Melinda Santos Beccari

A VAPB e a Esclerose Lateral Amiotrófica

VAPB and Amyotrophic Lateral Sclerosis

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Dedicatória

Aos meus queridos pais, meus irmãos, e meu noivo David.

Epígrafe

"When life gets tough—and it surely will, because if you are not failing a few times, that means you are not pushing yourself hard enough—what you need is an unshakable commitment to persevere. Perseverance is patience with a purpose, when you willingly and proactively take your turn because you know why you should."

— Dan Clark

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Resumo

A Esclerose Lateral Amiotrófica (ELA) é uma doença crônica, progressiva e neurodegenerativa causada pela morte dos neurônios motores. O diagnóstico destes pacientes pode levar até 12 meses para acontecer, sendo que estes vão à óbito entre 3-5 anos do início dos sintomas. Há, porém, grande variabilidade de quadro clínico, com alguns pacientes falecendo com menos de 1 ano do início dos primeiros sinais, e outros que sobrevivem por décadas. A identificação da ELA8, causada por uma mutação *missense* no gene VAPB (c.C166T, p.P56S), tem contribuído significativamente com o conhecimento dos mecanismos moleculares por trás da ELA. A literatura recente tem evidenciado que a diminuição dos níveis de VAPB está presente em modelos celulares e murinos da doença, e também em amostras de pacientes, sugerindo que esta proteína teria papel central na doença e uma contribuição significativa para a morte dos neurônios motores. O presente trabalho buscou três objetivos principais: (1) o diagnóstico molecular através de um painel de sequenciamento de nova geração que inclui os genes *SOD1, FUS, TARDBP, SETX, SPG11, FIG4* e *VAPB*; (2) a avaliação dos níveis de RNAm de *VAPA, VAPB* e *EPHA4* em pacientes de ELA8, controles familiares e outros pacientes de ELA, com o intuito de investigar possíveis papéis destes genes na doença; e por f'm, (3) o desenvolvimento de um ensaio quantitativo para as proteínas VAPA, VAP' e VAPC baseado em cromatografia líquida acoplada à espectrometria de massas em tandem (LC-MS/MS), para a posterior avaliação de VAPB como possível biomarcador em ELA, e de suas isoformas VAPA e VAPC como modificadores da doença. Para a análise genômica, foram avaliados 67 pacientes, sendo que 31 (ou 46%) apresentaram a mutação c.C166T em *VAPB*; 4 pacientes (6%) em *SOD1*, sendo que um destes apresentou uma mutação também em *FIG4*; 1 paciente (1.5%) foi identificado uma mutação patogênica em *FUS*; outro, duas mutações deletérias em *trans* em *SPG11.* Os níveis de RNAm de *VAPB, VAPA* e *EPHA4* não são estatisticamente distintos entre pacientes e controles; porém, os níveis de *EPHA4* estavam significativamente elevados em dois pacientes de início bulbar da doença. Para o desenvolvimento do método quantitativo por LC-MS/MS, foram escolhidos 8 peptídeos inequívocos para análise, estabeledos dos parâmetros de corrida, e desenvolvidos dois padrões internos (linhagens SILAC e VAPB recombinante) para a quantificação. Esta ferramenta desenvolvida poderá auxiliar não apenas os estudos moleculares que envolvem os mecanismos por trás ELA8, responsável por uma elevada taxa dos casos familiais brasileiros, mas também poderá determinar o potencial de VAPB como biomarcador para Esclerose Lateral Amiotrófica.

Abstract

Amyotrophic Lateral Sclerosis is a chronic, progressive neurodegenerative disorder caused by the death of motor neurons. Diagnosis can take up to 12 months, with no molecular marker to expedite this process. In this scenario, patients die within 3 to 5 years of symptom onset, although a large clinical variability is seen, with severe patients dying less than one year after onset, and others surviving for decades. The identification of ALS8, caused by a missense mutation in the VAPB gene (c.C166T; p.P56S), has contributed significantly to the knowledge of molecular mechanisms behind ALS. Recent literature has evidenced that the decrease of VAPB levels is present in cellular and murine models, and al'o in patient samples, suggesting a central role in motor neuron death in ALS. The present work sought three main objectives: (1) a molecular diagnosis through a NGS sequencing panel including the *SOD1, FUS, TARDBP, SETX, SPG11, FIG4* e *VAPB* genes; (2) analyze the expression levels of *VAPA, VAPB* and *EPHA4* in patients, family controls and other forms of ALS, in order to investigate t'eir possible roles in ALS8; and (3) the development of a targeted quantitative mass spectrometry based assay, gold standard in protein quantification due to its precision and sensitivity, for the VAPA, VAPB and VAPC proteins, seeking the analysis of VAPB as a potential biomarker in ALS and of its isoform's potential roles as modifiers in the disease. The genomic analyses revealed that out of 67 patients, 31 presented the ALS8 mutation in *VAPB*, 4 patients (6%) presented a mutation in *SOD1*, with one patient carrying a second mutation in *FIG4*; 1 (1.5%) patient was identified with a pathogenic mutation in *FUS*; and another presented two pathogenic mutations in trans in the *SPG11* gene. Thus, we were able to diagnose over half of the patients included in this study with a panel of only 7 genes. *VAPB, VAPA* and *EPHA4* mRNA levels are not statistically different between patients and controls; however, *EPHA4* was shown to be highly elevated in two bulbar-onset non-ALS8 patients. For the development of the LC-MS/MS targeted assay, 8 surrogate peptides were chosen for analysis, run parameters were established, and two internal standards for quantification were developed (SILAC cell lines and recombinant VAPB). This tool will prove to be useful not only towards elucidating the molecular mechanisms behind ALS8, one of the most prevalent forms of familial ALS in Brazil, but also to determine VAPB's potential as a biomarker for ALS.

Capítulo 1: Introdução - A Esclerose Lateral Amiotrófica e as Proteínas VAP.

1.1. Esclerose Lateral Amiotrófica: Aspectos clínicos, epidemiológicos e genéticos.

A Esclerose Lateral Amiotrófica, conhecida também como ELA ou ALS, do inglês *Amyotrophic Lateral Sclerosis*, é uma doença neurodegenerativa causada pela morte de neurônios motores superiores do córtex motor e inferiores do tronco cerebral e da medula espinhal. A perda destes neurônios leva à atrofia e fraqueza muscular, fasciculações e espasticidade. Classicamente, os primeiros sintomas surgem por volta dos 60 anos de idade, e os pacientes vão à óbito, em média, de 2 a 5 anos após o início dos mesmos. Porém, observa-se grande variabilidade clínica entre pacientes, evidenciada por tempos de progressão bastante distintos (5-10% dos pacientes apresentem sobrevida maior do que 10 anos), envolvimento de diferentes tipos de neurônios motores, idade variável de início dos sintomas (evidenciado por casos juvenis e de início bastante tardio), e a ocorrência ou não de mais casos na família (Swinnen & Robeberecht 2014). A incidência mundial da ELA está estimada em 1.90 casos por 100.000 indivíduos, e uma prevalência de 4.48/100.000 (Chiò *et al.*, 2013); no Brasil, os últimos dados populacionais foram obtidos em 1998, estimando uma incidência de aproximadamente 1.5 casos / 100.000 habitantes (ABrELA).

As formas principais de apresentação clínica são: (1) pacientes com início espinhal da doença - que correspondem a aproximadamente 70% dos casos - cujos sintomas iniciais se manifestam nos membros superiores e/ou inferiores; (2) pacientes de início bulbar (aproximadamente 25% dos casos), apresentando sintomas iniciais como disfagia, disartria, dispneia, e posteriormente sintomas nos membros; e (3) pacientes com envolvimento

respiratório como primeiro sintoma, correspondendo a apenas 5% dos casos (Kiernan *et al.*, 2011). O diagnóstico destes pacientes é apenas clínico e para tanto são usados os critérios definidos no El Escorial (Brooks *et al.*, 2000), com apoio da eletroneuromiografia.

Os pacientes de ELA são geralmente classificados em casos esporádicos, ou SALS (*Sporadic ALS*), que corresponderiam a aproximadamente 90% dos casos, ou em casos familiais, com mais de um membro da família afetado (FALS - *Familial ALS*), constituindo os demais 10%. Os casos familiais têm contribuído significativamente para a descoberta de novos genes associados à doença, levando à elucidação de diversos mecanismos moleculares responsáveis pela morte dos neurônios motores. Recentemente, as novas plataformas de sequenciamento têm contribuído também para a identificação de genes em casos esporádicos.

Apesar do número crescente de estudos e descobertas na área, são identificadas causas genéticas em apenas dois terços dos casos familiais e aproximadamente 10% dos casos esporádicos. Para os 33,3% restantes dos casos familiais ainda não foi encontrado um gene responsável; para os casos esporádicos tem-se como hipótese uma ação conjunta entre fatores genéticos associados e ambientais de risco, a própria variação no envelhecimento, ou estilos distintos de vida (Marangi *et al.*, 2014). Um estudo de gêmeos mostrou que a herdabilidade em ELA é de aproximadamente 60%, evidenciando a necessidade de se descobrir outros fatores de risco que possam contribuir para a doença (Al-Chalabi *et al.*, 2010).

Além de casos de origem monogênica, foram descobertos também pacientes que apresentavam mutações em em dois ou mais genes associados à ELA, evidenciando a possibilidade de uma etiologia oligogênica para a doença (van Blitterswijk *et al.*, 2012; Cady *et*

al., 2015). Casos familiais com indícios de penetrância incompleta e uma parcela dos casos esporádicos poderiam ser causados pela ocorrência de 2 ou mais variantes genéticas com efeitos aditivos ou sinergísticos deletérios.

Há, atualmente, grande interesse também em descobrir possíveis fatores modificadores de fenótipo. Membros de uma mesma família, com a mesma mutação causadora, apresentam grande variabilidade de idade início e tempo de progressão da doença.

Atualmente, 58 genes foram de alguma forma associados à ELA (dados revisados em Marangi *et al.*, 2014). Destes 58, 32 são considerados genes "principais", ou causadores; 19 são considerados como genes de susceptibilidade, e 7 como modificadores de fenótipo. As vias principais de atuação dos genes causadores genes são bastante distintas, incluindo: processamento de RNA (como *TARDBP*, *FUS*, *TAF15*, *TBK1*, *C9ORF72* entre muitos outros); stress oxidativo (*SOD1*); tráfego de endossomos e sinalização celular (*VAPB*, *FIG4*, *OPTN*); vias de degradação protéica (*UBQLN2*, *VCP*, *SQSTM1*); e até mesmo remodelamento de cromatina (como *CREST*).

O primeiro gene associado à Esclerose Lateral Amiotrófica foi encontrado em 1993: *Cu^{2+}/Zn Superoxide Dismutase*, ou *SOD1*, é responsável por aproximadamente 20% dos casos familiais e 2-7% dos esporádicos (Rosen *et al.*, 1993). Segundo os dados encontrados no banco ALSoD (http://alsod.iop.kcl.ac.uk/) (Abel *et al.* 2012), um repositório de mutações encontradas em pacientes de ELA, já foram descritas 182 mutações distintas neste gene como responsáveis pela doença. A proteína SOD1 é responsável pela eliminação de diversas espécies reativas de oxigênio (ROS) intracelulares, que são formados pelas reações metabólicas normais da célula (como fosforilação oxidativa). Quando mutada, há um

desequilíbrio entre a criação e remoção de ROS, gerando um estresse oxidativo celular, que por fim acarreta na morte dos neurônios motores (dados revisados em Ferraiuolo *et al.*, 2011). Por ter sido o primeiro gene descoberto e por ser uma frequente causa da doença, *SOD1* foi um dos genes mais estudados em ELA. O modelo murino mais usado para ensaios pré-clínicos é o camundongo transgênico que carrega a mutação humana G93A em *SOD1*.

Durante muito tempo, acreditava-se que mutações em *SOD1* eram a principal causa da Esclerose Lateral Amiotrófica. Porém, foi descoberto recentemente que uma mutação no gene *C9ORF72* - mais especificamente, uma expansão intrônica de hexanucleotídeos GGGGCC (G4C2) no gene seria responsável por aproximadamente 40% dos casos familiais, e 10% dos casos esporádicos (De Jesus-Hernandez *et al.*, 2011; Rohrer *et al.*, 2015). Esta expansão trouxe uma forte evidência de ligação entre Demência Frontotemporal (FTD) e ELA: diversos estudos sugerem que ELA e FTD são, na verdade, dois extremos de um espectro. A classificação de FTD é feita em análises pós-mortem de tecidos, que apresentam inclusões muito semelhantes às encontradas em neurônios motores de pacientes de ELA. Além disso, 15% dos pacientes de ELA apresentam alguma forma de demência frontotemporal, caracterizada por alterações comportamentais e de fala (Robberecht & Williams, 2013).

O número normal de repetições G4C2 é bastante variável, mas 90% da população européia possui entre 2 e 10 repetições. Pacientes de ELA, FTD ou FTD-ELA possuem de centenas a milhares de repetições. Não existem evidências concretas para o fenômeno de antecipação clínica, e nem uma correlação entre o tamanho da repetição do hexanucleotídeo e a gravidade do quadro clínico. Quanto ao mecanismo de atuação desta mutação, os dados parecem bastante controversos. Existem evidências para ganho tóxico de função, através da formação de agregados intracelulares de RNA, que podem sequestrar proteínas essenciais, e

também pela formação de proteínas DPR (proteínas de repetição de dipeptídeos), que também formam inclusões intracelulares, quanto para perda de função, através da perda da proteína codificada por *C9ORF72* e uma diminuição significativa dos 3 transcritos do gene (Rohrer *et al.*, 2015).

Genes envolvidos com mecanismos de processamento de RNA possuem também papel fundamental em ELA. O primeiro deles a ser descrito foi *TARDBP*, que codifica a proteína TDP-43 é responsável por aproximadamente 4% dos casos familiais e 1% de casos esporádicos (Chiò *et al.*, 2012). *TARDBP* tornou-se um gene candidato em ELA devido à presença de TDP-43 nas inclusões citoplasmáticas de um número significativo de pacientes; investigações mostraram, posteriormente, a presença de mutações neste gene em casos familiais e esporádicos (Sreedharan *et al.*, 2008). A maior parte das mutações neste gene são *missense*, e encontram-se no exon 6, que codifica um domínio C-terminal rico em glicina (Mackenzie et al., 2010).

Logo após a descoberta do *TARDBP* como gene causador, foram identificadas mutações no gene *FUS* (*Fused in Sarcoma*) (Vance *et al*. 2009, Kwiatkowski *et al.*, 2009), cuja proteína compartilha forte homologia com TDP-43. Como em *TARDBP*, a maior parte das mutações afeta o domínio C-terminal, que contém o sítio de ligação para moléculas de RNA. Assim como o *TARDBP*, mutações em *FUS* correspondem a aproximadamente 4% dos casos familiais de ELA e 1% dos casos esporádicos (Mackenzie *et al.*, 2010).

Em 2004, nosso grupo identificou uma mutação no gene *VAPB* (c.C166T, p.P56S) que era responsável por uma forma familial de ELA no Brasil (denominada de ELA8), o que trouxe um elevado interesse no estudo de mecanismos por trás das funções das proteínas VAP. A ELA8 apresenta herança autossômica dominante, sendo caracterizada por uma progressão

lenta, fasciculações, câimbras e tremores posturais (Nishimura *et al.*, 2004). Desde que foi descrita pela primeira vez, mais de 127 pacientes foram diagnosticados com ELA8 no Centro de Pesquisas Sobre o Genoma Humano e Células Tronco, correspondendo a aproximadamente 48% dos casos lá atendidos. Outros pacientes de ELA8 (com a alteração P56S) foram encontrados em países distintos - mais especificamente, no Japão (Millecamps *et al.*, 2010) e na Alemanha (Funke *et al.*, 2010) - que possuem haplótipos distintos das famílias brasileiras, sugerindo origens distintas da mutação. No Reino Unido, foi encontrado um paciente com outra alteração em VAPB, T46I (Chen *et al.*, 2010), a qual também seria patogênica (Chen *et al.,* 2010; Lua *et al.*, 2011).

Estudos funcionais iniciais utilizando modelos de superexpressão evidenciaram o acúmulo de VAPB mutada no retículo endoplasmático (RE), e estas inclusões ubiquitinadas sequestrariam também VAPA e VAPB selvagens, sugerindo um mecanismo dominante negativo para a doença (Teuling *et al.*, 2007). Inclusões citoplasmáticas são marcas típicas de doenças neurodegenerativas. Porém, modelos murinos de superexpressão de VAPB selvagem e mutada não apresentam fenótipo algum de degeneração dos neurônios motores, apesar de serem encontradas tais inclusões (Qiu *et al.*, 2013). Em contrapartida, foi visto que a expressão de VAPB está reduzida na medula espinhal de pacientes esporádicos e em modelos murinos mutantes para *SOD1*, indicando que a diminuição nos níveis desta proteína seria um mecanismo comum associado à degeneração dos neurônios motores (Teuling *et al.*, 2007). Estes dados sugerem que o disparo da doença pode estar na redução, e não no aumento dos níveis de VAPB (Tudor *et al.*, 2010).

1.2. As Proteínas VAP

As VAPs, do inglês "*Vesicle-Associated Membrane Associated Proteins*" são proteínas de membrana tipo II ancoradas no retículo endoplasmático (RE). Existem três proteínas VAP conhecidas: a VAPA (do inglês, *VAMP Associated Protein A*), com 242 aminoácidos e 28kDa, codificada por gene de mesmo nome localizado no cromossomo 18; VAPB e VAPC, codificadas a partir do *splicing* alternativo do gene *VAPB*, localizado no cromossomo 20. VAPA e VAPB possuem três domínios altamente conservados: um domínio globular N-terminal que compartilha 22% de identidade em sua sequência com uma proteína denominada "*major sperm protein*", presente em *C. elegans* (chamado, então, de domínio MSP); um domínio *coiled-coil*; e um domínio transmembrana C-terminal (TMD), que permite a homo ou heterodimerização das proteínas VAP (Lev *et al.*, 2008). VAPA e VAPB apresentam 63% de

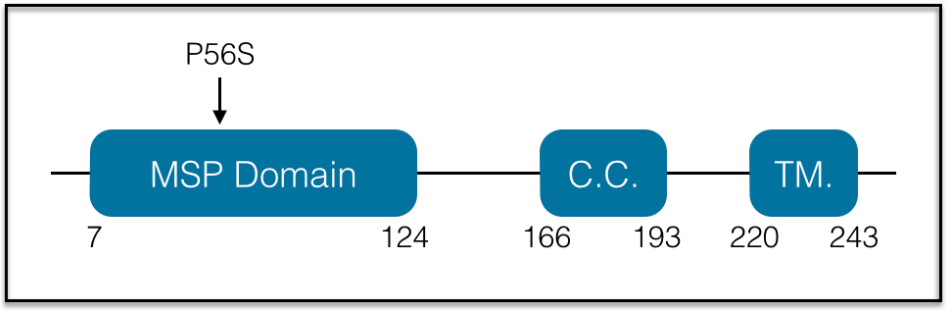

Figura 1.1. Estrutura da proteína VAPB. Estão representados os domínios Major Sperm Protein (MSP), no qual se localiza a mutação característica de ELA8 (P56S), o domínio coiled-coil, e o domínio transmembrana. A proteína VAPA possui os mesmos três domínios principais.

identidade entre si, sendo que a identidade para os domínios MSP é de 85%. A estrutura de VAPB está representada na Figura 1.1. As VAPs foram descritas como participntes do tráfego intracelular de membranas, do processo de UPR (do inglês, *Unfolded Protein Response*), da organização de microtúbulos, mas suas funções mais conhecidas são o transporte de vesículas e metabolismo de lipídeos (Lev *et al.*, 2008; Teuling *et al.*, 2007). Outras funções de VAPB têm sido descritas na literatura, incluindo seu envolvimento no controle de qualidade de

proteínas pelo retículo endoplasmático, chamado de *ER Quality Control* ou ERQC (Moustaqim-Barrette *et al.*, 2013), localização mitocondrial (Han *et al.*, 2012), homeostase de Cálcio (Morotz *et al.*, 2012; De Vos *et al.*, 2012), e proliferação tumoral em câncer de mama (Rao *et al.*, 2012). Estas funções foram descritas para VAPB devido ao grande interesse na mesma por sua participação conhecida em ELA. Entretanto a alta similaridade entre VAPA e VAPB e sua heterodimerização sugere a possibilidade da atuação da VAPA também nestes mecanismos. A sobreposição de funções entre as duas proteínas ainda é desconhecida.

O trabalho recente de Tsuda e colaboradores (2008) evidencia a capacidade do domínio MSP de atuar como molécula sinalizadora: este domínio é clivado, secretado, e atua como ligante de receptores de efrina (EphR). Em drosófila, viu-se que o domínio MSP é capaz de modular a atividade destes receptores, o que resultaria em um mecanismo não-autônomo de ativação da morte do neurônio motor. Essa atividade sofre interferência da mutação P58S em drosófilas (equivalente à P56S em humanos), pois leva à formação de inclusões citoplasmáticas, impedindo a secreção do MSP. Ainda, outro achado importante deste trabalho foi a identificação de fragmentos de VAPB no soro humano, através da técnica de *Western Blot* (Tsuda *et al.*, 2008).

Um dos possíveis mecanismos de atuação do domínio MSP incluiria agir como molécula competidora das efrinas. Quando a ligação EphR-efrina é impedida por um antagonista (como o domínio MSP), não ocorre a ativação das vias *downstream* deste receptor. Sabe-se que receptores de Eph têm papel fundamental na formação sináptica e sinalização por glutamato através do agrupamento de receptores NMDA (N-metil-D-Aspartato). Três conjuntos de evidência sugerem que o MSP pode regular este processo: 1) Receptores Eph se associam diretamente com receptores de glutamato NMDA e regulam o

agrupamento destes receptores (essencial para sua ativação) em neurônios em cultura (Dalva *et al.*, 2000); 2) A perda de função de dVAP em drosófila ou a superexpressão de P58S neste modelo estão associadas a um aumento do agrupamento destes receptores de glutamato e aumento das amplitudes de mEJPs (potenciais excitatórios) nas junções neuromusculares (Tsuda *et al.*, 2008); 3) MSP e o receptor de Eph VAB-1 em *C. elegans* regulam a função do receptor NMDA durante a maturação de oócitos neste modelo (Corrigan *et al.*, 2005).

Dando suporte a estes estudos, Van Hoecke e colaboradores (2012) mostraram que um tipo específico de receptor Eph, o *EPHA4*, atua como importante modificador da Esclerose Lateral Amiotrófica. Os níveis de expressão deste receptor modulam o fenótipo em modelos murinos e de zebrafish, onde os neurônios motores mais vulneráveis apresentavam maiores níveis de *EPHA4*. Em humanos, a expressão mais elevada está ligada a um pior prognóstico, e mutações de perda de função neste gene estão associadas com maior sobrevida (Van Hoecke *et al.*, 2012). Ou seja, quanto maior a atuação do *EPHA4*, pior é o prognóstico do paciente.

