-parenchymal cells regulate liver organoid development through differential modulation of Wnt and TGF-β

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Abstract

Background: Liver organoid technology holds great promises to be used in large-scale population-based drug screening and in future regenerative medicine strategies. Recently, some studies reported robust protocols for generating isogenic liver organoids using liver parenchymal and non-parenchymal cells derived from induced pluripotent stem cells (iPS) or using isogenic adult primary non-parenchymal cells. However, the use of whole iPS-derived cells could represent great challenges for a translational perspective.

Methods: Here, we evaluated the influence of isogenic versus heterogenic non-parenchymal cells, using iPS-derived or adult primary cell lines, in the liver organoid development. We tested four groups comprised of all different combinations of non-parenchymal cells for the liver functionality in vitro. Gene expression and protein secretion of important hepatic function markers were evaluated. Additionally, liver development-associated signaling pathways were tested. Finally, organoid label-free proteomic analysis and non-parenchymal cell secretome were performed in all groups at day 12.

Results: We show that liver organoids generated using primary mesenchymal stromal cells and iPS-derived endothelial cells expressed and produced significantly more albumin and showed increased expression of CYP1A1, CYP1A2, and TDO2 while presented reduced TGF-β and Wnt signaling activity. Proteomics analysis revealed that major shifts in protein expression induced by this specific combination of non-parenchymal cells are related to integrin profile and TGF-β/Wnt signaling activity.

Conclusion: Aiming the translation of this technology bench-to-bedside, this work highlights the role of important developmental pathways that are modulated by non-parenchymal cells enhancing the liver organoid maturation.

Keywords: Organoid, Liver, iPS, Hepatocyte, 3D culture

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Anexo 4: co – autoria de artigo publicado na revista Biofabrication

3D bioprinting of liver spheroids derived from human induced pluripotent stem cells sustain liver function and viability *in vitro*.

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Anexo 5: co – autoria de artigo publicado na revista European J. of Medical Genetics

ARTICLE IN PRESS

European Journal of Medical Genetics xxx (xxxx) xxxx

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: www.elsevier.com/locate/ejmg

DNA methylation fingerprint of monozygotic twins and their singleton sibling with intellectual disability carrying a novel *KDM5C* mutation

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ARTICLE INFO

Keywords:
KDM5C
DNA methylation
Intellectual disability
XLID

ABSTRACT

Mutations in *KDM5C* (*lysine (K)-specific demethylase 5C*) were causally associated with up to 3% of X-linked intellectual disability (ID) in males. By exome and Sanger sequencing, a novel frameshift *KDM5C* variant, predicted to eliminate the JmjC catalytic domain from the protein, was identified in two monozygotic twins and their older brother, which was inherited from their clinically normal mother, who had completely skewed X-inactivation. DNA methylation (DNAm) data were evaluated using the Illumina 450 K Methylation Beadchip arrays. Comparison of methylation levels between the three patients and male controls identified 399 differentially methylated CpG sites, which were enriched among those CpG sites modulated during brain development. Most of them were hypomethylated (72%), and located mainly in shores, whereas the hypermethylated CpGs were more represented in open sea regions. The DNAm changes did not differ between the monozygotic twins nor between them and their older sibling, all presenting a global hypomethylation, similar to other studies that associated DNA methylation changes to different *KDM5C* mutations. The 38 differentially methylated regions (DMRs) were enriched for H3K4me3 marks identified in developing brains. The remarkable similarity between the methylation changes in the monozygotic twins and their older brother is indicative that these epigenetic changes were mostly driven by the *KDM5C* mutation.

1. Introduction

At least 21 *KDM5C* (*Lysine Demethylase 5C*) pathogenic mutations were identified in individuals with XLID, representing around 3% of the XLID cases (Abidi et al., 2008; Jensen et al., 2005; Rujirabanjerd et al., 2010; Santos-Rebouças et al., 2011). There is substantial clinical heterogeneity, however, short stature, microcephaly, hyperreflexia and aggressive behavior are usually reported in males (Gonçalves et al., 2014; Jensen et al., 2005), whereas female carriers rarely exhibit mild ID or learning difficulties (Rujirabanjerd et al., 2010). KDM5C is an epigenetic regulator that removes di- and trimethyl groups from lysine 4 of Histone 3 (H3K4me2/3) (Brookes et al., 2015; Tahiliani et al., 2007). In neurons, KDM5C is recruited to CpG islands at gene promoters targeted by H3K4me3 (Iwase et al., 2016). All *KDM5C* mutations were associated with reduced enzymatic activity, suggesting that the pathophysiological mechanism is a consequence of the protein loss

Abbreviations: DMRs, Differentially methylated regions; DNAm, DNA methylation; GEO, Gene expression Omnibus; H3K4, lysine 4 in histone H3; HM450K, Human Methylation 450 BeadChip microarrays; ID, Intellectual disability; MDS, Multidimensional scaling; MSET, Modular Single-set Enrichment test; OFC, Occipitofrontal circumference; P1, Monozygous twin 1; P2, Monozygous twin 2; P3, Older brother; shelves, regions defined as the 2 kb outside of a shores; shores, regions with lower CpG density that lie within the 2 kb up- and down-stream of a CpG island; TSS1500, region from transcript start site to 1500 nt upstream of transcript start site; XLID, X-linked intellectual disability

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https://doi.org/10.1016/j.ejmg.2019.103737
Received 29 October 2018; Received in revised form 25 June 2019; Accepted 11 August 2019

Please cite this article as: João V.S. Guerra, et al., European Journal of Medical Genetics, https://doi.org/10.1016/j.ejmg.2019.103737