Diversos trabalhos dão embasamento à hipótese de que a redução de VAPB presente em pacientes esporádicos e em diferentes modelos de ELA pode ter um papel fundamental no disparo da doença. Inicialmente, o trabalho de Teuling e colaboradores mostrou que os níveis de VAPB estão reduzidos em pacientes com ELA esporádica e no sistema nervoso central de camundongos transgênicos portadores da mutação SOD1G93A, principalmente nos estágios mais avançados da doença (Teuling *et al.*, 2007). Anagnostou e colaboradores (2008) analisaram a expressão de 11 genes associados à ELA através da técnica de PCR em tempo real e observaram que os transcritos correspondentes à *VAPB* estavam reduzidos significativamente na medula espinhal de pacientes com ELA esporádica (22% em relação

aos controles), sendo que esta diminuição é mais marcante em pacientes de rápida progressão da doença (Anagnostou *et al.*, 2008). O trabalho de Mitne-Neto e colaboradores (2011) em neurônios motores derivados de células-tronco pluripotentes induzidas (iPSC), geradas a partir de fibroblastos de pacientes e controles familiares, mostrou que (1) há a diminuição de VAPB nos extratos celulares provenientes de pacientes de ELA8, e (2) há uma falha no aumento de expressão de VAPB ao longo do processo de diferenciação das iPSCs em neurônios motores. Acredita-se, portanto, que os pacientes de ELA8 apresentariam quantidades reduzidas de VAPB ao longo de seu desenvolvimento, e estes níveis se tornam críticos para a sobrevivência dos neurônios motores a partir da 4ª ou 5ª décadas de vida, idade de início dos sintomas (Mitne-Neto *et al.*, 2011). Mais recentemente, Deidda e colaboradores (2013) avaliaram os níveis de VAPB em pacientes esporádicos, analisando leucócitos de sangue periférico e líquido cefalorraquidiano. Através de *Western Blots*, foi constatado que em pacientes de ELA de início bulbar a banda correspondente ao MSP estava significativamente reduzida (ou ausente) no líquido cefalorraquidiano destes pacientes. Não foi detectada uma alteração clara nos níveis de VAPB em pacientes de início espinal da doença; porém, sabe-se que existem limitações para a técnica utilizada neste trabalho (Deidda *et al.*, 2013). *Western Blots* são capazes apenas de detectar mudanças drásticas nos níveis protéicos, sendo que manos proeminentes, porém significativas, também podem alterar as funções de vias metabólicas. A diminuição encontrada em pacientes de início bulbar pode estar associada à rápida progressão da doença observada nestes pacientes. Dessa forma, faz-se necessário uma avaliação mais robusta e precisa dos níveis de VAPB.

1.3. A Proteômica e a Cromatografia Líquida Associada à Espectrometria de Massas *em tandem* (LC-MS/MS)

A habilidade de identificar a sequência de aminoácidos e determinar a estrutura de proteínas é tema central nas ciências biológicas. As proteínas são as moléculas “efetoras" das funções celulares, e a compreensão de sua estrutura e função permite um maior entendimento de redes biológicas complexas (Domon *et al.*, 2006). O termo “Proteômica”, cunhado nos anos 90 pela junção de “Proteína" com “Genômica”, designa a área da bioquímica cujos esforços estão direcionados para identificar e quantificar todas as proteínas de um proteoma, analisando expressão, localização celular, interações, e modificações pós-traducionais (PTMs) como funções de tempo, espaço e tipo celular. Estas investigações são mais complexas do que sequenciar um genoma. Existem aproximadamente 100.000 formas protéicas possíveis codificadas pelos aproximados 20.235 genes humanos (Zhang *et al.*, 2013).

O progresso desta área acompanhou o desenvolvimento de novas tecnologias para separação e análise de proteínas e peptídeos, como a cromatografia líquida associada à espectrometria de massas em tandem (LC-MS/MS). A cromatografia líquida a alta pressão (ou HPLC, do inglês *High-Pressure Liquid Chromatography*) é a técnica utilizada para a separação de proteínas ou peptídeos em uma amostra biológica complexa, com base em suas propriedades físico-químicas. Um espectrômetro de massas permite que sejam medidas a razão massa-carga (m/z) de moléculas, além da determinação de propriedades estruturais de peptídeos como a sequência de aminoácidos ou o sítio de ligação e o tipo de modificação pós-traducional presente naquela molécula. Uma das vertentes mais usadas na proteômica é a abordagem *bottom-up*, onde as proteínas são digeridas em peptídeos (geralmente com

tripsina), separadas cromatograficamente e analisadas por espectrometria de massas. Para a determinação da sequência e/ou estrutura de um peptídeo é empregada a espectrometria de massas *em tandem:* após a determinação inicial da razão massa-carga, íons específicos são selecionados e submetidos a um processo de fragmentação por um gás de colisão. A análise destes perfis de fragmentação permite, então, o detalhamento estrutural dos peptídeos em questão (Domon *et al.*, 2006).

As estratégias baseadas em LC-MS/MS podem ser divididas em duas principais categorias: estratégias não-direcionadas, ou *shotgun*, e estratégias direcionadas, ou *targeted*. A primeira baseia-se na análise de peptídeos presentes em uma amostra gerados a partir de digestão enzimática. Após a análise inicial, são automaticamente selecionados tipicamente os 10-15 peptídeos mais abundantes daquela aquisição e estes são submetidos ao processo de fragmentação por colisão. Os espectros gerados são analisados para obtenção das sequências de cada peptídeo. Este processo repete-se ao longo de toda a separação cromatográfica. Porém, a complexidade de amostras biológicas e o grande intervalo dinâmico das proteínas limitam a reprodutibilidade e sensibilidade desta abordagem estocástica, algo crítico quando o alvo é quantificação. As estratégias *shotgun* são utilizadas então, para análises qualitativas, onde se busca compreender quais proteínas estão presentes em cada amostra. Análises quantitativas são também possíveis, mas com um grau de precisão mais baixo quando comparado às estratégias direcionadas (Gallien *et al.*, 2012).

As abordagens direcionadas consistem na definição de uma lista de proteínas a serem analisadas. Este processo engloba a construção de um método de LC-MS/MS voltado à análise apenas daquele conjunto. Para isso, são determinados peptídeos inequívocos para cada proteína (*surrogate peptides*, ou peptídeos cuja sequência remete apenas à proteína em

questão), e a detectabilidade dos mesmos é analisada após um processo de proteólise. Outros parâmetros importantes são seu comportamento cromatográfico, e a otimização do processo de fragmentação de cada peptídeo para análise. O tempo de aquisição do espectrômetro é voltado para o registro de cada alvo durante seu tempo cromatográfico de eluição, elevando significativamente a precisão e exatidão das quantificações (Lesur *et al.*, 2015). Este tipo de análise requer instrumentos de alto desempenho, que permitem a mensuração de um alto intervalo dinâmico de proteínas, com alta sensibilidade para detectar peptídeos em baixíssimas concentrações e seletividade suficiente para lidar com um ruído bioquímico significativo (Gallien *et al.*, 2012).

A espectrometria de massas tem sido também utilizada para a descoberta (com estratégias *shotgun*) e avaliação (através de estratégias *targeted*) de moléculas diagnósticas em diversas matrizes. No caso da Esclerose Lateral Amiotrófica, diversos marcadores têm sido descritos, mas evidências definitivas não foram obtidas por falta de significância estatística de muitos estudos (dados revisados em Tarasiuk *et al.*, 2012). A importância de se encontrar um biomarcador para esta doença reside no fato de que o diagnóstico atualmente é apenas clínico, e demora em torno de 9-12 meses para acontecer. Para pacientes que possuem uma sobrevida média de 2-5 anos, este tempo é precioso para terapias paliativas e até mesmo para *clinical trials*. As buscas atuais por terapias envolvem fatores (compostos ou tipos celulares) capazes de reduzir a progressão da doença, através da manutenção dos neurônios motores ali presentes. Dessa forma, quanto mais precoce o diagnóstico, maior a chance de sucesso destas terapias. O biomarcador ideal para ELA seria sensível e específico para um diagnóstico precoce, até mesmo antes do início dos sintomas clínicos (onde mais da metade dos neurônios motores foram perdidos) e seria alterado ao longo da progressão da

doença, indicando estágios mais ou menos avançados, ou uma progressão mais rápida ou mais lenta (Tarasiuk *et al.*, 2012). Idealmente, este marcador seria também de fácil acesso e avaliação robusta.

1.4. Hipótese, Objetivos e Justificativa

Dados recentes da literatura sugerem que a VAPB tem um papel sinalizador importante, e mostram que a mesma estaria reduzida não só em pacientes de ELA8, mas também em outras formas da doença. A VAPB se tornou, portanto, um importante alvo de estudos para a elucidação do mecanismo por trás da morte dos neurônios motores. A carência de um biomarcador e a demora no diagnóstico da ELA, aliados às questões acima citadas, justificam o desenvolvimento de uma metodologia de quantificação de VAPB para que, em seguida, seja possível avaliar seu potencial como marcador molecular para Esclerose Lateral Amiotrófica.

A hipótese deste trabalho é a de que a redução de VAPB levaria a uma menor disponibilidade do MSP como sinalizador; a consequência disto seria uma excitotoxicidade gerada pela estimulação dos neurônios motores por glutamato através de receptores NMDA, que por sua vez estão agrupados e em pleno funcionamento através da sinalização via efrinas-EphR.

Sendo assim, os objetivos principais deste projeto englobaram três áreas - Genômica, Transcriptômica e Proteômica - com o objetivo de analisar VAPB no contexto de diferentes formas de Esclerose Lateral Amiotrófica. Cada área será abordada em um capítulo distinto, e cada capítulo agrupará os principais resultados obtidos. Maiores informações metodológicas (protocolos) estão detalhadas como material suplementar (II, III e IV).

Para cada área, os objetivos traçados estão descritos a seguir.

1) Genômica:

- Triagem de mutação em genes envolvidos em ELA para determinação de um diagnóstico molecular. Os genes analisados - *SOD1, FUS, TARDBP, SETX, SPG11, FIG4* e *VAPB* - estão disponíveis num painel de sequenciamento de nova geração na plataforma MiSeq (Illumina). A mutação P56S em *VAPB* também foi analisada por outras duas técnicas - *High Resolution Melting* e/ou digestão enzimática por HaeIII. Este diagnóstico permitirá o posterior agrupamento desses pacientes nas etapas seguintes de experimentação e análise, e permitirá o aconselhamento genético das famílias incluídas no estudo.

2) Transcriptômica:

- Análise da expressão de *VAPA*, *VAPB* e *EPHA4* em amostras de sangue periférico de pacientes de ELA8, controles familiares e pacientes com outras formas de ELA através da técnica de PCR em tempo real, para avaliação da possível influência da expressão destes três genes no quadro de ELA, principalmente em ELA8.

3) Proteômica:

- Desenvolvimento ferramentas para quantificação das proteínas VAPA, VAPB e VAPC baseadas em estratégias de proteômica direcionada, através de cromatografia líquida acoplada à espectrometria de massas em *tandem*, para análise de amostras biológicas obtidas de pacientes de ELA (familiais e esporádicos). Esta ferramenta permitirá avaliar se VAPB pode ser usada como biomarcador para Esclerose Lateral Amiotrófica, e se as outras proteínas VAP possuem alguma influência no fenótipo dos pacientes.

Conseguimos encontrar um número substancial de pacientes com a mutação c.C166T em *VAPB*, e também pacientes com mutações patogênicas em *SOD1, FUS,* e *SPG11.* Todas estas alterações foram encontradas em casos familiais de ELA. Outras alterações de significância desconhecida também foram encontradas, mas necessitam de maiores investigações. Os níveis de RNAm de *VAPA, VAPB* e *EPHA4*, a princípio, não são diferentes entre pacientes; porém, os dois pacientes de nível bulbar analisados possuíram um aumento substancial de *EPHA4*, mostrando a necessidade de maiores investigações deste receptor nesta forma específica de ELA. Conseguimos também estabelecer um método de quantificação de VAPA, VAPB e VAPC por cromatografia liquida acoplada à espectrometria de massas em tandem, e padrões internos para normalização da quantificação protéica.

Chapter 2: Mutation screening in Brazilian ALS patients - an analysis of 7 genes in familial and sporadic cases.

Abstract

Amyotrophic Lateral Sclerosis is the most common form of motor neuron disease. Around 10% of cases are classified as familial (FALS), with more than one affected family member, while the remaining 90% are classified as sporadic (SALS). In order to better understand the genetic causes of ALS in patients of Southeast Brazil, a screening of 7 genes - *SOD1, FUS, TARDBP, SETX, SPG11, FIG4* and *VAPB* - was carried out in a cohort of 67 patients (49 familial, 11 sporadic and 7 of unknown family history) and 11 unaffected family members. Of these, 31 (46%) presented the c.C166T (p.P56S) mutation in the *VAPB* gene, being thus diagnosed with ALS8 [1]; 4 patients (6%) carried mutations in *SOD1* (c.G50C; c.T116G; and c.T380C in two patients), with one of them also carrying a seemingly damaging mutation in *FIG4* (c.G2440T); 1 patient presented a previously known *FUS* mutation (c.C1558T) (1.5%); and 1 patient presented two mutations in trans in the *SPG11* gene (c.6477+4A>G and c.6365_6387del) (1.5%). Mutations of unknown clinical significance in *SETX* and *SPG11* were also found. A next-generation sequencing panel for the MiSeq (Illumina) platform was used as the screening method. None of the sporadic patients had a causal mutation discovered by the present panel. Considering solely the familial individuals, we were able to close up over half of our ALS cases. The main reason for the larger proportion of familial cases in this cohort is due to patient referral to our center for genetic investigation. The high rate of P56S mutation in the *VAPB* gene shows the spreading of this mutation in Brazil and highlights the importance of our findings describing this mutation 11 years ago. Additionally, previous haplotype studies with distinct ALS8 families suggested that P56S has a founder effect and that patients are clustered in Southeast Brazil. Although the present work does not reach all ALS related genes, we were able to give a diagnosis to 33% (6/18) of the non-ALS8 familial patients. The unique genetic diversity found in Brazil, generated by its mixed colonization features the importance of studies focusing the search for new genes in this population.

Introduction

Amyotrophic Lateral Sclerosis in a neurodegenerative disorder caused by the death of upper motor neurons from the motor cortex and lower motor neurons from the brain stem and spinal cord. About 10% of cases are familial, in which more than one family member is affected, and the other 90% are classified as sporadic. Despite the ever growing number of studies and discoveries in the field, genetic causes are found in only 66.6% of familial cases and approximately 10% of sporadic cases (Marangi et al, 2014). Environmental risk factors are unknown. Additionally, patients carrying more than one mutation in ALS-causing genes have been described (van Blitterswijk et al., 2012; Cady et al., 2015), suggesting the possibility of a complex oligogenic etiology for ALS as well. To date, around 58 genes have been described for ALS: 32 as disease-causing or main genes; 19 are considered susceptibility genes; and 7 are considered disease modifiers (Marangi et al., 2014).

The Human Genome and Stem Cell Research Center (CPGH-CEL), up until 2015, has received about 262 ALS patients, with 127 of them carrying the P56S mutation in the *VAPB* gene, which is responsible for ALS type 8. The ALS8 was described in 2004 as an atypical slow-progressive form of ALS (although many distinct clinical outcomes were observed in patients with this same *VAPB* mutation) (Nishimura et al., 2004), and a haplotype analysis suggested the possibility of a founder effect for these Brazilian patients (Nishimura et al., 2005). Investigations on family history carried out by the patients themselves has given more evidence for the founder effect hypothesis by showing that the patients are, in some degree, all part of one family tree (unpublished data). These families are located throughout southeast Brazil, mainly in the state of Minas Gerais (Figure 2.1-A). Other ALS8 patients have been described throughout the world (Millecamps et al., 2010; Funke et al., 2010, Chen et al., 2010),

but these show distinct haplotypes (and a new mutation, T46I) when compared to the Brazilian patients. These data suggest a distinct origin for worldwide ALS8 cases throughout the world, but a particular founder for the ALS8 patients from the Brazilian population.

The remaining 135 patients seen in the CPGH-CEL (who are not carriers for the P56S mutation) are mostly from southeast Brazil as well, with the largest number of them located in the state of São Paulo (Figure 2.1-B). Out of these 135 patients, clinical data was found for 82, showing that 45 (54.9%) are familial cases, and 37 (45.1%) are sporadic. The elevated number of familial cases is most likely due to their being referred to our center for genetic investigation.

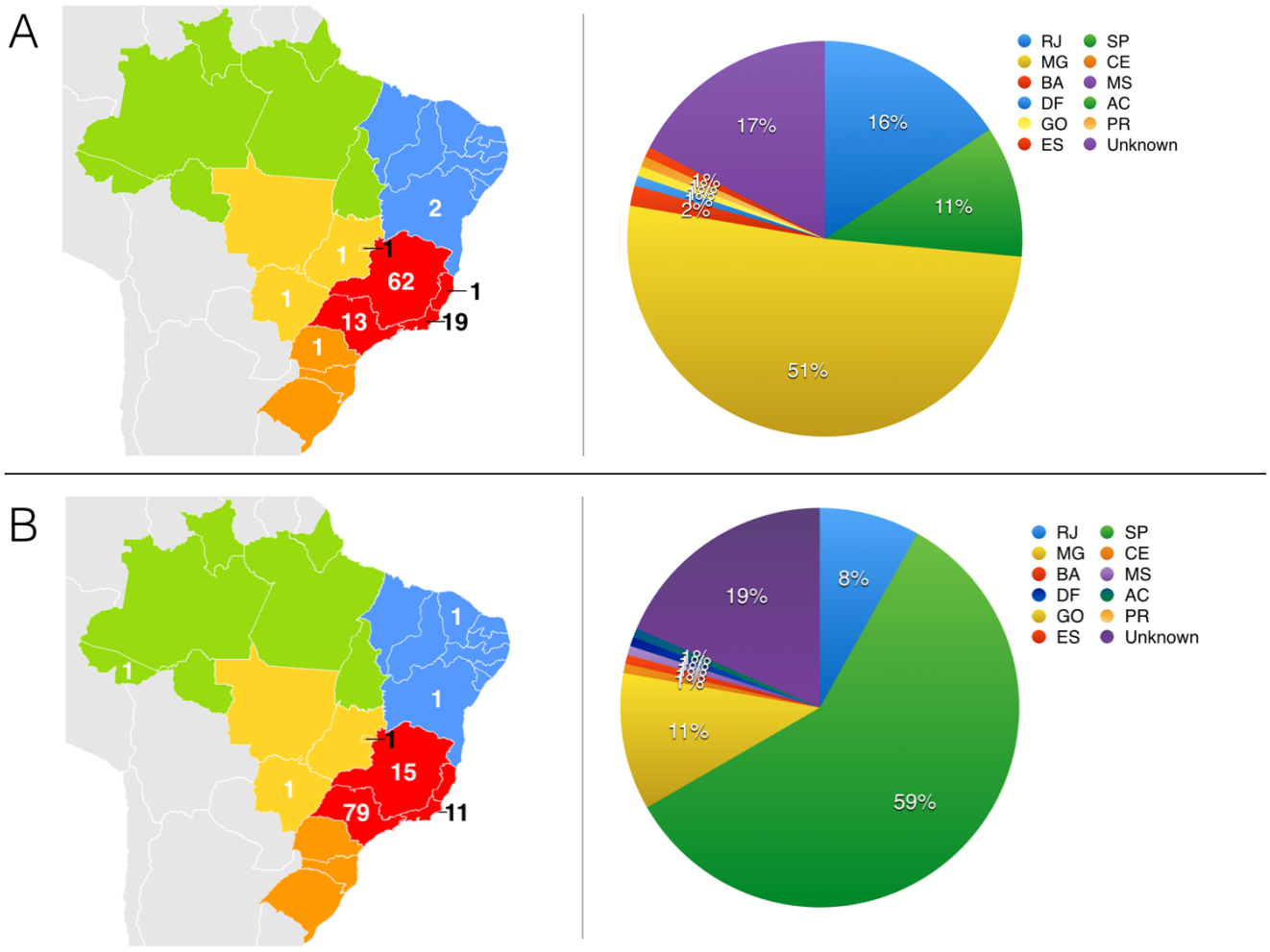

Figure 2.1: A) Geographical distribution of ALS8 patients throughout Brazil. Data shows residence location. B) Geographical distribution of other ALS cases throughout Brazil. Data shows residence location.

We aimed to investigate possible genetic causes for molecularly undiagnosed ALS cases in our population, using DNA samples from 78 patients and family controls which were screened for mutations in the following genes: *SOD1, FUS, TARDBP, SETX, SPG11, FIG4* and *VAPB*. The initial screening was made for the P56S mutation in *VAPB*; if the sample was negative for this mutation, they were submitted to a NGS panel (using the MiSeq - Illumina platform) available in the CPGH-CEL which includes all aforementioned genes. Out of 67 patients, 31 (46.3%) carried the P56S mutation (being therefore diagnosed with ALS8), 4 patients presented mutations in *SOD1*, 1 patient (1.5%) presented a mutation in the *FUS* gene, and 1 patient presented two mutations in trans in the *SPG11* gene. These mutations were found in patients with a known family history. Mutations were also found in the *SETX* and *SPG11* genes, but their clinical significance remains unknown.

Results

Screening for the P56S mutation in *VAPB*

DNA samples were initially analyzed for the P56S mutation in the *VAPB* gene using one of two methods: enzymatic digestion with HaeIII followed by agarose gel separation; or genotyping through High Resolution Melting (HRM) in the LightCycler480 (Roche) platform. The mutation c.C166T alters the HaeIII restriction enzyme's site, so a simple analysis using PCR, digestion and electrophoresis is accurate and cost-effective for diagnosis. An alternative method for genotyping, named HRM, relies on the melting temperature of PCR amplicons, which are determined mostly by size, GC-content and sequence. A point mutation is enough to alter this temperature, allowing for a time-effective diagnosis using real-time qPCR platforms. Since 40-50% of cases referred to our center are carriers of the ALS8 mutation, an initial

screening allows for a quick diagnosis and avoids an unnecessary expensive and time-consuming NGS analysis. Out of the 67 patients analyzed, 31 were carriers of the c.C166T (P56S) mutation, being diagnosed with ALS8.

Screening of *SOD1, FUS, TARDBP, SETX, SPG11,* and *FIG4* genes

A NGS panel including 7 ALS genes (*SOD1, FUS, TARDBP, SETX, SPG11, FIG4* and *VAPB*), available at the CEGH-CEL, was used for screening of those patients (and family controls) which were non-carriers of the c.C166T mutation. The NGS platform used was MiSeq-Illumina. A summary of the mutations found can be seen in Table 2.1.

A total of 36 patients were analyzed; 18 known familial cases, 11 sporadic patients, and 7 of unknown family history. Four patients (familial cases) carried mutations in the ***SOD1*** gene, (a total of three mutations was found: 1) **p.G17A** (c.G50C) in exon 1; 2) **p.L39R** (c.T116G) in exon 2; and 3) **p.L127S** (c.T380C) in exon 5. All three mutations were described as pathogenic in the Human Genome Mutation Database (HGMD - http://www.hgmd.cf.ac.uk/ac/index.php), all causative for ALS. Interestingly, of the two patients (first degree cousins) who carried the **p.L127S** (c.T380C) *SOD1* mutation, one also carried a missense mutation in ***FIG4*** (c.G2440T - **p.D814Y**). No entry for this mutation was found in HGMD; however, *in silico* analyses by PolyPhen2 and Mutation Taster predicted a high probability of pathogenicity in this mutation. Further investigations should be carried out to better understand the clinical significance of this variant.

A mutation in ***FUS*** - **p.R520C** (c.C1558T), exon 15 - was found in another familial ALS patient. It is described as pathogenic for ALS in the HGMD database, and falls within a known hotspot for pathogenic mutations in the gene (exons 13-15). Mutations in FUS are mostly

missions changes that cluster in two regions: exon 13-15, which encode for a Arg-Gly-Gly-rich region and the nuclear localization signal of FUS, and exons 3, 5 and 6, which encode for the Gln-Gly-Ser-Thr-rich and Gl-rich regions (Mackenzie et al., 2010).

The ***SPG11*** gene, coding for the spatacsin protein, is described as causative for hereditary spastic paraplegia type 11, a motor neuron disease affecting lower limbs caused by the degeneration of corticospinal axons. This gene has also been described as causative for an autosomal recessive form of ALS - meaning either homozygous or compound heterozygous mutations must be found in order to attain a diseased phenotype. In one particular female familial ALS patient with an atypical early onset (31y) and slow progression, two mutations in trans were found: a 23bp deletion in exon 34 (c.6365_6387del / **p.A2122fs**) leading to a frameshift; and a intronic mutation, c.6477+4 A>G, described in the HGMD database as leading to a splicing defect. The fact that these mutations were found in trans suggests that there is an absence of normal spatacsin, which could lead to the clinical phenotype observed in this patient. However, this patient's sister, who presents a rapidly-progressive bulbar-onset phenotype, only carries one mutation in the *SPG11* gene - the splicing variant; and out of all 7 genes tested, none presented any significant alterations. Although unlikely, these two sisters might present either different motor neuron diseases (suggested by their very distinct clinical phenotypes), or present different mutations in other genes involved in autosomal recessive ALS.

Mutations of unknown clinical significance were also found in our patient cohort. A sporadic case presented a mutation in the ***SETX*** gene (p.K801E / c.A2401G); no descriptions regarding pathogenicity were found, and *in silico* analyses were discordant - according to PolyPhen2, this mutation is possibly damaging with a score of 0.952 (the highest being 1), but

according to the Mutation Taster algorithm, it is only a polymorphism. As reported by the ExAC (Exome Aggregation Consortium) database, which contains exome data from 60706 unrelated individuals, the frequency for this mutation is of 0.00061. Further investigations are necessary to confirm the pathogenicity of this mutation. One particular patient (of unknown family history) presented a heterozygous deletion in the ***SPG11*** gene (c.529_533del / **p.177_178del**), with a known HGMD entry, which leads to a frameshift. As previously stated, mutations in this gene are usually found in homozygosity or with another mutation (compound heterozygote), so the clinical significance of this particular alteration is unknown.

Gene	DNA	Protein	Exon/Intron	HGMD Entry	Effect
SOD1	c.G50C	p.G17A	exon 1	CM983777	Pathogenic
SOD1	c.T116G	p.L39R	exon 2	CM983778	Pathogenic
SOD1	c.T380C	p.L127S	exon 5	CM014099	Pathogenic
FIG4	c.G2440T	p.D814Y	exon 21	-	Probably damaging
FUS	c.C1558T	p.R520C	exon 15	CM091090	Pathogenic
SPG11	c.6477+4 A>G	-	intron 33-34	CS080744	Pathogenic
SPG11	c.6365_6387del	p.A2122fs	exon 34	-	Damaging
SPG11	c.529_533del	p.177_178del	exon 3	CD071362	Damaging
SETX	c.A2401G	p.K801E	exon 10	-	?

Table 2.1: A summary of all mutations found in the present study. All mutations were found in familial cases. Gene name is followed by gene alteration, protein changes, gene location and HGMD entry. Known mutations in ALS patients are described as "Pathogenic"; mutations classified as damaging by in silico analyses are termed "damaging" or " probably damaging".

Discussion and Perspectives

Brazil is known for being a large, culturally diverse country. There are significant regional differences, mainly due to historical reasons such as distinct colonizations, which generated an admixture of populations unique to their geographical location. As a consequence, the genetic diversity is also considerable. Our study focused on patients from

southeast Brazil, comprising patients from the states of São Paulo, Minas Gerais, and Rio de Janeiro. The elevated rate of ALS8 found here highlights the importance of our laboratory's findings describing this mutation 11 years ago (Nishimura et al., 2004), and also the necessity of searching for new ALS-associated genes in this population, which might help uncover novel and important mechanisms associated to the disease.

Despite this substantial amount of genes, we were able to diagnose 63% of our familial cases evaluating only 7 of them. In this cohort, it is important to highlight that 33% are of non-ALS8 familial cases. Mutations considered rare in ALS might be more common in this population, tracing an interesting unique profile.

Despite the interesting findings, the results found in this study cannot be considered representative of the Brazilian population as a whole. The ideal study would involve analyzing samples from all over the country, and would involve a larger number of genes analyzed. There are, however, many barriers to such a study, such as a small number of specialized centers with physicians capable of diagnosing ALS, which are mostly located in the southeast and south of Brazil. Additionally, the rapid disease progression prevents some patients from reaching any of the available centers. Obtaining a representative cohort for this kind of study is indeed a challenge, however, a feasible one which could increase the understanding of ALS as a whole.

Chapter 3: Analysis of *VAPA*, *VAPB* and *EPHA4* expression in Amyotrophic Lateral Sclerosis 8.

Abstract

Amyotrophic Lateral Sclerosis is a neurodegenerative disease caused by the death of motor neurons. Patients show very distinct clinical outcomes, disease progression and site of onset, which led to the study of many potential disease modifiers. Amongst these is the ephrin receptor EPHA4, which is shown to have an important role in patient prognosis. ALS type 8 is an autosomal-dominant form of ALS characterized by a slow progression, fasciculations and postural tremors, caused by the c.C166T (p.P56S) mutation in the *VAPB* gene. VAPB protein is known to be downregulated in many distinct cellular and mouse models of ALS, and also on patient samples. *VAPB* expression has been shown to be downregulated in the spinal cord of sporadic patients, suggesting that the level of VAPB might be an important player in this disease. VAPB protein's N-terminal domain, MSP, interacts with the EPHA4 receptor, suggesting this receptor has a potential role in disease progression. VAPA is VAPB's paralog protein, and shares high identity with VAPB, suggesting it might also have a role in ALS8 in order to compensate wild-type VAPB reduction. This work sought to analyze the expression levels of *VAPA, VAPB* and *EPHA4* in ALS8 patients, family controls, and ALS patients without VAPB mutations, in order to better understand the implications of their levels in disease pathogenesis. Here we show that in peripheral blood leukocytes, no significant change is observed in *VAPA, VAPB* and *EPHA4* expression in the analyzed groups. However, two bulbar-onset patients presented an elevated *EPHA4* expression. Interestingly, recent works presented an absence of MSP protein in sporadic ALS patients, particularly in those with a bulbar disease onset; considering the previously reported relationship between MSP and EPH, ligand and receptor, these results suggest the necessity of further investigation of the correlation between *VAPB, EPHA4* and bulbar-onset ALS.

Introduction

Amyotrophic Lateral Sclerosis is a lethal degenerative disease of the human motor system. In 2004, the c.C166T mutation in the *VAPB* gene (p.P56S) was described as being responsible for an autosomal dominant form of ALS, called ALS8, which is characterized by atypical slow disease progression, fasciculations, tremors, and a complete penetrance (Nishimura et al., 2004). Despite carrying the same mutation, ALS8 patients present diverse clinical phenotypes, such as typical ALS, atypical slow-progression ALS, and spinal muscular atrophy (SMA).

The VAPB protein N-terminal domain, MSP, which is cleaved and secreted, is known to act as a ligand for Eph receptors (particularly, with *EPHA4*), being thus an ephrin competitor (Tsuda et al., 2008).The ephrin-EphR connection allows the clustering of NMDA receptors, which are then activated by glutamate; excessive glutamante activation is known to be toxic for motor neurons. When the eprhin-EphR connection is interrupted, the downstream activation is also turned off.

EPHA4, one of 9 types of EPH-A receptors, is described as a potent disease modifier in animal models and humans, (Van Hoecke et al. 2012). According to this work, the most vulnerable motor neurons had the highest expression of *EPHA4*, and this expression also correlated with shortened survival. It is known that the VAPB protein is downregulated in ALS8 iPSC-derived motor neurons (Mitne-Neto et al., 2011), and also in many distinct models of ALS and sporadic patients (Teuling et al., 2007, Anagnostou et al., 2008, Deidda et al., 2013). Taking all these data into consideration, the downregulation of VAPB expression (resulting in a reduction of available MSP) could lead to an overactivation of *EPHA4*, and consequently contribute to motor neuron degradation by a glutamate excitotoxicity mechanism. This

hypothesis suggests a potential role of the *EPHA4* gene expression in the clinical outcome of ALS patients.

The VAPA protein, an important paralog of VAPB, shares the same structure and domains as VAPB, with the two presenting a 67% identity (85% identity of the MSP domain). The VAP proteins are known for their important roles in lipid metabolism, ER morphology, membrane trafficking, among others; however, no exclusive function for VAPA or VAPB has been described. Its similarity to VAPB suggests that VAPA could have an important role as protective factor in ALS8, covering for at least part of the functions lost with the reduction of VAPB levels due to the P56S mutation.

The present work sought to identify potential roles of *VAPB*, *VAPA* and *EPHA4* expression in Amyotrophic Lateral Sclerosis 8, and compare them to other forms of ALS. For such, we performed TaqMan assays using a probes for these three genes (and *GAPDH* as an endogenous control) on cDNA generated from peripheral blood leukocyte RNA (details can be found in supplemental section III). Patients included in this study were previously diagnosed with ALS8 (slow-progressive forms, with some patients presenting over 15 years since disease onset), bulbar-onset ALS or limb-onset ALS. *VAPA* and *VAPB* mRNA levels showed no significant changes between groups, despite some variation at the individual level. *EPHA4* expression is elevated in two patients with bulbar-onset ALS.

Results

VAPA, VAPB and *EPHA4* expression are not significantly different among ALS patients and controls - but is *EPHA4* in bulbar-onset ALS an exception?

For all analyses, samples were divided as follows: Controls (samples were obtained from unaffected relatives), Pre-Symptomatic ALS8 patients (carriers of the p.P56S mutation in VAPB without clinical symptoms), Symptomatic ALS8 patients, and “Other ALS” (a small cohort of samples from ALS patients who do not carry the p.P56S mutation). ALS8 mutation carriers were thus divided in order to possibly identify any changes occurring in expression after disease onset.

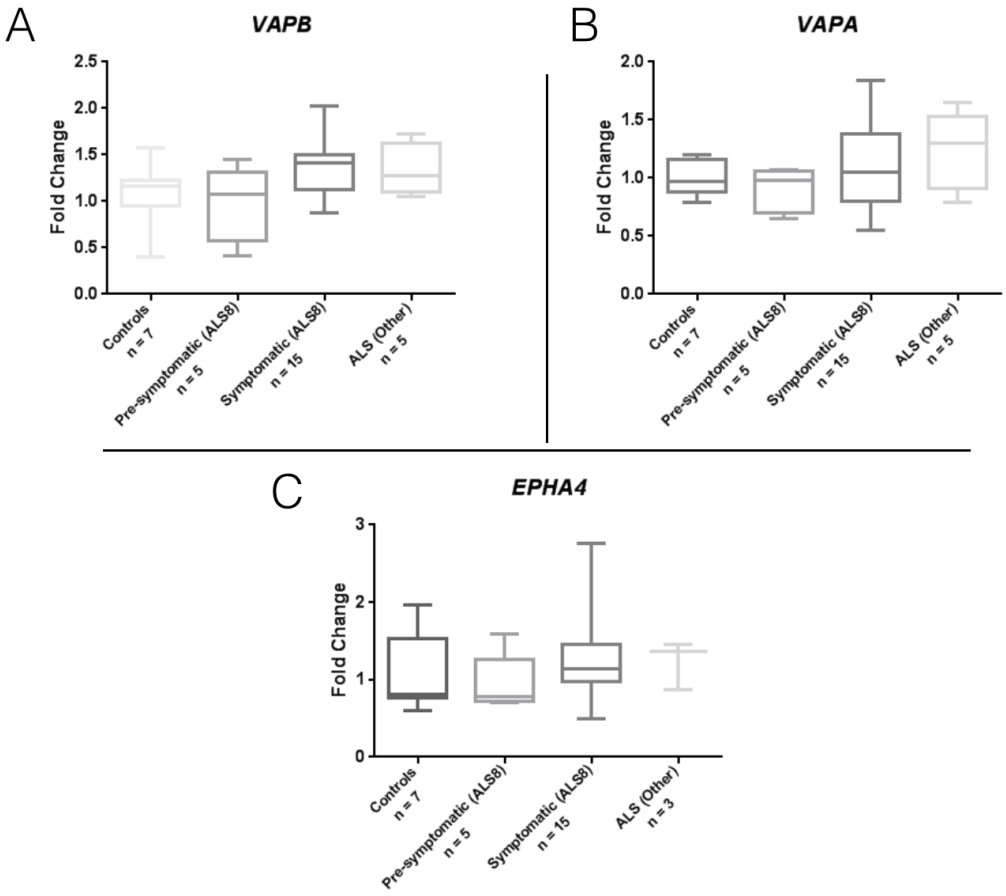

Figure 3.1. *VAPB, VAPA* and *EPHA4* expression in peripheral blood leukocytes of ALS patients and family controls. A: VAPB expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.0891. B: VAPA expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.3366. C: EPHA4 expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.2560.

However, as seen in Figure 3.1, overall expression of the three analyzed genes is not statistically significant. A tendency towards a higher expression of VAPB is seen in ALS8 patients after symptom onset and in the group “other ALS”, but a larger number of pre-symptomatic patients should be analyzed in order to confirm these results.

The two bulbar-onset patients were not included in the *EPHA4* analysis because their fold change values were outliers when compared to the others. They were analyzed separately, as seen in Figure 3.2.

We also sought to identify possible changes in gene expression comparing two extremes: patients with very slow disease progression (as evaluated by the attending neurologist) versus those with a known fast progression, in order to verify *VAPA, VAPB* and *EPHA4* expression as potential disease modifiers (Figure 3.3).

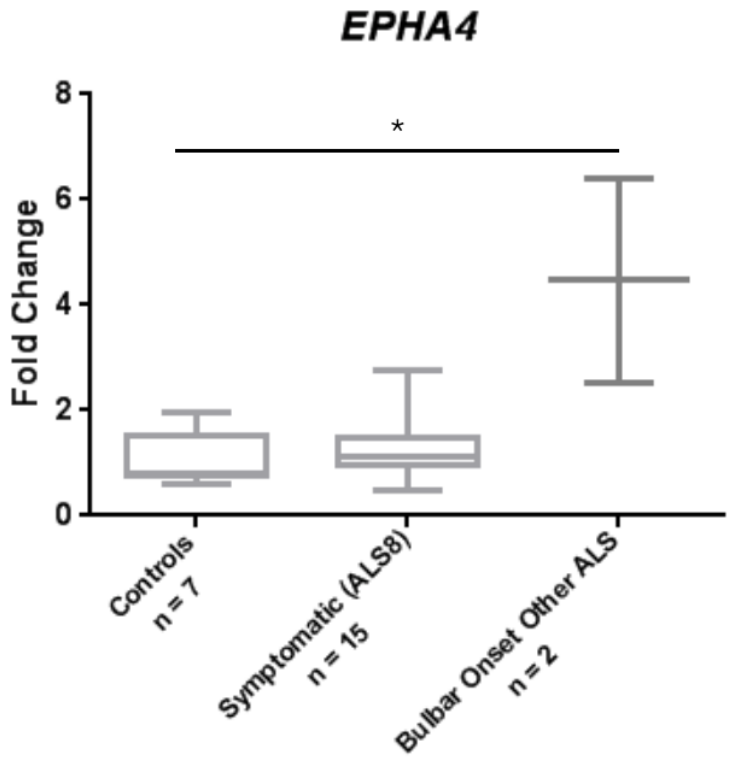

Figure 3.2. *EPHA4* expression in peripheral blood leukocytes of AL8 symptomatic patients, bulbar-onset ALS patients, and family controls. Kruskal-Wallis p value = 0.0333.

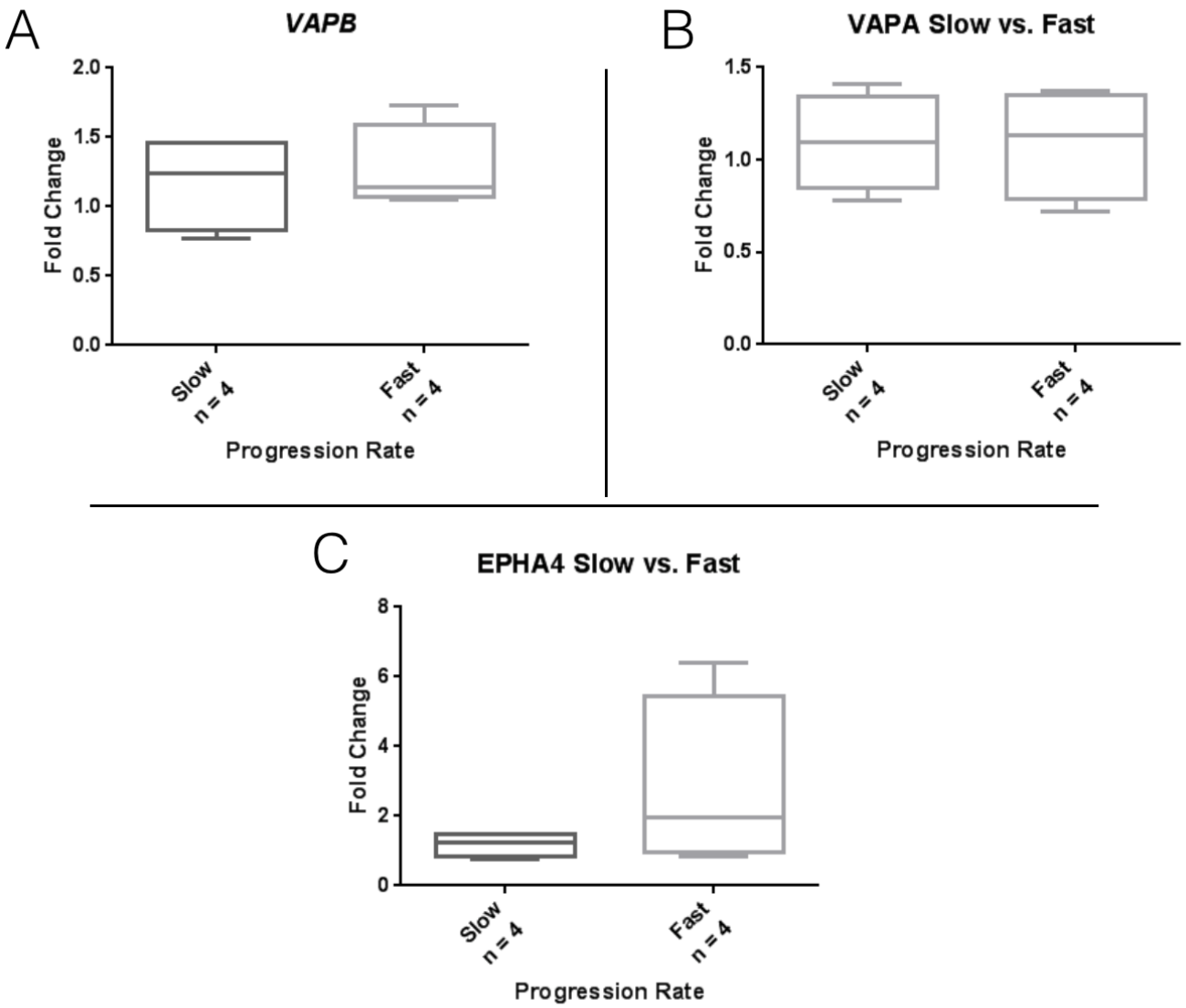

Figure 3.3. *VAPB, VAPA* and *EPHA4* expression, comparison between slow-progressive and fast-progressive ALS. **A:** VAPB expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.6286. **B:** VAPA expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.8286. **C:** EPHA4 expression, shown using normalized fold change values. Kruskal-Wallis p value = 0.4571.

Discussion and Perspectives

The recent literature has shown a downregulation of VAPB on the expression level (Anagnostou et al., 2008) and on the protein level (Teuling et al., 2007, Mitne-Neto et al., 2011, Deidda et al., 2013) in distinct models and on sporadic patients, which suggests an overall downregulation in ALS. Analyzing the *VAPB* expression levels in peripheral blood leucocytes

(PBL) of ALS patients, particularly ALS8, however, has not confirmed these results. These data might be seen initially as contradictory; however, the fact that these results are derived from expression levels of distinct tissues must be taken into consideration. Our data (derived from expression in PBL) and the one published by Anagnostou et al. in 2008 (derived from cerebrospinal fluid expression) suggest that *VAPB* expression levels are very distinct among tissues, and that VAPB downregulation might be specific only to those directly affected in ALS. Supporting this evidence, the work published by Deidda et al. in 2013 shows that a fragment corresponding to the MSP domain (in Western Blots) is absent in CSF obtained from sporadic ALS patients, especially in bulbar-onset ALS, while this same change is not seen in the PBL-derived levels. Thus, the ideal results would be obtained by analyzing motor neurons and adjacent tissues directly, which is now possible with patient derived iPSC cells.

Regarding the levels of the *EPHA4* receptor, the literature describes that the higher the expression, the worse the prognosis (Van Hoecke et al., 2012). Interestingly, the two patients with the highest levels of *EPHA4* are affected by a bulbar-onset ALS, which is known to have a fast progression. Although an "n" of two does not allow us to draw any conclusions, our results suggest that the correlation between bulbar onset ALS and *EPHA4* expression should be further investigated. Interestingly, Deidda et al. (2013) showed that the reduced levels of MSP found in CSF samples of ALS patients were mostly found in those with a bulbar disease onset, which could also contribute to the aforementioned excitotoxicity motor neuron degeneration process.

Ideally, these three genes would not only be analyzed in matrices affected in ALS, but also by dividing patients into groups according to disease stage and progression, aiming their analysis as potential disease modifiers. However, these groupings and classifications are not

trivial, especially among ALS8 patients, which do not show a linear disease progression; while some patients lose walking ability after many years (over 15) of symptom onset, and have apparently stabilized, others don't. In opposition, some patients are still walking even after 30 years after the first symptoms, while others die within the same 30 years. A large cohort of extreme cases (very fast and very slow progression) could be the starting point for these analyses.

In conclusion, our negative results show the importance of tissue selection for gene expression analyses, since the affected tissue in ALS is very specific. Also, gene expression levels do not necessarily reflect on protein levels, and, in a large number of cases, the proteins are the true effector molecules (and not the mRNA). Methods allowing for a precise and sensitive method of protein quantification should bring interesting insights into the molecular pathogenesis of Amyotrophic Lateral Sclerosis. *EPHA4* receptors and MSP levels should be further investigated in bulbar-onset ALS patients, since recent data suggests that they might be indeed correlated and contributing significantly to this phenotype.

Chapter 4: An LC-MS/MS method for the evaluation of VAPB as a protein biomarker for Amyotrophic Lateral Sclerosis

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a chronic, progressive neurodegenerative disorder caused by the death of upper and lower motor neurons. Diagnosis can take up to 12 months, and there are currently no biomarkers capable of expediting this process. In this condition, patients die within 3 to 5 years of symptom onset. The identification of a new form of ALS, called ALS8, has contributed significantly to the knowledge of disease mechanisms. Recent literature has shown that a downregulation of this protein is seen in different disease models, suggesting that this reduction in VAPB levels could have significant contribution to motor neuron death and a central role in ALS development. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), emerges as a promising alternative for an accurate measurement of VAPB levels. Disease severity, rapid progression and slow diagnostic process justify the development of a targeted quantitative assay to evaluate VAPB's potential role as a biomarker in ALS. In this context, we selected surrogate peptides for the analysis of VAPA, VAPB and VAPC by LC-MS/MS, which were synthesized, purified, and validated by shotgun MS experiments using cellular extracts. These were also used for the establishment of the LC-MS/MS assay parameters. As internal standards, isotopically labeled proteomes were established using HeLa and HEK293T cells (SILAC). The first was enriched for VAPA and VAPB by plasmid transfection. Recombinant VAPB was produced and a purification protocol was also established.

Introduction

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease caused by the death of upper and lower motor neurons. It has a worldwide incidence of 1.9 per 100.000, and a prevalence of 4.48 in 100.000 (Chiò et al., 2013). Since the discovery of a mutation in *VAPB* as the cause for ALS type 8 (Nishimura et al., 2004), there has been an elevated interest in studying the roles of the VAPB protein in ALS.

Studies using distinct models have shown a potential role for VAPB in disease pathogenesis, and that its influence is in the downregulation, not overexpression, of the protein. Transgenic mice overexpressing mutant and wild-type VAPB show no phenotype (Tudor et al., 2010; Qiu et al., 2013), while knock-out mice for VAPB show a mild motor deficit (Kabashi et al., 2013). In *SOD1* G93 transgenic mice, with no mutations in VAPB, there is a significant reduction of this protein in the spinal cord (Teuling et al., 2007). Also supporting this hypothesis on the impact of VAPB reduction, Anagnostou et al. showed that the expression of *VAPB* mRNA is significantly decreased in the CSF of sporadic ALS patients (Anagnostou et al., 2008). An important study using iPSC-derived motor neurons (all carrying the P56S mutation in VAPB) showed a failure of VAPB expression increase during the differentiation process; while control cells showed an increase of VAPB expression in each stage, VAPB-P56S cells failed to do so (Mitne-Neto et al., 2011). These works suggest a potential role of VAPB downregulation in the course of ALS onset and progression. The most recent study of VAPB levels in ALS patients was published in 2013, looking at CSF and peripheral blood leukocytes through Western Blot analyses (Deidda et al., 2013). They were able to see band corresponding to the full-length 27kDa protein and a 14kDA fragment corresponding to the MSP domain, which is known to be secreted (Tsuda et al., 2008). Interestingly, the MSP-VAPB band was significantly

reduced in the CSF samples of bulbar-onset patients (Deidda et al., 2013). Western Blots are not, however, precise in terms of quantitative analyses, being capable of measuring only fold-changes in protein levels. Perhaps significant, albeit smaller, changes occur, but are undetectable by this method.

One of the most promising techniques for biomarker discovery and analysis is the LC-MS/MS, or liquid chromatography coupled to tandem mass spectrometry. It allows the sequencing of peptides and determination of protein structures and post-translation modifications, giving important insight into the biological effector molecules - the proteins. One of the most accurate and sensitive methods for protein quantification is targeted mass spectrometry, in which a group of peptides act as surrogates for their proteins, and specific parameters are established for the analysis of these peptides in complex biological matrices such as plasma, CSF, and cellular extracts (Lesur et al., 2015). This work aimed to develop a quantitative LC-MS/MS based assay for the quantification of VAPB, and of the other members of the VAP protein family, VAPA and VAPC, in order to understand their roles in Amyotrophic Lateral Sclerosis. The Q-Exactive (Thermo) mass spectrometer is ideal for these evaluations due to its initial quadrupole mass filter (increasing selectivity) and an orbitrap mass analuzer for high precision and resolution.

A total of eight peptides was selected as surrogates for the three proteins. They were synthesized, purified by HPLC and later used to refine chromatography and mass spectrometry parameters using the Skyline software (MacLean et al., 2010). Two internal standards for quantification were developed: SILAC cell lines, with entire labeled proteomes from HeLa and HEK293T cells; and a recombinant VAPB protein.

Results

The steps necessary for the development of a targeted quantitative LC-MS/MS method are summarized in Figure 4.1 and described below.

Surrogate peptide selection, synthesis and purification

The method designed in this work aims at the identification and quantification VAP proteins - VAPA, VAPB and VAPC. VAPA and VAPB share a similar structure, containing the MSP domain (which is predicted to be secreted), a coiled-coil domain and a transmembrane domain. VAPC carries a partial MSP domain. Tryptic peptides were selected from the MSP

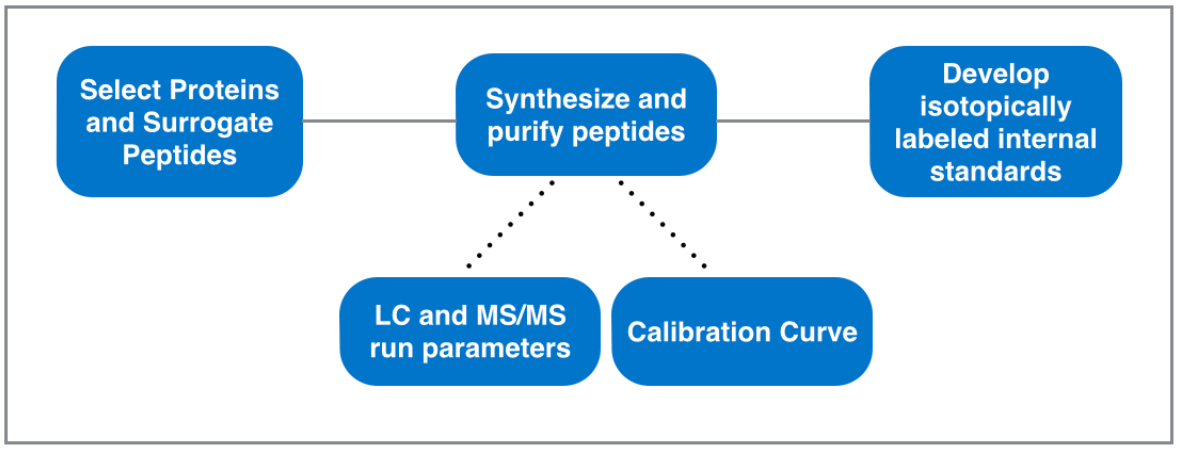

Figure 4.1: Summary of the step required for the establishment of a targeted quantitative LC-MS/MS method.

domain, which is predicted to be secreted, and from other protein regions, as illustrated in Figure 4.2. Peptide sequence is summarized in Table 4.1.

These peptides were then synthesized by GenScript, and purified using HPLC. The purification details are in Supplemental Section IV.

Surrogate peptide validation by shotgun mass spectrometry

In order to determine peptide detectability by LC-MS/MS, HeLa and HEK293T cell extracts were analyzed by shotgun mass spectrometry in the Q-Exactive (Thermo); data was

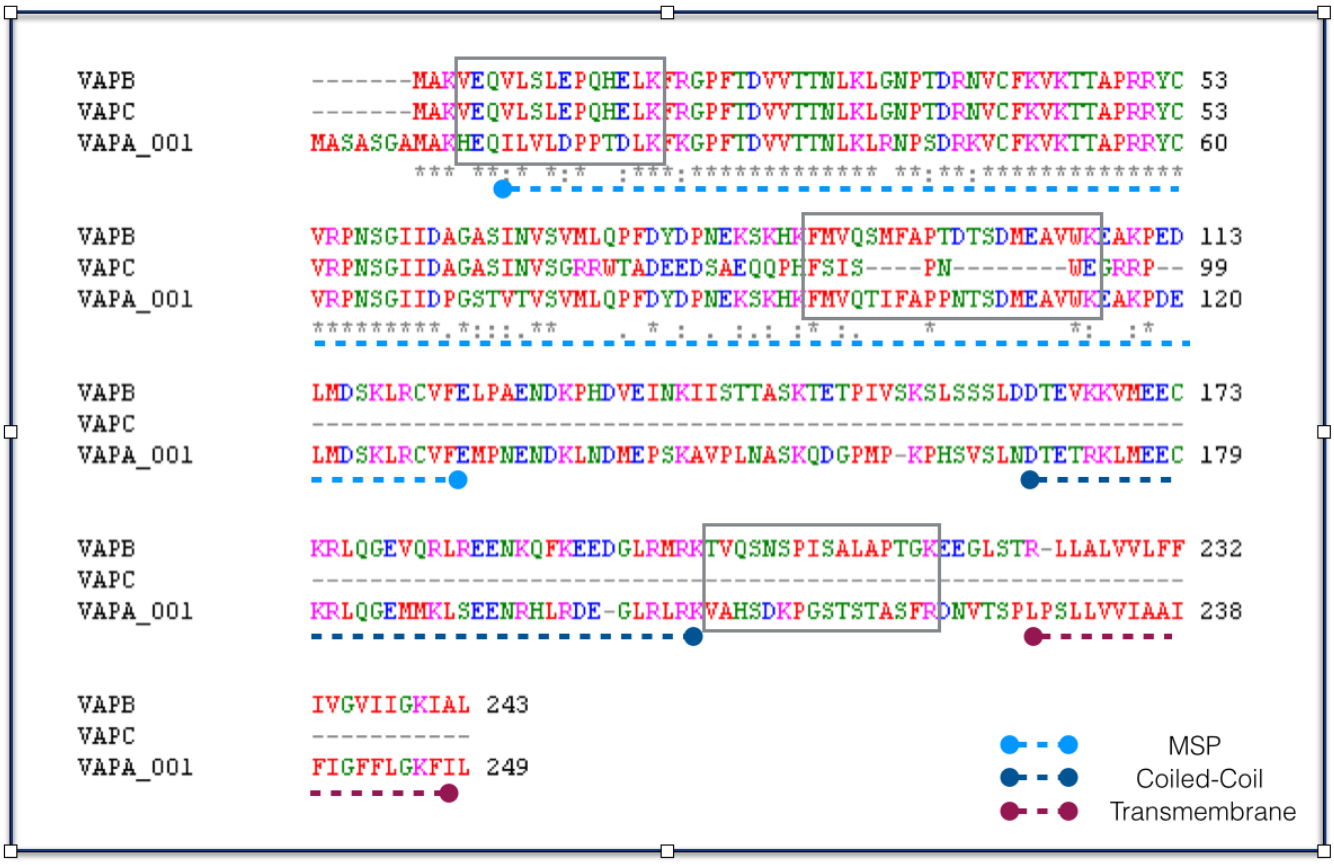

Figure 4.2. A ClustalW2 alignment of VAPA, VAPB and VAPC, illustrating a total of 8 selected peptides for VAPA, VAPB and VAPC, and the three domains for VAPA and VAPB. VAPA_001 corresponds to the isoform 1 of the VAPA protein.

Protein	Peptide Sequence
VAPA*	HEQILVLDPPTDLK
VAPA*	FMVQTIFAPPNTSDMEAVWK
VAPA*	VAHSDKPGSTSTASFR
VAPB*	VEQVLSLEPQHELK
VAPB*	FMVQSMFAPTDTSDMEAVWK
VAPB	TVQSNSPISALAPTGK
VAPC	WTADEEDSAEQQPHFSISPNWEGR
VAPC	YCVRPNSGIIDAGASINVSGR

Table 4.1: Amino acid sequence of VAPA, VAPB and VAPC surrogate peptides for targeted method development. (*) shows peptides validated by shotgun mass spectrometry.

extracted by the MaxQuant software (Cox et al., 2008). Using this method, 5 of the 8 selected peptides were identified - all three peptides from VAPA, and VEQ(…) and FMVQ(…) peptides from VAPB.

The SRM Atlas (http://www.srmatlas.org), a compendium of targeted proteomics assays to detect and quantify protein in complex proteome digests, is a publicly available database of the measurements of natural and synthetic peptides conducted on a triple-quadrupole mass

spectrometer. It is a resource for selected-reaction monitoring (SRM)-based proteomic workflows. Searches in this database revealed data for two additional peptides included in this work: TVQ(…) (VAPB) and WTA(…) (VAPC). Taking this into consideration, the MS detectability of 7 of the 8 chosen surrogate peptides were validated.

Synthesis of Isotopically-labeled internal standards: SILAC proteomes and recombinant VAPB

Internal standards (IS) are essential in quantitative experiments, and their addition in initial steps eliminates procedural errors. Ideally, IS are added to the sample in the first steps of the preparation, accounting for variation in sample processing steps - namely, protein extraction, sample purification, and trypsin digestion. In mass spectrometry, where mass shifts are detectable, isotopically labeled internal standards are ideal (Villanueva et al., 2014). ^{13}C and/or ^{15}N labeled versions of the proteins of interest can be synthesized through many methods; these are stable and naturally occurring isotopes. For the purposes of this work, SILAC HeLa and HEK293T cell lines were established (both lines showing expression of VAPA and VAPB), followed by transfection with pFlag-CMV2 plasmids containing either the coding sequence for *VAPA, wtVAPB* or *mutVAPB* for an enrichment of VAPA and VAPB in cell extracts; also, for the development of an isolated labeled VAPB, the *VAPB* gene was cloned in a pET15b vector (Novagen), inserted into BL21 codon+ E. coli cells, expressed and purified.

SILAC HeLa and SILAC HEK293T Cell Line Establishment and Transfection

A metabolic labeling strategy, termed SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture), was used for the development cells containing isotopically labeled proteins.

According to this method, two cell cultures are maintained, and while one is cultivated with unlabeled amino acids (normal culture media), the second is cultivated with ^{13}C and ^{15}N labeled amino acids. They are then mixed and analyzed by mass spectrometry. These cells are maintained in culture for at least 5 cell doublings, allowing for the labeling of up to 97% of cellular proteins (Ong and Mann, 2006).

Trypsin digestion is a standard protocol in proteomics and this specific enzyme cleaves proteins after lysines and arginines (with few exceptions). For this work, labeled lysine (K) and arginine (R) were used; therefore, all peptides generated by digestion will contain at least one labeled amino acid. They will generate 6 m/z and 10 m/z mass shifts, respectively. SILAC cell lines have two main purposes: (1) to be used as a comparative culture method, in which the light or heavy strains receive a specific treatment, and the other serves as a control group - the proteome changes are then analyzed by shotgun mass spectrometry (Ong and Mann, 2006); and (2), as an internal standard containing an entire labeled proteome, a “spike-in”, for normalization in quantitative experiments (Geiger et al., 2011).

HeLa cells were maintained in culture for at least 5 cell doublings. Proteins were then extracted and analyzed by shotgun mass spectrometry in the Q-Exactive, and data extraction was performed using MaxQuant. Out of a list of approximately 2800 proteins, 20 were randomly chosen (if present in all 6 samples analyzed) to calculate a labeled/unlabeled peptide ratio. These proteins showed a 98% proteome labeling. HEK293T cells were cultivated using the same conditions; however, after data extraction, the labeling incorporation ratio was calculated using a different method - this time, all proteins were taken into consideration, and the incorporation was seen through the “heavy” peptide intensity/“light” peptide intensity ratio. The calculated incorporation ratio was 82.3%, which suggests that free amino acids possibly

present in the HyClone serum could interfere with labeled amino acid incorporation. For future experiments, commercial dialyzed fetal bovine serum will be used.

As previously stated, VAPA and VAPB are both present in HeLa and HEK293T cell extracts. In order to enrich one of the cell extracts with the proteins of interest, pFlag-CMV2 plasmids containing *VAPA, wtVAPB* or *mutVAPB* coding sequences (described in Prosser et al., 2008 and Mitne-Neto et al., 2011) were transfected into HeLa cells using lipofectamine 2000 (Invitrogen - Life Technologies). To determine optimal time for protein expression, a transfection protocol performed and cells were maintained in culture for either 24, 48 or 72 hours after transfection. The results are shown in Figure 4.3.

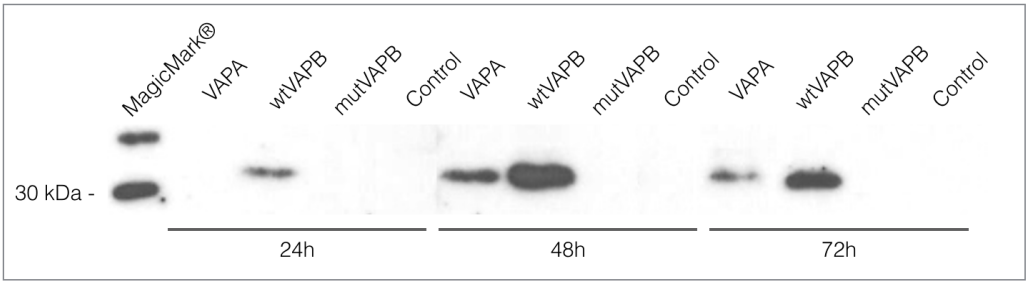

Figure 4.3: Western Blot results of HeLa cell transfection with pFlag-CMV2 plasmids using an anti-Flag antibody (Sigma). The molecular weight used was the Magic Mark (Invitrogen). Bands can be seen clearly 48h after transfection, when optimal expression was obtained. The differences in band intensity for VAPA and VAPB suggest differences in protocol efficiency.

It is interesting to note that there is a significant difference in fragment intensity between VAPA and VAPB 48h after transfection. The same total amount of DNA was used in these experiments; however, transfection protocols suggest plasmid DNA should be at a 0.5µg/µl concentration, while the concentrations here obtained were of 0.4µg/µl for *VAPB* and 0.2µg/µl for *VAPA,* possibly influencing protocol efficiency. Also, no fragments were identified for mutant *VAPB* (carrying the c.C166T, or p.P56S mutation), suggesting that the abnormal protein was either degraded or accumulated in insoluble fractions during protein extraction, as previousle described in Kanekura et al., 2006, Teuling et al., 2007, and Mitne-Neto et al., 2011.

Cloning and Purification of VAPB protein

The ideal internal standard for mass spectrometry-based quantitative experiments is that which possesses the same physicochemical properties as the analyte of interest (Villanueva et al., 2014). Although VAPB is present in the cell extracts of the established cell lines, the large amount of proteins and peptides of this proteome largely increase sample complexity when used as a spike-in. Therefore, we sought to express and purify the VAPB protein through recombinant DNA technology.

First, the coding sequence for VAPB was amplified using the pFlag-CMV2-*VAPB* plasmid as template DNA. Primers were designed containing restriction enzyme sites on their 5' ends (NdeI sequence for the forward primer and BamHI sequence for the reverse). After amplification, PCR product and pET15b vector (Novagen) are purified, digested by the NdeI and BamHI enzymes (New England Biolabs), and ligated in a 3:1 PCR product:vector ratio using T7 ligase (Promega). This product is dialyzed and ready to be inserted into competent bacteria. The pET15b was chosen for two main reasons: (1) it inserts a His-tag in the N-terminal portion of the protein; (2) the vector shows strong and controlled expression through a system similar to the *lac* operon: only in the presence of isopropyl-beta-D-1-thiogalactopyranoside (IPTG) expression will occur.

The first *E. coli* strain to be transformed using this plasmid is the electrocompetent DH5alfa (Invitrogen - Life Technologies). This strain was used for plasmid amplification and subsequent sequence confirmation by Sanger sequencing.

Next, *E. coli* strains were transformed (BL21, CD43 and BL21-Codon Plus (DE3)-RIPL), and tests for purification protocols were initiated. We decided to continue experimentation with the BL21-Codon Plus (DE3)-RIPL strain (Stratagene), which contains extra copies of the

tRNAs that most frequently limit translation of heterologous proteins in *E. coli,* in order to avoid limitation of the forced-high level expression due to tRNA depletion. The bacteria were transformed by electroporation, plated, and 5 isolated colonies were selected for expression tests (Figure 4.4). They were grown in M9 minimal medium, since it will be the one used during the stages of stable isotope incorporation.

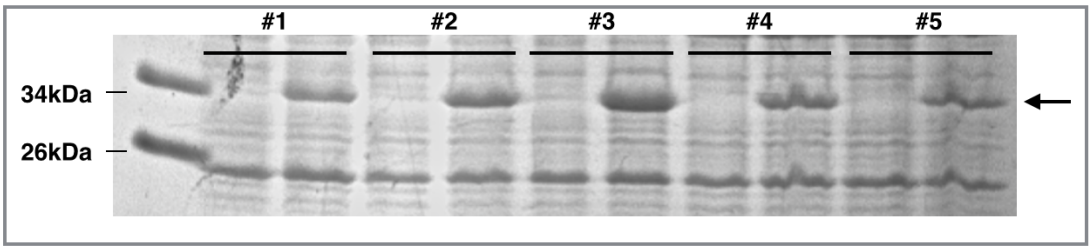

Figure 4.4. SDS-Page of VAPB expression test of 5 selected colonies. Order on gel: PageRuler (Thermo); colony #1 before induction; #1 after induction; #2 before induction; #2 after induction; #3 before induction; #3 after induction; #4 before induction; #4 after induction; #5 before induction; #5 after induction. The VAPB fragment is shown by the arrow, and is present only after expression induction.

After extensive testing, a purification protocol for the recombinant VAPB protein was established, using the Ni-NTA Superflow Cartridges (Qiagen). Protocol details can be found in the Supplemental Section IV. Figure 4.5 (SDS-Page and Western Blot) show the final results of this protocol.

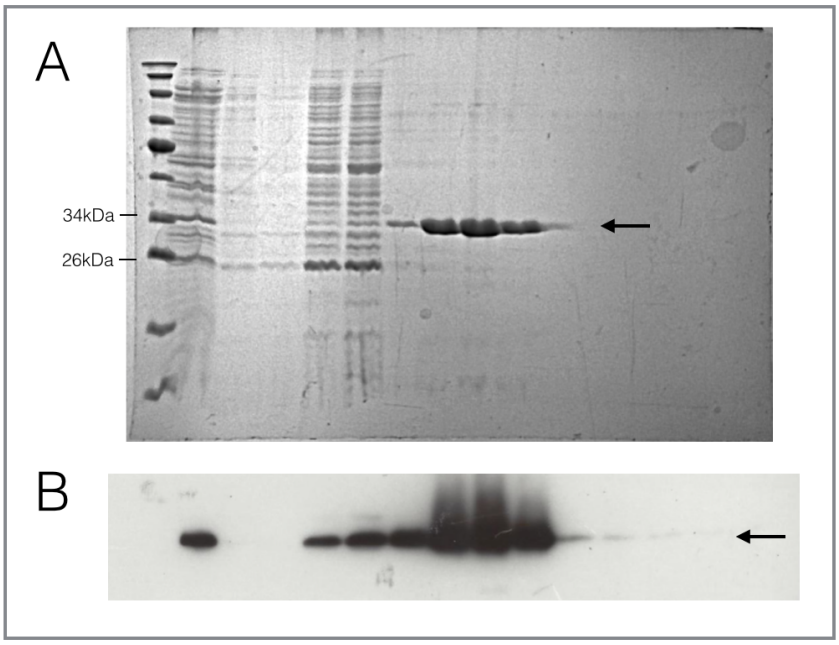

Figure 4.5: SDS-Page (A) and correspondent Western Blot (B) showing the different fractions obtained during the VAPB purification process. Marker used for the SDS Page: PageRuler (Thermo). Antibody used for VAPB: described in Mitne-Neto et al., 2011.

Targeted LC-MS/MS method parameters

All method development experiments described here were carried out in a **Q-Exactive**

(Thermo) Mass Spectrometer. Coupled to it is the NanoAcquity UPLC instrument (Waters). Experiment analyses were performed using the Skyline software (MacCoss Labs) (MacLean et al., 2010). The strategy chosen for this work was PRM (Parallel Reaction Monitoring), in which all products of a target peptide are monitored under high resolution and high mass accuracy conditions (Peterson et al., 2012).

The first step towards building a targeted method is building a spectral library of all peptides of interest. For this, the 8 purified peptides were mixed and diluted to a concentration of approximately 100fmol/µl. About 1µl was injected for a standard DDA run, and the resulting spectra were used to build the spectral library in Skyline. The VAPA, VAPB and VAPC FASTA sequences were pasted into Skyline, which automatically digests proteins using previously defined parameters, and the peptides generated were filtered in order to keep in that method file only those which were previously chosen. For each peptide, 3 transitions were selected, based on those fragments which produced the most intense ion signals in the fragmentation spectrum. The chromatography conditions resulted in reproducible peptide retention times, and narrow elution peaks. Therefore, the same conditions were kept throughout all experiments.

Next, the optimal collision energy for each peptide was tested in a series of runs. The chosen collision energy was that which produced the largest peak area for that same peptide. An example can be seen below in Figure 4.6, which shows different runs for the peptide VEQVL(…). The peak shoulder observed for this peptide could be related to proline isomerization, which generates cis- and trans-observable forms.

Another important parameter tested was the radio frequency value for the S-Lens of the equipment. The S-Lens is a progressively spaced stacked ring ion guide, whose radio frequency (RF) captures and efficiently focuses ions in a tight beam, increasing sensitivity. The

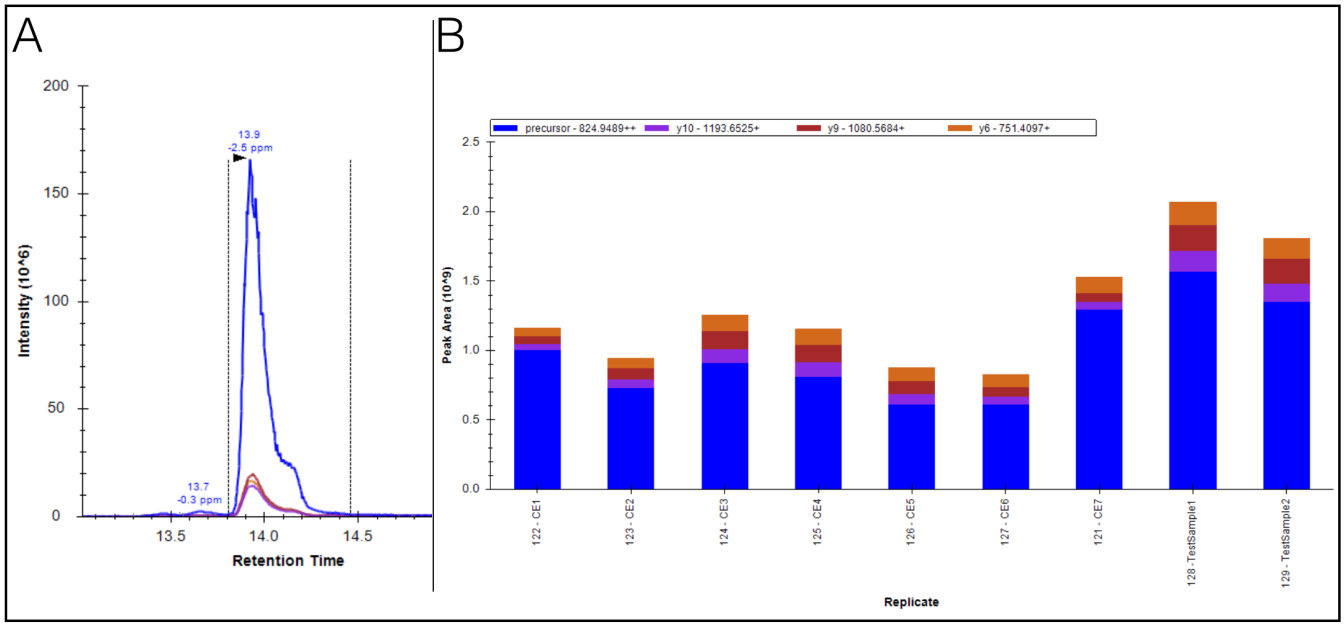

Figure 4.6. Peptide VEQ(…) extracted ion chromatogram (A), showing intensity and retention time for one replicate, and peak intensities (B) obtained in each run. The peak area shown in blue corresponds to precursor intensity, while the purple, red and orange areas represent the fragment ions intensities. The last two replicates are two peptide mixtures tested with the chosen collision energy value.

values of 50, 55 and 60 were tested, and for most peptides, the RF value of 55 represented a slight increase in peak area, so that value was maintained in further experiments.

The initial isolation window set for the quadrupole mass analyzer plays an important role on mass filtering and suppression of the biochemical background. We tested the values of 1.4m/z, 2 m/z, and 4 m/z, but no significant difference was seen between the different values set. Therefore, we kept the default value of 2 m/z for the isolation window of the quadrupole.

The 8 VAP peptides are mostly hydrophobic in nature due to their sequence. The peptide pool solution was being diluted in water, which resulted in signal intensity loss along the run series, probably because peptides due to peptide precipitation. Due to this setback, different solutions for peptide dilution were tested: DMSO (dimethyl sulfoxide) 2%, DMSO 5%, TFE (trifluoroethanol) 2% and TFE 5%, all with TFA (trifluoroacetic acid) to avoid nonspecific interactions. However, the intensity loss issue persisted, so further tests were performed by spiking HEK293T cell extracts with the peptides, these in a final concentration of 50fmol/µl.

The last parameters tested were the **AGC (Automatic Gain Control) target** - which corresponds to the number of ions collected before being before orbitrap injection - and **MaxIT (Maximum Injection Time),** the amount of time that ions are allowed to accumulate in the ion trap mass analyzer when AGC is off. A combination of 4 AGC target values and 3 MaxIT values was tested in a randomized manner, with all combinations tested in triplicates. The AGC target values analyzed were: $5x10^4$, $2x10^5$, 10^6, and $5x10^6$; for MaxIT, the values were 25ms, 100ms and 200ms. A summary of results can be seen in Figure 4.7.

Despite the initial fluctuation in peak intensity, as shown by the replicate peak areas, the MaxIT 200ms parameter (marked with an asterisk in Figure 4.7) showed an evident increase when compared to 25 and 100ms. This high value of MaxIT compensated for the low detectability of these surrogate peptides, allowing more ions to accumulate in the C-trap before analysis in the Orbitrap. Even the highest value used for MaxIT did not allowing the reaching of typical AGC Target values for tryptic peptides. Even though this resulted in an increased duty cycle, it still allowed the collection of enough points from chromatograms.

Discussion and Perspectives

The physicochemical properties of the VAP proteins present a challenge for the standard biochemical protocols. As membrane proteins, their hydrophobicity requires adaptation of standard HPLC separation protocols, sample preparation steps, and even recombinant protein purification was a time-consuming process. Despite these difficulties, however, a robust method for the analysis of VAPA, VAPB and VAPC was established for an LC-MS/MS platform (the quadrupole-orbitrap Q-Exactive). The next steps involve sample analysis, starting with cellular extracts (which present a smaller dynamic range, and thus

require less sample preparation steps and results in less protein loss) such as those obtained from fibroblasts; and further down the road, the analysis of bodily fluids such as plasma as CSF could also bring important information on the role VAPB plays in ALS, and potentially VAPA and VAPC as well.

In summary, this LC-MS/MS tool will not only allow the analysis of VAPB as a potential biomarker for ALS; it will also allow different studies regarding the biology of VAP proteins as a group and the analysis of VAPB's roles in other diseases such as cancer (Rao et al., 2012). Given the large number of roles VAPB plays inside and outside the cell and the large number of protein interactions and pathways in which it is involved, this method should prove to benefit a vast array of experiments and help answer a large number of questions.

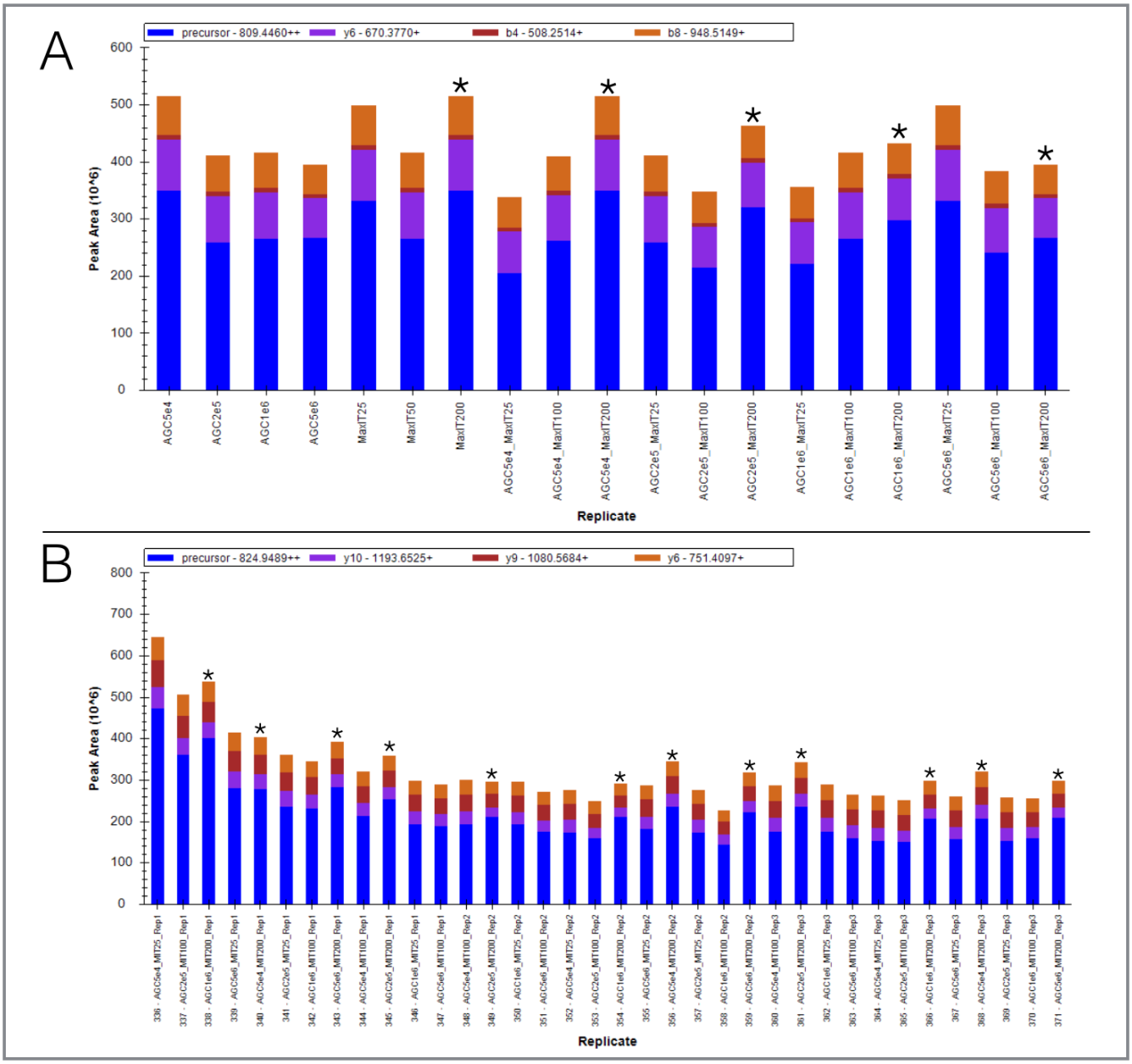

Figure 4.7. Peak Areas of the VEQ(…) VAPB peptide shown for each setting and their combinations (A), in the following order: AGC $5x10^4$, $2x10^5$, 10^6, and $5x10^6$; MaxIT of 25, 100 and 200 ms; AGC $5x10^4$ with MaxIT 25; AGC $5x10^4$ with MaxIT 100; AGC $5x10^4$ with MaxIT 200; AGC $2x10^5$ with MaxIT 25; AGC $2x10^5$ with MaxIT 100; AGC $2x10^5$ with MaxIT 200; AGC 10^6 with MaxIT25; AGC 10^6 with MaxIT100; AGC 10^6 with MaxIT200; AGC $5x10^6$ with MaxIT25; AGC $5x10^6$ with MaxIT100; AGC $5x10^6$ with MaxIT200. B: Peak areas shown separately for each replicate. All combinations and replicates including the MaxIT 200ms parameter are marked with a (*).

Capítulo 5: Discussão e Conclusões

A Esclerose Lateral Amiotrófica é uma doença geneticamente diversa, com os danos aos neurônios motores sendo causados por uma ação combinada de múltiplos processos patogênicos. Dependendo da população em estudo, as características genéticas são distintas, encontrando-se diferentes frequências para mutações conhecidamente causadoras. Os casos familiais, e mais recentemente, os esporádicos, têm contribuído significativamente para a descoberta de genes envolvidos no processo de degeneração do neurônio motor, sendo que muitos destes genes e seus produtos estão envolvidos em múltiplas vias deste processo.

A redução nos níveis de VAPB, proteína que possui diversos papéis intra e intercelulares, tem sido identificada em diversos modelos de ELA, incluindo modelos murinos e modelos *in vitro*, além de ser encontrada em pacientes esporádicos sem mutação em *VAPB,* o que sugere um papel mais central desta proteína na neurodegeneração. Os objetivos deste trabalho visaram buscar possíveis alterações em VAPB (ao nível de DNA, RNA e proteína) que poderiam ter um valor diagnóstico, e também analisar a expressão de genes cujos produtos conhecidamente interagem com esta proteína (como *EPHA4* e *VAPA*).

A análise genômica de pacientes de ELA - mais especificamente, de 67 pacientes, sendo que 49 são casos familiais - foi capaz de fornecer um diagnóstico molecular para aproximadamente 55% dos casos analisados (75% dos casos familiais). Só a mutação c.C166T em VAPB, considerada rara no mundo, foi encontrada em 63.2% dos casos familiais. Apesar de existirem atualmente mais de 30 genes descritos para a ELA, a análise de apenas 7 foi capaz de diagnosticar uma parcela considerável dos nossos casos, a grande maioria

oriunda do sudeste do Brasil. Análises maiores, contemplando pacientes de todas as regiões do país e um número maior de genes (incluindo a expansão em *C9ORF72*, responsável por uma parcela considerável de casos mundialmente) poderão traçar o perfil genético dos pacientes brasileiros, e nos trazer números atualizados sobre a incidência e prevalência da doença no país. O diagnóstico molecular tem a vantagem também de classificar os pacientes de acordo com a(s) mutação(ões) que possuem, o que futuramente poderá ter importância para o uso de terapias possivelmente específicas a determinados subtipos da doença. Além disso, ele permite o aconselhamento genético das famílias, abrindo a possibilidade de um diagnóstico pré-implantacional e evitando a propagação da mutação na família.

No nível transcricional, ao contrário do esperado pelos dados da literatura recente, não foi encontrada uma diminuição da expressão de *VAPB* no tipo celular analisado (leucócitos de sangue periférico). Os dados publicados em 2008 por Anagnostou *et al.* evidenciaram a redução de VAPB em pacientes esporádicos sem mutação neste gene; porém, esta redução foi observada em líquido cefalorraquidiano, sugerindo então que as alterações nos níveis de VAPB seriam tecido-dependentes. Deidda et al. (2013) suportam esta hipótese pois observaram, ao nível protéico, a ausência de uma banda correspondente ao domínio MSP de VAPB em Western Blots de líquido cefalorraquidiano, mas não nos de leucócitos de sangue periférico. Apesar de VAPB apresentar expressão ubíqua, existe variação conforme o tecido analisado. Outro aspecto a ser levado em consideração é a grande variabilidade de progressão dos pacientes de ELA, incluindo os de ELA 8. Análises feitas classificando os pacientes de acordo com sua progressão seriam ideais, mas esta categorização se torna um desafio quando o curso da doença não é linear.

O receptor *EPHA4*, conhecido como forte modificador de fenótipo em Esclerose Lateral Amiotrófica, interage com o domínio MSP de VAPB, que por sua vez age como competidor das efrinas. A ausência de MSP e/ou o aumento na expressão deste receptor poderiam contribuir para um maior agrupamento de receptores NMDA, e consequentemente provocar uma maior ativação dos neurônios motores por glutamato, gerando um mecanismo de excitotoxicidade. Nas duas amostras de pacientes de ELA de início bulbar que analisamos, há um aumento considerável na expressão desse gene (3-6 vezes em relação aos controles). Não é possível concluir que este receptor está de fato elevado em ELA bulbar devido ao baixo número de amostras deste grupo, mas estes resultados evidenciam a necessidade de se investigar o *EPHA4* a fundo em ELA bulbar, a qual apresenta rápida progressão. Esses dados aliados à ausência de MSP vista por Deidda *et al.* (2013) principalmente em ELA bulbar sugerem que o mecanismo aqui proposto pode ser um forte contribuinte para a rápida degeneração dos neurônios motores nesses casos.

Uma abordagem completa para esta questão requer o desenvolvimento de um método quantitativo preciso para análise protéica de VAPB nos pacientes de ELA, visando compreender se esta proteína pode ser considerada um biomarcador para a doença. Aqui, fomos capazes de desenvolver um método sensível e específico para VAPA, VAPB e VAPC por LC-MS/MS, que permitirá analisar os níveis destas proteínas em diversos tecidos.

Inicialmente, será analisado extrato celular de fibroblastos obtidos de pacientes (disponíveis a partir de outros estudos do nosso laboratório). Esta matriz possui um intervalo dinâmico menor entre as proteínas, requerendo menos passos de preparação de amostra (e, consequentemente, menos perda de proteína), o que tornaria a análise mais simples num primeiro momento. Em seguida, protocolos de análise para plasma e líquor também poderão

ser desenvolvidos, para que seja possível realizar as quantificações em matrizes de mais fácil acesso. Esta ferramenta permitirá também analisar a VAPB no contexto de outras doenças, como o câncer, onde ela foi descrita em níveis elevados e com papel importante na proliferação tumoral (Rao et al., 2012), além da elucidação das funções biológicas das proteínas VAP.

Glossário / *Glossary*

ELA: Esclerose Lateral Amiotrófica / ALS: *Amyotrophic Lateral Sclerosis*

ELA8: Esclerose Lateral Amiotrófica tipo 8 / ALS8: *Amyotrophic Lateral Sclerosis type 8*

FALS: *Familial ALS*

SALS: *Sporadic ALS*

VAPA: *Vesicle-Associated Membrane Protein (VAMP) - Associated Protein* A

VAPB: *Vesicle-Associated Membrane Protein (VAMP) - Associated Protein* B

VAPC: *Vesicle-Associated Membrane Protein (VAMP) - Associated Protein* C

MSP: *Major Sperm Protein Domain*

TMD: *Transmembrane Domain*

C.C.: *Coiled-Coil Domain*

EPHA4: Receptor de Efrina A4 / *Ephrin Receptor A4*

NMDA: Receptor N-Metil-D-Aspartato / *N-Methyl-D-Aspartate Receptor*

ABrELA: Associação Brasileira de Esclerose Lateral Amiotrófica

CPGH-CEL: Centro de Pesquisas Sobre o Genoma Humano e Células Tronco / *Human Genome and Stem Cell Research Center*

LC-MS/MS: Cromatografia Líquida acoplada à Espectrometria de Massas em tandem / *Liquid Chromatography coupled to tandem Mass Spectrometry*

DDA: Aquisição Dado-Dependente */ Data-Dependent Acquisition*

SRM: *Selected Reaction Monitoring*

PRM: *Parallel Reaction Monitoring*

AGC Target: *Automatic Gain Control Target*

MaxIT: *Maximum Injection Time*

Suplemento I: Artigo publicado em 2015: Revisão sobre ELA e células-tronco.

Stem Cells for Amyotrophic Lateral Sclerosis Modeling and Therapy: Myth or Fact?

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Stem Cells for Amyotrophic Lateral Sclerosis Modeling and Therapy: Myth or Fact?

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease whose pathophysiology is poorly understood. Aiming to better understand the cause of motor neuron death, the use of experimental cell-based models increased significantly over the past years. In this scenario, much knowledge has been generated from the study of motor neurons derived from embryonic stem cells and induced pluripotent stem cells. These methods, however, have advantages and disadvantages, which must be balanced on experimental design. Preclinical studies provide valuable information, making it possible to combine diverse methods to build an expanded knowledge of ALS pathophysiology. In addition to using stem cells as experimental models for understanding disease mechanism, these cells had been quoted for therapy in ALS. Despite ethical issues involved in its use, cell therapy with neural stem cells stands out. A phase I clinical trial was recently completed and a phase II is on its way, attesting the method's safety. In another approach, mesenchymal stromal cells capable of releasing neuroregulatory and anti-inflammatory factors have also been listed as candidates for cell therapy for ALS, and have been admitted as safe in a phase I trial. Despite recent advances, application of stem cells as an actual therapy for ALS patients is still in debate. Here, we discuss how stem cells have been useful in modeling ALS and address critical topics concerning their therapeutic use, such as administration protocols, injection site, cell type to be administered, type of transplantation (autologous vs. allogeneic) among other issues with particular implications for ALS therapy. © 2015 International Society for Advancement of Cytometry

Key terms

Key terms: amyotrophic lateral sclerosis; stem cells; cell therapy; iPSC; ESC; MSC

Introduction

Amyotrophic Lateral Sclerosis (ALS), or Lou Gherig's Disease, is a neurodegenerative disorder characterized by the death of motor neurons, with symptoms including fasciculation, spasticity, muscle atrophy, and weakness. These signs emerge when axons retract, and denervation of muscles occurs. Initially, the retraction is compensated by more resistant neurons, which sprout and reinnervate some muscles; however, this mechanism eventually fails, and the neuronal cell bodies become visibly abnormal and die (1). In addition to motor neuron symptoms, 15% of patients present also Frontotemporal Dementia (ALS-FTD), with the neurons in the prefrontal and temporal cortexes being affected in the disease (2). The criteria used for diagnosis are mainly clinical, as reported in the *El Escorial*, created in 1994 (3) and revised in 2000 (4). Electromyography data is now considered of equal importance as clinical features, and the signs required for an ALS diagnosis are revised in the Awaji-Shima electrodiagnostic criteria (5).

Most cases are characterized by an adult onset of symptoms, usually in the fourth decade of life. Some juvenile cases have, however, been described, including patients with mutations in *ALS2* (6), *SPG11* (7), *SIGMAR1* (8), genes, and *SETX* (9).

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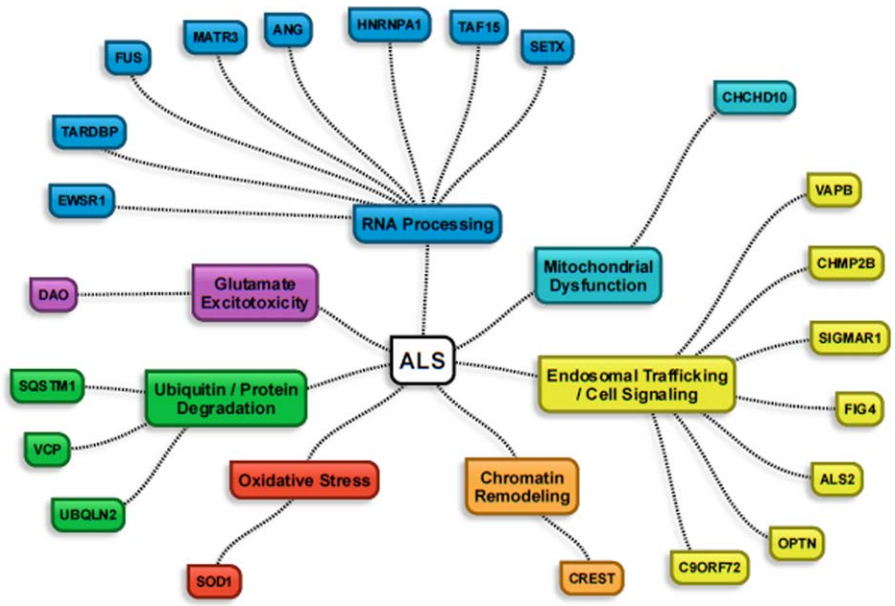

Figure 1. Genes currently known as associated with ALS classified by common molecular pathways involved in the disease pathophysiology. Adapted from Refs. (1) and (15). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The average survival ranges from 3 to 5 years after onset, but there is a great interfamilial and intrafamilial variability between patients, which suggests the existence of important disease modifiers that contribute to disease progression. Among them, the ephrin receptor EphA4 was described as one of the main modifiers in humans; the higher the expression level of this receptor, the more vulnerable the motor neurons (10).

Another observed correlation with disease progression involves T-regulatory cells (T-regs), which are critically involved in suppressing the inflammation induced by neurotoxic T-lymphocytes and microglia/macrophages in ALS. The analysis of affected patients' blood through flow cytometry (FC) recently showed that $CD4+CD25^{High}$ regulatory T-regs are reduced in patients with a rapid disease progression. Also, FoxP3 (a transcription factor required for T-reg function) levels were shown to be indicative of progression rates and thus could be used to identify rapidly progressing cases (11).

ALS is mainly classified into two groups: FALS (Familial ALS), for the inherited forms of the disease, and SALS for the sporadic cases, in which there are no affected relatives and where in the majority of cases it is not possible to identify a single causative agent. The familial cases have their historical importance in causative gene identification since through these discoveries much has been uncovered about ALS pathogenesis.

Around 33 genes or chromosomal regions have been linked to ALS, providing important clues on the pathophysiological mechanisms of the disease (ALSoD - http://alsod.iop.kcl.ac.uk). Recent studies using high-throughput technologies such as exome sequencing have identified not only novel mutations in these known ALS-causing genes, but also additional genetic causes of ALS. A search for de novo mutations in SALS, for instance, revealed mutations in CREST (12), a protein that associates with FUS; deletions in *SPG11*, a gene associated with hereditary spastic paraplegia, have been found in familial ALS cases as well (13), highlighting the importance of the elucidation of disease modifiers; and last, but not least, mutations in MATR3, a DNA/RNA-binding protein that interacts with TDP-43, have been found in FALS cases (14).

The main pathways in which ALS-associated genes are involved are oxidative stress, RNA processing, endossomal trafficking, cell signaling, glutamate excitotoxicity, proteostasis, and cytoskeleton composition ((15); Fig. 1). However, little is understood about how these pathways converge into the ALS phenotype. Even though they are described as causative for ALS, a large amount of ALS-causing genes are also responsible for other types of neurodegenerative diseases; in other words, the same mutations are able to lead to various phenotypes. Such clinical variability suggests that ALS is indeed a syndrome, and not one single disease (1). Given the fact that the most frequent gene alteration in ALS, *C9ORF72*, is responsible for only ~33% of familial cases and 8% of sporadic cases (16), environmental conditions could play an important role for disease development. Well-designed cohort studies are difficult and costly, but could reveal important information on potential risk factors.

Despite the increasing amount of genes associated with ALS and the consequent elucidation of molecular pathways, other pathophysiological mechanisms contributing to the ALS phenotype are poorly understood. Besides the known neurodegeneration, recent findings also suggest the involvement of an aberrant innate immune system. The percentages of CCR2 and CD62L on CD14+CD16- classical monocytes were significantly lower in ALS patients than in healthy controls (17).

This complex profile hinders progress for important clinical trials, which have been, in most cases, unsuccessful. However, stem cells seem to be the new light at the end of the tunnel, providing important models for molecular pathway studies, drug screening, and even cell therapy, with two clinical trials currently in phase II: one by NeuralStem (18,19), and another by BrainStorm (NCT01051882).

In this review, we will discuss the importance of distinct stem cell types for disease modeling, summarizing new data from embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) models, their uses, advantages and disadvantages, and how FC has significantly contributed to these cell-based in vitro studies. We also review and discuss important issues to be addressed on possibilities of cell therapy, including neural and mesenchymal stem cells.

FC as an Ally for Cellular and Molecular Studies

Among all available techniques for cellular marker evaluation, FC plays a major role in many aspects of cell-based ALS studies. It allows the search of characteristic ALS markers, such as mentioned above for CD4 lymphocytes and FoxP3, and may provide important insight into patient prognosis. This can help not only the design of a more personalized therapeutic approach, but also selection of patients for clinical trials based on similar progression rate and symptoms. A recent work from Marrali et al. (20) shows that NOX2 activity, evaluated from fresh blood assayed through a special FC protocol, is a modifier of ALS survival. This kind of assay is of special interest for clinical trials due to the accessibility of the studied tissue and its applicability in a high throughput screening. Despite the motor neurons being the main affected cells in ALS, as described above, other cell types can take part in this disease, showing the need for a multicellular evaluation. In this case, not only the cell surface markers are the target of those studies, but also the evaluation of intracellular patterns. As presented by Pickles et al. (21) depositions of protein aggregates in mitochondria is age dependent in mouse models and are found in the lymphoblast fraction of ALS1 patients.

Regarding cell therapy, one great promise is the use of neural stem cells (NSCs) derived from the patient's own iPSC. By sorting the cells of interest, one can select and inject only the desired cell type, and exclude possible tumorigenic (or other harmful) cells.

In short, the importance of FC in this field of study, contributing not only to the understanding of ALS's pathophysiological mechanisms, but also to the elucidation of possible treatments will be discussed in this review as we describe cellular models and proposed cell therapies.

Cell-Based Models for Understanding ALS

Many transgenic murine models for ALS studies have been described, providing important information on the mechanisms behind motor neuron death. Even though they are very useful and informative, models that mimic human diseases are, biologically speaking, still very limited, since they present very distinct disease progression and drug responses. Consequently, to understand the precise molecular pathways behind ALS pathogenesis and the effects of different drugs and other potential therapies, recent efforts have been focusing on human cell-based disease models derived from pluripotent stem cells. The difficulties of reaching the cells affected in neurological and psychiatric diseases, for studies in vitro or in vivo, highlights the importance of cell-based models. Pluripotent stem cells provide models that are more flexible since they may generate different types of specialized neurons as well as supporting glial cells. Some disadvantages, however, accompany different sources of pluripotent stem cells. The pros and cons of ESCs and iPSCs as disease models are discussed below.

Embryonic Stem Cells

ESCs, first isolated in 1998 by Thomson (22), have the capacity to self-renew indefinitely in culture while maintaining a potential to generate any of the hundreds of cell types in the body. The same plasticity that permits ESCs to generate so many different cell types also makes their control quite a challenge (23). Many important and decisive differentiation factors have been discovered, and simple protocols for ES differentiation into motor neurons are available. The first successful differentiation method for mouse ESC-derived motor neurons was described in 2002 (24), and the first protocol for human cells in 2005 (25), all of which turned possible due to previous discoveries of important factors such as Sonic hedgehog (26), Shh factor, and the elucidation of the sequence of differentiation events that culminate in different neuronal populations (27). Later in this same year, in a well-designed study, a human ES lineage was transfected with plasmids encoding GFP placed under the control of a MN-specific enhancer within the 5′-regulatory region of the gene encoding the transcription factor Hb9. After treatment with Shh and retinoic acid (RA), activated Hb9-driven GFP expression permitted the isolation of MN by fluorescence-activated cell sorting, excluding undifferentiated cells and thus making this differentiation protocol a more reliable process (28).

ESCs were firstly used to generate animal models. Clement et al., used mouse ESCs to create chimeric mice composed of mixtures of normal cells and cells that express a human mutant SOD1 polypeptide. Interestingly, non neuronal cells that did not express mutant SOD1 presented delayed degeneration and significantly extended survival of mutant-expressing motor neurons (29).

The differentiation of ES cells into motor neurons or other cell types such as glial cells have also been used as important models for ALS studies, elucidating the main disease mechanisms (cell-autonomous and noncell-autonomous) involved in motor neuron death.

In 2008, two important studies were developed using ES-derived motor neurons. Di Giorgio et al. showed that other cells besides motor neurons play an important role in ALS disease progression. Human motor neurons were cocultured with mice glial cells carrying the G93A mutation in the *SOD1* gene, and were shown to be sensitive to the toxic, noncell-autonomous effect of these cells (30). Marchetto et al.

published a similar work simultaneously, coculturing human ESC-derived motor neurons with astrocytes carrying the G37R mutation in *SOD1*; this coculture caused a 50% reduction in the number of motor neurons when compared with controls. The toxicity of these astrocytes is believed to be generated by an increased inflammatory response, and also the activation of NOX2, consequently producing oxygen radicals. The addition of antioxidants was able to reverse this process and specifically, apocynin could rescue the motor neuron survival in the presence of *SOD1* mutant astrocytes (31).

In these studies, glial cells carried *SOD1* mutations. Since most ALS cases are sporadic, Haidet-Phillips et al. sought to study the effects of FALS and SALS-derived astrocytes cocultured with motor neurons. These astrocytes were derived from neural progenitor cells (NPCs) obtained from postmortem tissues, and cocultured with mouse ESC-derived motor neurons. All patient-derived astrocytes showed great toxicity to these motor neurons, selectively killing them in this coculture model system (32).

Supporting the idea of a toxic environment contributing to this motor neuron disease, the work published by López-González et al. in 2009 showed that transplanting mouse ESC-derived motor neurons into an ALS mouse *SOD1* model (carrying the G93A mutation) was not beneficial. Although the mice showed an initial recovery, the engrafted motor neurons died shortly after transplant, and the disease progression resumed its course (33).

Even though strong noncell autonomous effects are reported in diverse studies, cell autonomous effects also take place in ALS. Wada et al. (2008) described a human ESC-based model for ALS, in this work, the differentiated motor neurons expressed the mutated form of *SOD1*, and were not initially cocultured with other neuronal cell types. Interestingly, the G93A mutation does not affect the differentiation process. What the authors observed was a significantly higher death rate among terminally differentiated motor neurons, showing an intrinsic mechanism (cell-autonomous) behind motor neuron death in ALS. This work also involved the coculture of these differentiated cells with conditionated medium of astrocytes carrying the same mutation, confirming also a noncell-autonomous astrocyte-dependent cell survival mechanism (34).

ESC-based models can be of great importance not only for the understanding of pathophysiological mechanisms behind ALS, but also for the screening of potential therapeutic molecules. Recently Yang et al. (2013) have derived motor neurons from mouse ESCs, and performed a small-molecule screening comparing survival of wild-type motor neurons and those carrying a human transgene with the G93A *SOD1* mutation. A compound named kenpaullone stood out, not only by keeping motor neurons alive for several weeks (in the absence of added trophic support), but also by maintaining neuritic processes, synapses, and normal electrophysiological characteristics (35). Olesoxime and Dexpramipexole (36,37), two drugs that recently failed a phase III trial, were also tested in this study, and were shown to be less effective. These two drugs had never been tested in in vitro models, only in vivo, which supports the concept that both types of study need to be performed to obtain a deeper knowledge of the drug's therapeutic potential, and avoid clinical trial failure.

In summary, cell models based on ES cells have shown that the mere substitution of motor neurons as a cell therapy would not be enough to circumvent the neurodegeneration; besides the cell-autonomous mechanisms, the toxic environment, provided by glial cells, is harmful to motor neurons, and contribute to their death. Even though these findings represent a great advance in ALS understanding, they have been limited to only one gene, *SOD1*. This disease involves multiple pathways, and most likely has multiple etiologies. Even in cases of ALS in which the causative mutation is unknown, proteins such as TDP-43, FUS, and others, are found in cytoplasmic aggregates, suggesting an underlying convergence of cellular processes and pathological effects (38). ESC-based models for other frequent mutations, especially the expansions on *C9ORF72*, may brighten our knowledge as to other potential mechanisms to be aimed for therapy.

Despite these many advantages, the ethical issue related to the destruction of embryos still remains. Although ESC may be obtained for disease modeling and drug screening, a large-scale use of ESC still seems hardly feasible for treatment of all ALS patients

Induced Pluripotent Stem Cells

The development of iPSC in 2006 allowed remarkable changes in stem cell science (39). It made possible to obtain pluripotent stem cells directly from a patient's adult cells [mainly fibroblasts, although other sources have been used (40)], bypassing the large ethical issues behind ESCs and embryo destruction (39) and allowing the study of patient-specific ALS mutations, without the need for genetic engineering cells. Although ESCs with ALS mutations could be obtained through preimplantation diagnosis in familial cases with known mutations, only iPSC obtained from patients allow the analysis of sporadic cases. Since then, many important discoveries with iPSC-derived motor neurons and glial cells have enhanced our (still currently little) understanding of ALS pathophysiology.

The diverse stages of iPSC differentiation into mature motor neurons are well characterized. After iPSC establishment, cells are usually induced to form embryonic bodies and subsequently neural precursor cells (NPCs). Factors such as Shh and RA are used to induce differentiation into mature motor neurons which can be distinguished by the expression of some markers such as Hb9 (Fig. 2).

Somatic cell reprogramming is a stressful process, since it goes against the natural course of a cell's life. The iPSCs generated carry with them imprinting marks from their tissue of origin, and consequently present some altered genetic activity due to the reprogramming process. This possible genetic variation raises concerns about the validity of these models. However, researchers have shown that there is great similarity between iPSCs and ESCs, demonstrating telomere renewal during cell reprogramming into iPSCs and telomere shortening on differentiation into somatic cells (41), teratoma

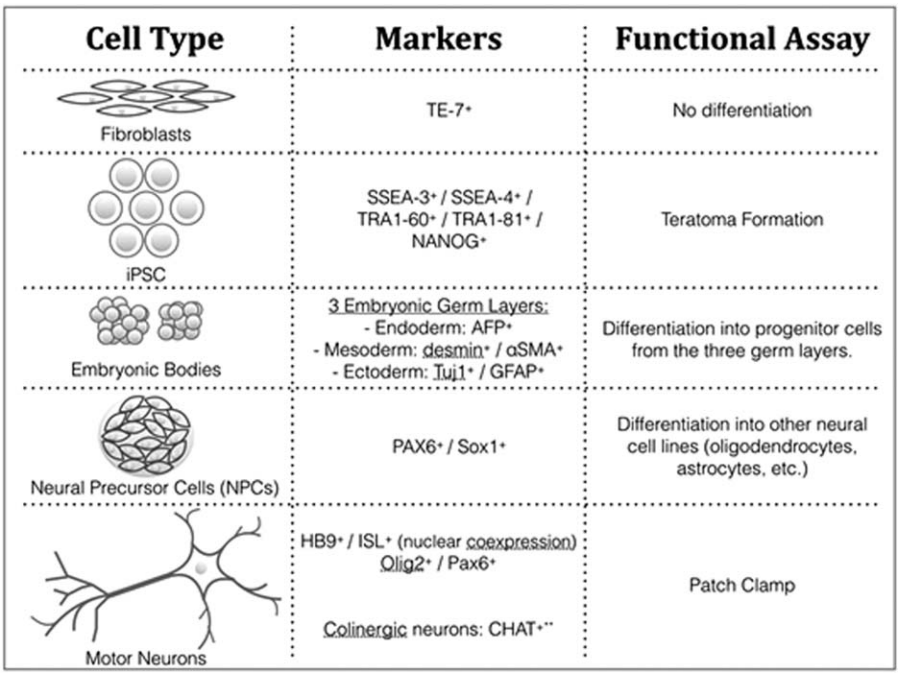

Figure 2. Characterization of cell type, markers, and functional assay of the stages of iPSCs differentiation into motor neurons. Adapted from Refs. (46,151, and 152).

formation, the possibility of derivation into the three kinds of embryonic tissues, epigenetic marks, and transcripts levels (42,43). This similarity suggests that iPSC cells could potentially be considered patient-specific ES cells, and consequently be used without rejection, and eliminate ethical issues.

Another important aspect to consider in the process of iPSC formation is the clonal variation inside iPSC strains, especially when the reprogramming technique used is integrative (44). Recently a shRNA mediated knockdown of the Mbd3 protein was reported (a regulator of gene expression and chromatin remodeling activities (45), generating a highly efficient, nonintegrative method for mouse cells. This represents an important breakthrough which eliminates many important discussed issues. However, this strategy must be tested on human cells as well. The main techniques used for reprogramming somatic cells (e.g., fibroblasts) into a pluripotent state, and their respective reprogramming efficiency, are demonstrated in Figure 3.

The doors opened by iPSC models have brought many interesting discoveries for ALS. Dimos et al. (2008) developed the first strain of ALS-related iPSC. The pluripotent cells derived from an ALS patient's fibroblasts were used to obtain motor neurons with a *SOD1* mutation (46). However, they could not identify any phenotype studying those cells. Afterward, an ALS (47) model developed in our laboratory by Mitne-Neto et al. (48), based on ALS8 patients' cells, has demonstrated that there is a reduction on the VAPB protein levels along motor neuron differentiation from iPSCs. These results complement other studies that show VAPB reduced levels in SALS patients and in *SOD1* mice (49,50). These and other findings (51,52) strengthen the evidences that iPSC-derived motor neurons can recapitulate the disease phenotype. Interestingly, in this model the typical cytoplasmic inclusions found in overexpression *VAPB* systems or in patients carrying other mutations such as *TARDBP* (53,54) was not observed. Additionally, patient and control iPSC-derived motor neurons showed perinuclear localization of VAPB (Fig. 4), suggesting that late-onset disease models might not show a full phenotype in these early stages (discussed below). Studies using MNs derived from iPSCs of ALS patients with C9ORF72 hexanucleotide expansion, have correlated many aspects seen in vivo and in vitro as neuronal hyperexcitability (55). Besides the identification of intranuclear RNA foci, Sareen et al. (51) showed that antisense oligonucleotides against the expanded transcript could improve the expression profile of mutant cells.

Cocultivation of mutation-carrying astrocytes and motor neurons was also investigated. Serio et al. (2013) showed that, while astrocytes derived from ES cells and carrying a SOD1 mutation showed a toxic effect on motor neurons (previously discussed; (34)), astrocytes derived from iPSCs carrying a TDP-43 mutation presented no toxicity for patient iPSC-derived motor neurons (56). The authors reported that the astrocytes derived from iPSC of patients with ALS carrying a TDP-43 mutation die earlier than control iPSC derived astrocytes. The different results observed in cellular and animal models show a clear necessity of understanding the ALS pathophysiology in deeper levels (56,57).

Some of the apparently conflicting results that arise when using iPSCs-derived models for ALS might be due to the fact that ALS is a late-onset disease, usually occurring between the fourth and fifth decades of life. iPSC originate from an

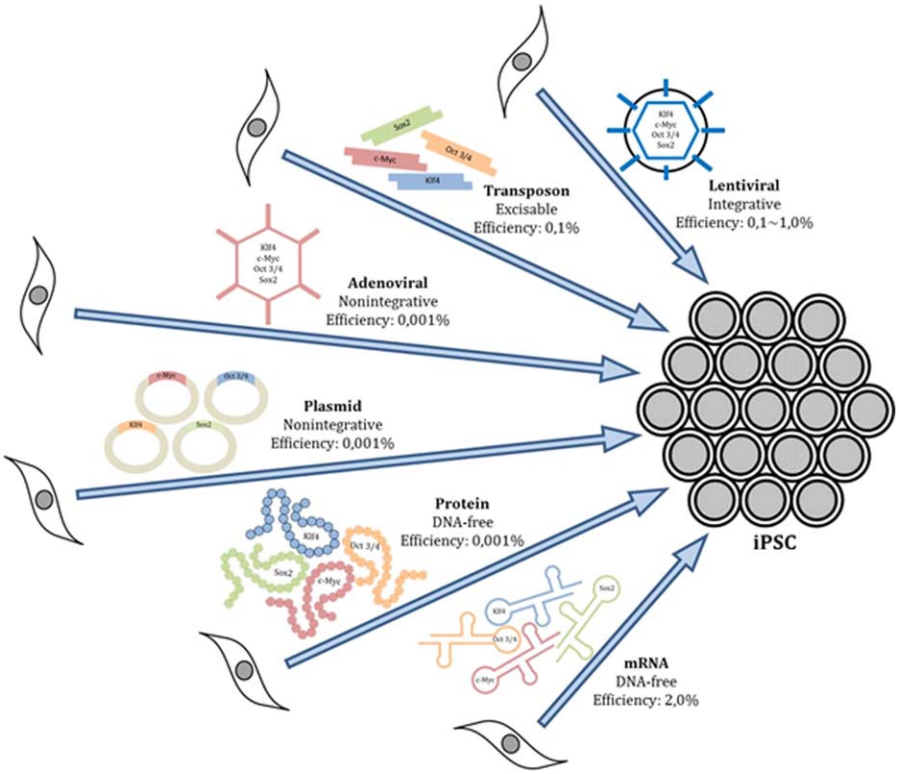

Figure 3. Main reprogramming strategies to transform somatic cells into iPSC, classified as integrative, excisable, nonintegrative, or DNA-free reprogramming vectors. Efficiency of transformation is also shown. Copyright CHS Press. Reproduced with permission (Ref 153).

"identity reset," taking the cells back to an embryonic stage. Given the short life span of motor neurons in vitro, this may disguise some of the signs observed in tissues from affected individuals and animal models that would appear afterwards. One possible solution to this issue is to graft motor neurons or even neuronal progenitor cells derived from iPSC in mice models, which could extend the survival of these cells; following these steps, a late recovery of the grafted cells from the mice might allow the visualization of ALS-related signs only seen in in vivo models and postmortem patient tissues. Another interesting (and possibly, more efficacious) strategy to induce aging-related features in iPSC-derived lineages is to

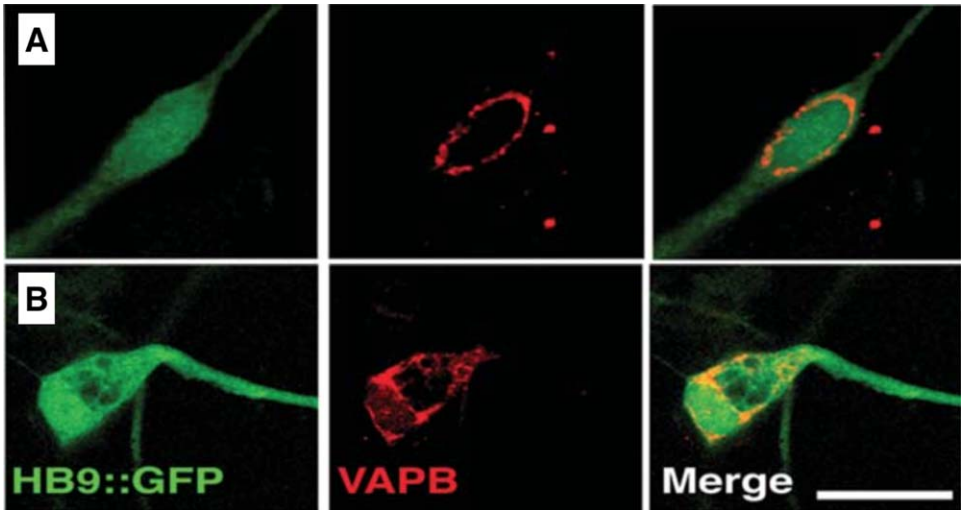

Figure 4. VAPB localization on iPSCs derived motor neurons (A) Perinuclear VAPB distribution on a GFP-positive motor neuron derived from a control iPSC clone and (H) from an ALS8. Bar = 20 μm (48). Copyright Oxford University Press. Reproduced with permission (Ref 48).

express progerin, a truncated form of Lamin A associated with Progeria (premature aging). This was recently reported for dopaminergic neurons used as a model for Parkinson's disease generating many physiological signatures of the disease (58).

The progress in iPSC is currently extending to drug screenings. Recently, an iPSC-derived ALS model was generated from fibroblasts of patients carrying a mutation in the *TARDBP* gene (52). The resulting motor neurons showed many correlated aspects of the disease, including shorter neurites and a decrease in neurofilament mRNA levels. These motor neurons were also used for testing the efficacy of treatment with anacardic acid. An overall attenuation of the phenotype occurred and this result is most likely linked to the resulting down-regulation of *TARDBP* expression after drug administration (52).

Another recent iPSCs study focused on electrophysiological alterations observed in motor neurons derived from patients with mutations in *SOD1*, *FUS*, and *C9ORF72* (55). These cells presented a neuronal hiperexcitability that is usually observed in these ALS forms in vivo and in vitro. In this same work, the authors used retigabine (a drug already used as an anticonvulsant) to mitigate the disease-related signs in the three types of generated motor neurons, making it so far the study with the broadest drug screening in an iPSC-based model of ALS.

iPSCs could also be used to better understand the molecular pathways involved in sporadic ALS cases, which are the vast majority. Recently, iPSC-derived motor neurons derived from SALS patients were treated with 1757 different compounds in a high-throughput drug screening. In 20% of cases, MNs showed a de novo TDP-43 aggregation, and a correspondent postmortem tissue obtained from one of the patients validated this molecular phenotype. These TDP-43 cells were then used for drug screening, and some FDA-approved drugs such as Digoxin ameliorated this particular phenotype (59). With these high throughput screening advanced technology, and with an ever increasing efficiency of iPSC generation and differentiation, a personalized medicine approach for sporadic patients could become a real possibility. Patient MNs could be derived in vitro, analyzed for ALS hallmark phenotypes, and consequently submitted to a drug screening that would reveal a possible effective treatment, which in its turn would hopefully stop disease progression.

Cell Therapy

Stem cells have been considered for therapy purposes in a number of different diseases for which no efficient treatments are available. For ALS therapy, in particular, the most studied types of cells are NSC and mesenchymal stromal cells (MSC).

Neural Stem Cells NSC

One of the most actively studied cells for therapy in ALS are the NSCs. When dividing, these cells give rise to other NSCs and/or progenitor cells which progressively differentiate into neurons, astrocytes, or oligodendrocytes (60). They are characterized by the expression of markers such as Sox2, GFAP, Nestin, and Musashin 1 and 2, but no specific combination has been found to definitely distinguish NSCs from NPCs (78).

A great number of studies evaluating the therapeutic potential of NSCs in ALS models were performed (61,62). Recently, a well designed meta-analysis using the SOD1 (G93A) transgenic mouse model was performed by a consortium of 11 independent ALS investigators. In these experiments, transplanted NSCs (both mouse and human) were capable of slowing the onset and the progression of clinical signs. Prolonged survival was also seen in ALS mice. The beneficial effects of transplanted NSCs were mediated by a variety of processes, including the cells' ability to produce trophic factors, preserve neuromuscular function, and reduce astrogliosis and inflammation, which differs from the primary hypothesis of motor neuron regeneration (63).

The NSCs have also been used in a recent phase I clinical trial by NeuralStem, completed in 2012. A total of 12 patients were divided in two groups receiving 5 unilateral or 5 bilateral (10 total injections) each, with ~10.000 cells per injection at the lumbar level. These patients were followed for 18 months after treatment, and there was no evidence of disease progression due to the intervention (18). The cell delivery apparatus was patented by NeuralStem. In this delivery procedure, a guide needle is inserted into the spinal parenchyma, followed by the insertion of an injection needle through the guide needle horizontally into the spinal parenchyma; then, the injection needle is withdrawn while delivering substrate into the spinal cord (NeuralStem patent # US2010198189A1). A second phase I trial was concluded in 2013, attesting the safety of cervical and thoracolumbar spinal cord injections with the same cells (64). The phase II dose escalating trial (NCT01730716) was recently concluded in July with an estimated publication of preliminary results in 2015.

iPSC Therapy–Can It Happen?

The idea of transforming adult cells into NSCs is very attractive for therapy. iPSCs can be obtained from the patients' cells, since they share the same MHC molecules as the patient's immune system the risk of rejection is greatly reduced, and the ethical issues concerning the source of ESCs is avoided.

A recent work generated and purified a specific NSC population from human iPSCs, based on high aldehyde dehydrogenase activity, low side scatter, and integrin VLA4 positivity by FC. After intrathecal or intravenous injections of these cells into SOD1-mutated mice, NSCs migrated and engrafted into the central nervous system of the treated ALS mice, which exhibited improved neuromuscular function and motor unit pathology and significantly increased life span. These positive effects are linked to multiple mechanisms, including production of neurotrophic factors and reduction of microgliosis and macrogliosis. According to this study, minimally invasive injections (intravenous) of iPSC-derived NSCs could exert a therapeutic effect in ALS (65).

Despite preclinical evidences, the therapeutic use of cells generated from iPSC is still a highly debated issue. First, the safety of this method should be ascertained, since these cells

have a well-known tumorigenic potential (39). Another important issue concerning iPSCs is that there are many reprograming protocols with different efficiency (66,67). Recent publications including novel transcription factors and chemical compounds (68,69) that substitute a reprogramming factor illustrate the lack of a gold standard protocol ensuring both reprogramming efficiency and safety for therapeutic uses. In addition, a protocol for separation of differentiated and nondifferentiated must be established, since nondifferentiated cells represent serious risk due to their oncogenicity.

Thus far, iPSCs have mostly been employed for the derivation of ALS models, which allow a better understanding of mechanisms behind motor neuron death, and also for drug screening, aiming the selection of compounds that might have the ability to reduce or stop disease progression and/or postpone symptom onset (70). Aiming to circumvent the issues of therapeutic use of iPSCs, Wook Han and coworkers used a combination of transcription factors (Brn4, Sox2, Klf4, and c-Myc) to induce mouse fibroblasts to directly acquire a NSC identity. These induced NSCs (iNSCs), exhibit cell morphology, gene expression, epigenetic features, differentiation potential, self-renewing capacity, as well as in vitro and in vivo functionality similar to those of wild-type NSCs (71). Adult cells might now be used as potential sources of NSCs without the need of an iPSC reprogramming stage.

Mesenchymal Stromal Cells

The terms "stromal stem cells" and "MSCs," established respectively by Owen in 1988 (72) and Caplan in 1991 (73), refer to a group of cells thought to have a common progenitor in the mesenchyme (74). In 1999, the capacity of these bone-marrow derived mesenchymal stromal/stem cells (MSCs) to differentiate into adipogenic, chondrogenic and osteogenic lineages was firstly demonstrated (75). Subsequently, some parameters had to be established for the correct classification of these cells.

According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, there are three minimum criteria for defining human mesenchymal stem cells: (1) MSCs must be plastic-adherent when maintained in standard culture conditions; (2) MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14, or CD11b, CD79a or CD19, and HLA-DR surface molecules, and (3) MSCs must differentiate to osteoblasts, adipocytes, and chondrocytes in vitro (76,77). MSCs have been isolated from almost all adult tissues, including bone marrow (78), adipose tissue (79), umbilical cord tissue (80), and others. Since these cells are obtained from adult tissue sources, the application of MSCs does not involve ethical concerns (81,82), and because ALS does not affect MSC expansion and differentiation potential (83), cells can be obtained from the own patient, thus avoiding rejection.

Classic Model of Tissue Regeneration Versus MSC Transdifferentiation

The prominent regenerative potential of these adult stem cells in organs such as liver (84), muscle (85) and heart (86) has been widely studied. However, when it comes to neurodegenerative diseases, NSCs may have a therapeutic advantage over MSCs, since the latter have mesodermal origin and thus their ability to transdifferentiate into neuronal cells of ectodermal origin is questionable (87).

Neurogenin 1 (Ngn1) is a proneural basic helix-loop-helix transcription factor, reported to be sufficient for reprograming the mesodermal cell fate of human MSCs into a neuronal fate (88). In a recent and well-designed experiment, bone marrow-derived MSCs transduced with a retroviral vector encoding Ngn1 (MSCs- Ngn1) were intravenously injected in an ALS murine model carrying a *SOD1* mutation, and the effectiveness of non-neural (unprocessed) and neural-induced MSCs, Ngn1-expressing MSCs (MSCs-Ngn1) was compared, aiming future ALS treatment. In this study, the MSCs-Ngn1, but not the unprocessed MSC, exhibited a neuron-like morphology, and showed expression of the neuron-specific proteins NeuN, NF200, and MAP2 after induced terminal differentiation in vitro. Unlike the unprocessed MSC, the transplantation of MSCs- Ngn1, prevented the loss of ventral motor neurons, and increased cervical and lumbar neuron survival in 38 and 24%, respectively (89).

Furthermore, MSCs-Ngn1 delayed disease onset if transplanted during preonset ages, whereas unprocessed MSCs failed to do so. If transplanted near the onset ages, a single treatment with MSCs-Ngn1 was shown to be sufficient to enhance motor functions during the symptomatic period, whereas unprocessed MSCs required repeated transplantation to achieve similar levels of motor function improvement (89), showing the importance of an early (and, if possible, presymptomatic) diagnosis.

Differently from the results obtained by a previous study of this same group (88), most grafted cells in the spinal cord of the SOD1 mice did not express neuronal, microglial or astrocyte markers, showing that transplanted MSCs-Ngn1 had the potential to efficiently migrate from the circulation into the CNS regions, but persisted as undifferentiated cells (89). These results suggest that the beneficial effect of the MSC-Ngn1 cells in SOD1 mice were due to a higher tropism for the affected areas in the neuroaxis, differently from what happens to unprocessed MSCs (demonstrated by a migration assay), thereby increasing the amount of released factors and thus extending neuroprotective effects.

Released Factors from MSCs

Besides tissue regeneration stem-cell based tissue repair is currently believed to occur through the paracrine secretion of factors. The list of these MSC-released factors increases constantly and it is known that they exert diverse biological functions, such as immunomodulation, antiscarring, chemoattraction, improvement and support of angiogenesis, antiapoptotic effects, or even proliferation/differentiation potential (90) The secretion of these biomolecules could promote the regulation of postinjury tissue remodeling, activation of endogenous repair/regeneration mechanisms, and attenuation of undesirable immune response (91–97).

It has also been shown that specific subpopulations of MSCs secrete some neuroregulatory molecules, such as brain

derived neurotrophic factor (BNDF), nerve growth factor (98), and other molecules that, despite not being classified as "neuroregulatory," act on neuronal cells (such as GLP-1; (99,100)). In the CNS, these molecules can promote beneficial effects such as stimulation of intrinsic autocrine survival, neurite outgrowth, and axon guidance. They also promote a correct connectivity in the brain and spinal cord, influence dendrite, and synapse formation, and influence proliferation, migration, and differentiation of stem cells in the developing adult nervous system (reviewed in Ref. 101)).

The neurotrophic factor, BNDF, tested in preclinical studies, and clinical trials was shown to be safe for therapy, but it failed as a potential therapeutic drug (101). Some hypotheses were raised to explain these results. However, it is not clear whether BNDF is able to cross the blood-brain barrier; and on the other, it is possible that the BNDF molecule itself was not the ideal growth factor to be tested clinically (101).

Another MSC-secreted molecule was tested in three Phase III clinical trials. IGF-1 (also classified as neuroregulatory) was injected subcutaneously in patients, but also failed to produce consistent and meaningful effects. After injections, the decaying of motor neuron function was reduced, but not bulbar function, and the patients did not show any vital capacity increase. One hypothesis to explain these results was the gravitational effect acting on the medication, since injections were at the spinal level of intrathecal delivery (102).

Aiming a continuous release of the neurotrophic factor in the CNS, a recent work used an interesting strategy. After human MSCs were transfected with the GLP-1 gene, whose protein has neuroprotective (99,100) and antioxidant (103) properties in the brain, the encapsulation of these cells into a biopolymer material was performed to prevent rejection when transplanted into mice (104). The intracerebroventricular injection of GLP-1 producing MSC capsules in presymptomatic SOD1 (G93A) mice significantly prolonged survival, delayed symptom onset and weight loss, and led to significant improvements in motor performance tests compared to vehicle treated controls (105).

MSC Based Therapy–Clinical Trials

Based on the growing number of studies indicating the safety and feasibility of cell therapy with MSCs isolated from bone marrow (106,107), a Phase I clinical trial was performed with 10 ALS patients. Autologous bone marrow-derived MSCs were transplanted into the spinal cord at a high thoracic level and the patients were regularly monitored before and after transplantation by clinical, psychological, neuroradiological, and neurophysiological assessments. This study confirmed that MSC transplantation into the spinal cord of ALS patients is safe and that MSCs might have a clinical use for future ALS cell based clinical trials (108). No severe morbidity was associated with the surgical procedure. However, to maximize the safety of direct parenchymal injection, the authors suggest the use of a recently standardized microinjection platform (similar to the previously mentioned one patented by NeuralStem) to reduce eventual side effects in the surgery and facilitate MSC transplantation in the critical regions of the spinal cord (108,109). Hypothesizing that the main activity of the cells should be at the transplanted segments, the authors also suggest the application of cells in the cervical segments of the spinal cord to increase the potential benefit of MSCs therapy (108).

Another Phase I/II clinical trial was performed in the Hadassah Hebrew University Hospital (Jerusalem, Israel), with a total of 34 patients: 15 with intractable multiple sclerosis (MS), and 19 with progressive ALS. To maximize the potential therapeutic benefits by accessing the CNS through the cerebrospinal fluid and the systemic circulation, the autologous injections were combined intrathecally and intravenously. In addition, nine patients received MSCs labeled with ferumoxide (Feridex) to track cell migration after local grafting. Only mild adverse effects were reported during the 6-month follow-up. Meningeal irritation and aseptic meningitis was observed in one patient and the researchers attributed this effect to residual dimethyl sulfoxide in the culture medium. The mean ALSFRS score, an instrument for evaluating the functional status of ALS patients, remained stable during the first six months of observation and the EDSS score (method of quantifying disability in MS) declined gradually, indicating functional improvement. Magnetic resonance imaging (MRI) of the labeled MSCs indicated their possible dissemination from the site of inoculation to the occipital horns, meninges, and spinal cord parenchyma. To assess the immunological effects of MSC transplantation, lymphocyte subsets and cytokine production were also evaluated. Stronger changes than those induced by the conventional immunomodulatory medications were found in the treated patients, pointing to a down-regulation of activated lymphocytes and antigen-presenting cells after MSC transplantation

Amongst the initiatives with MSC trials, the leading trial is Brainstorm's, which uses NurOwn cells–bone marrow-derived MSCs taken from the patient and induced to produce large amounts of neurotrophic factors (www.brainstorm-cell.com). A phase I (NCT01051882) and a phase IIa (NCT01777646) trial have been recently concluded, and a phase II (NCT02017912) will be carried out with 48 patients in a multicentric study (www.clinicaltrials.org).

From Myth to Fact–Questions to be Addressed

In vivo function of MSCs and heterogeneity. The real in vivo function and physiological role of MSCs is still poorly understood. Recently, Waterman and coworkers observed that the stimulation of specific Toll-like receptors (TLRs) affects the immune modulating responses of human MSCs. The polarization of TLR4 or TLR3 receptors yielded two different MSCs lineages named MSC1 and MSC2, respectively. MSC1 mostly elaborate pro-inflammatory mediators, while MSC2 express mostly immunosuppressive ones (110). Taking these results into account, it is possible that MSCs might act differently according to the microenvironment status of the patient. Indeed, this "priming" of MSCs before transplantation is under consideration for some complex diseases such as cancer. MSC1-based therapy was capable of attenuate tumor growth

in vitro and in vivo while both MSC2-based and unprocessed MSC-based were not (111). Besides evaluating different outcome of these distinct populations, the "priming" of MSCs prior to injection allows a more standardized cell therapy.

Another issue to be considered is that the MSC isolation process itself results in a heterogeneous population composed by distinct cell types with different morphologies, differentiation potential and expression of surface markers. These differences are also related to their biological properties (112,113). Therefore, studies evaluating outcomes of MSC therapy with cells obtained from different donors are very important, especially due to the possibility of using autologous transplants for future treatments.

The cell sorting technique has been used to identify and purify subpopulations of MSCs (114–117). One particular subset of cells, namely, pericytes, which express the CD146 surface marker, could be of great use for ALS. Pericytes surround endothelial cells in capillaries and microvessels (118) and adventitial cells around larger vessels (114). The therapeutic potential of pericytes was attested for some conditions, such as muscle-linked diseases (119,120) and myocardium infarction (121). In these experiments, pericytes were shown to migrate and exert paracrine functions.

In the central nervous system, pericytes assist the formation and maintenance of the blood brain barrier, which, when impaired in neurodegenerative diseases, accelerates progression of symptoms (122). In ALS patients, a 50% reduction of perycites is found in the blood-spinal cord barrier (123). The evaluation of these cells' therapeutic effect in ALS models is very important and is currently being carried out in our laboratory. These are examples where FC and other cell-sorting techniques are key tools that allow us to better understand the biology of determined cell types, helping to better design an efficient cell therapy.

Cell quantity and injection protocols. Protocols used for cell transplantations in SOD1 mice are quite diverse, and one of the main variations is the number of cells per injection. It was shown that, in SOD1 mice, a high-dose transplant (3.9×10^5 cells) of neuronal hNT cells does not provide any additional benefit when compared with a lower-dose transplant (1.3×10^5 cells; (124), but when it comes to injecting mononuclear human umbilical cord blood cells into the same mouse model, a cell dose of 25×10^6 cells had the most beneficial effect on many therapeutic parameters than a lower (10×10^6) or higher dose (50×10^6) (125). Thus, the optimal amount of cells to be injected may vary according to the cell type.

Another important issue is the number of injections during the treatment period, allowing MSCs (or other cells used in therapy) to remain in contact with the damaged tissue for longer periods of time which could increase their beneficial effects. One previously mentioned study showed that two injections of 10^6 neural-induced MSCs in SOD1 mice led to a higher efficacy when compared with results obtained with a single dose (89). A triple injection of MSCs into the cerebrospinal fluid of SOD1 mice yielded significant therapeutic effects, including attenuated weight loss, enhanced motor performance, decreased motor neuron loss, and increased survival whereas a single transplantation did not alter disease progression (126).

Although there is increasing evidence that the optimal amount of cells and the transplantation method are crucial aspects for the therapy to succeed, only a few studies have been specifically designed to investigate these practical issues. Also, these studies illustrate that there are physiological differences among cells obtained from different tissues emphasizing the need to better understand the influence of the original niche in cell interaction and its role in determining what type of cell is the best to be administered.

Site of injection. Some clinical trials involving neurological diseases have shown that intraspinal injection of cells is a safe procedure (108,127). In another scenario, several clinical trials with non-neurological diseases have indicated that intravenous administration of MSCs is also a safe procedure (128,129). New approaches, such as the phase II clinical trial led by Brainstorm Company are also considering intramuscular injection of cells for ALS therapy. An important question to be addressed is which injection site for MSCs could yield the best results.

However, intraspinal (intrathecal) injections may facilitate the migration of MSCs through the neuroaxis since cells would be placed near the damaged tissue. However, intrathecal injections are a risky procedure and can lead to several side effects. Microinjection platforms, like the one patented by NeuralStem, are being developed and standardized to reduce eventual side effects from surgery and optimize the procedure, allowing the delivery of cells along the entire length of the spinal cord with only one injection (109).

Intravenous injection is a simple technique with few risks involved. Despite the lower risks, many cells may eventually end up homing to other tissues such as lungs and lymph nodes (127), interfering with systemic homeostasis and reducing the total number of cells available for engraftment in the damaged tissue (127). Even so, it has been shown that MSCs may migrate through the blood to the damaged areas of the CNS in response to inflammatory signals, and also that they are capable of exerting peripheral immunomodulating effects (130,131). This migration may be a consequence of the breakdown of blood vessels in the region where the tissue degenerates (89), or may occur through transiently formed intercellular gaps on the BBB (132).

Much attention has been given to spinal injections and intravenous injections, and only recently scientists are starting to show interest in injecting cells at the neuromuscular junctions. The motor neurons, before dying, retract, hampering these junctions, and, consequently, muscle function (133). If the neuronal protection is to start at the earliest stages possible, it may be interesting to promote such preservation on the sites that suffer the earliest injuries. The delivery route adopted for cell injection has an important role in

determining whether MSCs will be susceptible to early immunological recognition and rejection (134), and influences therapeutic efficiency, determining the success or failure of a particular treatment. To help circumvent these issues, methods for tracking the injected cells must be included in the research/therapy. MRI, positron emission tomography imaging and other cell imaging techniques are tools that could be extremely useful (135).

Donor age and autologous × allogeneic transplant. MSCs obtained from ALS patients' bone marrow have shown the same expansion and differentiation potential as cells obtained from healthy controls (83). Recently, possible correlations between certain biological markers and efficacy for autologous MSC transplant treatment was investigated. The levels of VEGF, ANG, and TGF-β were significantly higher in cultured autologous MSCs from ALS patients that responded to the therapy than in nonresponders patients (136).

However, in an autologous cell-based therapy for this late-onset disease, another factor must be taken into consideration: MSCs can suffer the effects of ageing (137), limiting the extension of autologous transplant, especially for late-onset diseases such as ALS. An overall decline in efficacy in cell therapy happens with the increase of both the age of the donor and the recipient (138,139). In addition, since a substantial number of ALS cases is genetic, the autologous transplant could be potentially harmful. Some of the proteins mutated in ALS are secreted (such as VAPB, for instance), raising the possibility of an additional motor neuron damage.

Heterologous transplantation is under investigation for NSCs (obtained from an 8-week-old aborted fetus) in ALS patients as mentioned before. In these trials, the immunosuppression protocol was designed based on a standard protocol used for whole organ transplantation, since there was no previous experience with immunogenicity of transplantation of allogeneic fetal-derived stem cells into the nervous system. In these studies, the majority of nonserious adverse events were attributed to the toxicities of the immunosuppressant drugs. Besides of being toxic to patients, steroidal immunosuppressants were found to complete abolish the therapeutic effect exerted by MSCs on a mouse model of liver cirrhosis (140). This specific issue must be better analyzed since it may hinder these trials.

Interestingly, it has been hypothesized that MSCs may have a certain degree of "immunological privilege," since they have been effective for allogeneic cell therapies and xenogeneic transplants as well (141,142). If MSCs have such strong immunomodulatory capacities, they might even be obtained from a young, healthy donor, and administered without any immunosuppressants, benefiting patients significantly.

It was observed that MSCs can modulate both innate and adaptive immunity by releasing molecules such as the Factor H (inhibiting the complement, and thus avoiding acute and hyperacute rejection mechanisms normally mediated through this system); prostaglandins and indoleamine dioxygenase, which suppress the effector functions of NK cells (that play a key role in the initiation and regulation of adaptive immune responses after transplantation); last but not least, PD1/PDL1, which inhibits B cell activation with a role in chronic rejection (134,143).

MSC transformation after in vitro expansion. MSC transplantation, like all cell therapies, requires the expansion of cells in culture before injection. Although it has been shown that MSC are less prone to genetic abnormalities and malignant transformation during multiple passages in vitro (144,145), other studies show that MSCs can undergo malignant transformation after in vitro expansion (146). A recent study demonstrated that MSCs already show a significant increase in chromosomal aberrations after their fifth passage (147). Thus, routine procedures to check the possible transformation, chromosomal abnormalities and telomere shortening of these cells should be included in preclinical and clinical trials as a quality control to ensure the safety of the method.

Since karyotype analysis before every round of injections is very laborious, focusing on a simpler method for observing DNA abnormalities would be very productive. Some attempts to do so are occurring in the field of cancer research with the high-resolution DNA FC (148), guaranteeing the safety of the cells to be injected in therapy. Cells containing DNA abnormalities have a higher transformation potential.

Final Considerations

Finding a therapy for ALS is a great challenge. Different pathways culminating into the same symptoms point out to more than one solution. Even so, many important steps have been made, and there is some consensus regarding the types of therapy best suited for ALS. Cell replacement therapies for motor neurons seem highly unlikely, due to the necessity of the correct engraftment and innervation, the maintenance of a nontoxic environment and the control of the noncell-autonomous pathways that could culminate into motor neuron death. Preclinical studies have been performed transplanting motor neurons into mice with ALS, but without much success. However, neuronal protection seems a very promising therapy. With neurotrophic factors being released by transplanted NSCs or MSCs, the disease onset might be delayed and/or disease progression might decelerate and, hopefully, stop altogether. With this in mind, it is necessary to plan carefully injection protocols, immunosuppressant regimen, injection sites and cell type to be administered to guarantee that these factors will not hamper the trials.

While the therapy with NSCs under investigation by the NeuralStem company seem promising for ALS treatment, one must not forget that the cells were obtained from an 8-week old aborted fetus. Before approval in clinical trials, some ethical questions and discussions may probably arise again. Since the only source of native NSCs is the nervous system, will we be able to use them in a larger scale for all patients? The advantages of MSCs, like the facility to obtain them without ethical issues, their immunomodulatory properties and their production of important neurotrophic factors, turn them into

the best-suited cells for therapy. However, a more profound knowledge of the "black-box" of MSC secretion must be understood before assuming their beneficial effects. What do these cells secrete in vivo, and how do these molecules act on motor neurons and their surrounding cells? Do cells obtained from patients have a potential harmful effect?

Another important factor that directly impacts all clinical trials and studies is the fact that the diagnosis for ALS may take up to 14 months (149). Therefore, it is imperative to diagnose the disease in the earliest possible stages. As suggested for Parkinson's disease and Alzheimer's disease, iPSC models could provide excellent tools and opportunities to investigate the course of changes during the disease progression, from the asymptomatic phases through the later stages when the pathology has become prominent (150). Early diagnosis would increase the chances of more successful clinical trials, since neuron degeneration may be well advanced when the symptoms starts.

In summary, preclinical studies with MSCs and NSCs show exciting results. There are, however, parameters that must be reconsidered when it comes to clinical trials in ALS patients, as mentioned above. If overlooked, they may delay therapeutic progress, and even contribute to the lack of positive results reported for most ALS trials. To eventually transform cell therapy for ALS from myth to fact, important issues addressed in the present review should be taken into consideration.

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Supplemental Section 2

Next-Generation Sequencing: MiSeq (Ilumina) Panel

Sample Prepatation Summary(extracted from "Nextera Enrichment DNA Sample Preparation Guide" - Ilumina).

The first step towards sample preparation is to fragment DNA and add adapter sequences do the 5' and 3' ends, which will allow PCR amplification in subsequent steps. The *Nextera Transposome* will then be responsible for generating the tagmented DNA molecules, which will be purified and amplified by PCR. In this step, two indexes are added for sample identification (allowing multiplexing), and adapters that allow not only the formation of clusters on the sequencing chip, but also the sequencing process itself. In order to guarantee quality results, the DNA library generated is purified in a process that separates the fragments that are too small and might hamper sequencing.

Next, probes are added to the DNA library in order to enrich it for the sequences of interest. If necessary, samples can be pooled in this step. Streptavidin beads are used to separate the probe-DNA hybrids, which is followed by 3 washing steps, and the elution of DNA. The steps of streptavidin capture and elution are repeated once more.

The DNA fragments are then amplified by PCR and quantified by real-time qPCR, for optimal clustering on the sequencing chip. They are then added to the chip, which contains sequences complementary to the adapters on the fragments, and amplified by bridge amplification, generating clone clusters for each sequence. A universal primer anneals to the adapter sequence in all DNA fragments, and sequence is captured by the fluorescent signal of ddNTPs being incorporated to the strand. After incorporation, the fluorophore is cleaved, and another ddNTP is incorporated, and so forth. After results are obtained, patient identification can be done using the attached index sequences.

***VAPB* ALS8 Genotyping by HaeIII digestion**

The c.C166T mutation in VAPB is located in the HaeIII enzyme cut site. PCR amplicons can be thus digested and analyzed by gel electrophoresis; patients carrying the heterozygous mutation will present two bands in the gel, while normal controls will only present one.

Protocol

1) *VAPB* Exon 2 PCR

The first step in the protocol is the amplification of the exon in which the mutation is located. Reaction mix (all reagents were purchased from Invitrogen - Life Technologies) and cycling temperatures are shown in Tables S1.1 and S1.2, respectively.

VAPB Exon 2 Primer Sequences: Forward: 5' CAG CTC TCT TTT CCA CAA ACC 3'. Reverse: 5' CTA CTG TCC AGG GGC CTT CT 3'.

2) HaeIII Digestion and Gel Electrophoresis

The digestion mix is prepared as shown in Table S1.3, and incubated at 37˚C for 90 minutes. After digestion, digestion products are run in a 3% agarose gel, 220V for 30-40 minutes.

Reagent	Volume (µl)	Final Concentration
10X PCR Buffer	1µl	1X
$MgCl_2$ 50mM	0.3µl	1.5 mM
dNTPs 2.5mM	1µl	0.25 mM
Forward Primer 20µM	0.5µl	1µM
Reverse Primer 20µM	0.5µl	1µM
Taq Polimerase 5U/µl	0.1µl	0.05U
H_2O PCR-grade	5.6µl	-
DNA	**1µl**	-
Total Reaction Volume	**10µl**	-

Table S1.1: Reaction Mix for *VAPB* exon 2.

Temperature	Time	
95˚C	5 min	
95˚C	30s	Steps 2-4: 30X
57˚C	30s	
72˚C	30s	
72˚C	10min	
4˚C	-	

Table S1.2: Cycling temperatures for *VAPB* exon 2.

Reagent	Volume (µl)
10X HaeIII M Buffer	1µl
HaeIII enzyme (10U/µl)	0.1µl
H_2O PCR-grade	3.9µl
PCR Product	5µl
Total Reaction Volume	**10µl**

Table S1.3: Digestion Mix.

***VAPB* ALS8 Genotyping by High Resolution Melting (LightCycler 480 - Roche)**

The High Resolution Melting (HRM) technique is a fast, sensitive method for discovering genetic variation. Currently, the main application for HRM is gene scanning, i.e., identification of heterozygotes in target-gene-derived PCR amplicons in a fast, cost-effective manner.

Double-stranded DNA possess characteristic melting temperatures, in which 50% of strands are dissociated. This temperature depends on sequence, length, GC content and complementarity between strands. Single-nucleotide variants, such as the mutation responsible for ALS8, are capable of shifting an amplicon's melting temperature.

Based on these principles, the HRM method involves the following steps: PCR, with the master mix containing a fluorescent dye (which will be incorporated into amplicons as DNA is synthesized); and a melting curve, in which temperature rises gradually and DNA strands start dissociating. In this last step, the fluorescence is captured and the melting temperature is calculated. The positive control and negative control used in the experiment allow a precise genotyping of patient samples.

Protocol

1) DNA quantification and dilution

DNA samples were first quantified using NanoDrop, and subsequently diluted to the concentration of 5ng/µl. Total DNA used per reaction is 25ng.

2) Primer Mix

VAPB exon 2 primers are diluted to a concentration of 4µM (pooled). 1µl of this mix is used per reaction. Their sequences are described in the "HaeIII Digestion - VAPB Exon 2 PCR" Section.

3) Reaction Mixture

The kit used for HRM reactions was the High Resolution Melting Master (Roche) (catalog number 04 909 631 001). Reaction was optimized for the concentrations shown in Table S1.4.

4) Run Settings

Cycling parameters are described in Table S1.5.

5) Analysis

Analyses were performed on "Gene Scanning" mode, using the LightCycler480 software default settings. Negative control was set to reference sample, while positive control was compared to patient samples for genotyping. Results are obtained as illustrated in Figure S1.1.

Reagent	Volume (µl)	Final Concentration
Master Mix (2x)	10µl	1X
MgCl2	2µl	2.5mM
Primer Mix 4µM	1µl	0.2µM
H_2O PCR-grade	2µl	-
DNA	5µl	25ng total
Final Reaction Volume	**20µl**	-

Table S1.4: Optimized reaction volumes for HRM genotyping of the c.C166T variant.

Step	Temperature	Time	Ramp Rate
Pre-Incubation	95˚C	10min	-
Amplification	95˚C	10s	-
	59˚C	15s	-0.5˚C per cycle until target temperature of 57˚C.
	72˚C	12s	
High Resolution Melting	95˚C	1min	-
	40˚C	1min	-
	75˚C	1s	1˚C/s
	95˚C	-	0.02˚C/s, with 25 acquisitions per ˚C.

Table S1.5: Optimized run settings for HRM genotyping of the c.C166T variant.

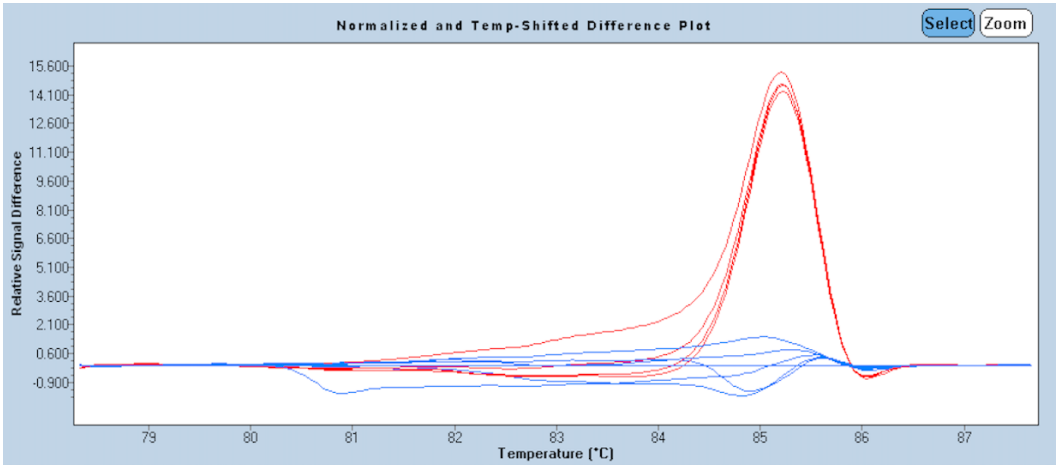

Figure S1.1: HRM results. The blue lines indicate samples that are similar to the reference sample (negative control). In red are shown patients carrying the c.C166T mutation, whose amplicon melting temperatures match the positive control.

Supplemental Section 3

Peripheral Blood Collection

Patient blood samples were collected using the PAXgene Blood RNA Tubes (PreAnalytiX - Qiagen), whose additives allow for the storage of blood and stabilization of intracellular RNA. About 2.5 ml of blood was collected for each tube, which were incubated at room temperature for two hours for cell lysis.

RNA isolation from Whole Blood

The PAXgene Blood RNA Kit was used for RNA isolation, according to manufacturer's protocols. The process begins with a centrifugation step to pellet nucleic acids in the PAXgene tube. This pellet is then washed, and proteins are subsequently digested using proteinase K. Lysates are filtered to remove cell debris, and DNase I is added to remove residual DNA. Remaining contaminants are removed in following washing steps before RNA is eluted.

cDNA Synthesis

For cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems - Life Technologies) was used, according to manufacturer's protocols.

Real-Time Quantitative PCR - TaqMan Assays

RT-qPCR assays using TaqMan are widely used due to the large gain in specificity when compared to techniques based on SYBR-Green reagents. The TaqMan probes consist of a fluorophore covalently attached to the 5' end of an oligonucleotide and a quencher at the 3' end. While in close proximity with the quencher molecule, the fluorophore will not emit any fluorescence. The oligonucleotide sequence is specific for a DNA region, and anneals to single-strand DNA. When the Taq

polimerase starts amplification, its exonuclease activity degrades the oligonucleotide probe, which frees the fluorophore and breaks its proximity to the quencher, emitting then a fluorescent signal. The detected fluorescence is proportional to the amount of free fluorophore, and consequently to the amount of DNA in the analyzed sample.

The probes used for the analysis of *VAPA*, *VAPB* and *EPHA4* are the following:

- *VAPB*: Hs00191003_m1;

- *EPHA4*: Hs00177874_m1;

- VAPA: Hs00427749_m1.

Reaction was prepared according to manufacturer's protocols.

Statistical analyses were performed using GraphPad Prism 6.

Supplemental Section 4

Peptide Purification Protocols

Peptides were synthesized by GenScript, but not purified. Therefore, the purification process was done in our laboratory. Two Onyx monolithic C18 columns (50 x 4.6 mm) were coupled in series, and eluted with a linear gradient of 3-30% in 20 minutes of acetonitrile (ACN) with 0.08% TFA, and the aqueous phase 3% ACN with 0.1% trifluoroacetic acid (TFA) in H_2O. The column and mobile phases were loaded on a Shimadzu equipment, consisting of two LC-10ADvp pumps, one DGU-14AM degasser, a SCL-10Avp system controller, a 10AF autosampler, a UV SPD-10A detector, and a CTO-10A column heater.

After this process, purity was evaluated by UPLC in the Waters I-Class equipment. The column used in this step was a Waters BEH C18, sub 2µ, 15cm, eluted with a binary gradient of 1-45% 0.1% TFA in ACN and 0.1% TFA in H_2O. Chromatographic peaks were integrated, and peptide peak areas were divided by the total areas obtained. Those peptides with >98% purity were stored at -30˚C, while those with lower results went through a second purification process, using the Waters I-Class equipment, a Phenomenex Gemini 5µ 110^A column, 250 x 4.6mm eluted with a gradient of ACN (1-30% in 30 minutes) and 20mM ammonium formate (pH = 10).

The VAPC peptide YCV(…) contains cysteines, which can generate bisulfide bonds. Therefore, this peptide was incubated with dithiothreitol (DTT) and alkylated with iodoacetamide, and subsequently re-purified.

Shotgun Mass Spectrometry Parameters

Initial shotgun analyses were performed using a Q-Exactive (Thermo) mass spectrometer, coupled to a Nano Acquity nano-UPLC system (Waters). Chromatographic separation was performed in a system equipped with a Symmetry C18 5µm (180µm x 20mm) capture column and a HSS T3 1.8µm (75µm x 150mm) analytical column, eluted in the following mobile phases: (A) 2% dimethyl sulfoxide (DMSO), 0.1% Formic Acid (FA) in H_2O; (B) 5% DMSO, 0.1% Formic Acid in ACN. Samples are injected in the chromatographic system, desalted in the capture column, and subsequently separated in the analytical column by an increasing gradient of (B). The Q-

Exactive was equipped with a nano-lectrospray ion source, a quadrupole mass filter for precursor ion selection, a C-trap for ion trapping and a HCD (Higher Energy Collisional Dissociation) collision cell, and an orbitrap mass analyzer. Shotgun acquisitions are obtained in the Full-MS / dd-MS2 acquisition mode (Top 15), which comprehends a full scan of masses without applying any fragmentation, followed by a data-dependent acquisition of the 15 most intense ions after fragmentation in the HCD cell. This process is repeated throughout all of the chromatographic run, as peptides are eluted from the column. Data analysis is performed in the MaxQuant software (Cox & Mann, 2008).

VAPA, VAPBwt and VAPB P56S plasmid amplification using DH5α competent cells (*E. coli*)

pFlag-CMV2 plasmids containing the coding sequences for *VAPA, VAPBwt* and *VAPBmut,* and containing the Flag tag sequence (C-terminal DYKDDDDK) (Prosser et al., 2008) were amplified from the transformation of a competent *E coli* strain (Library Efficiency DH5a - Life Technologies), according to manufacturer's protocols. Transformed bacteria were cultivated in a petri dish containing LB-agar medium and ampicilin for 24h. One colony from each dish was selected to grow in liquid LB medium, where they were cultivated overnight. The plasmids were extracted using the QIAprep Spin MiniPrep Kit (Qiagen). DNA sequencing was performed for coding sequence validation.

Cell Culture Protocols - SILAC and Cell Transfection

The following protocols were performed using HEK293T and HeLa cell lines.

SILAC

Cells were cultivated in DMEM SILAC Media (Life Technologies), supplemented with PenStrep (1%), FungiZone (1%; Life-Technologies), Arginine (34.2 nmol; labeled with N^{15} and C^{13} - Cambridge Laboratories, or unlabeled - Sigma), Lysine (28.7 nmol - labeled with N^{15} and C^{13} - Cambridge Laboratories, or unlabeled - Sigma), Glutamine (unlabeled - Sigma), and sodium pyruvate (1X; Life Technologies). Cells were cultivated in their respective medium (heavy or light) for 6 doublings.

Cell Transfection with Lipofectamine

10µg of plasmid DNA was diluted in 1ml of DMEM culture medium; in a second tube, 15µl of Lipofectamine 2000 (Invitrogen - Life Technologies) was diluted in 1ml of culture medium. After 5 minutes of incubation at room temperature, DNA and Lipofectamine are mixed and incubated for another 20 minutes at room temperature for liposome formation. In a $25cm^2$ bottle (T25) with approximately 90% confluence ($8\text{-}9x10^6$ cells), about 4.75µg of DNA (with 7.13µl of Lipofectamine) is added. Transfection occurs for 24h; the culture medium is subsequently changed, and 48h after transfection, cells are removed and proteins are extracted and quantified.

Protein Extraction, Digestion and Quantification

RapiGest Protocol for protein extraction and digestion.

Cell pellets are resuspended in 200µl of a 0.2% RapiGest (Waters) solution. RapiGest if a surfactant agent that denatures proteins. Proteins are then reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and digested with proteomic-grade trypsin for 12-16h. The excess RapiGest is hydrolyzed with the addition of trifluoroacetic acid (TFA) and the residues are removed by centrifugation.

Protein Quantification - Bradford Protocol

For protein quantity determination, the Bio-Rad Protein Assay (Bio-Rad) was used, according to manufacturer's protocols.

SDS-Page and Western Blot Protocols

SDS-Page

The poliacrylamide gels were prepared in two steps. First, the separation gels was prepared with 15% acrylamide concentration; after polymerization, the stacking gels was prepared (4% acrylamide), on top of the separating gels. A 45 minute run at 200V, 150W, 650mA is enough for VAPA or VAPB proteins to migrate to the middle of the gel.

Transfer to Nitrocellulose Membrane

If the gel is run for Western Blot Purposes, the proteins are transferred to a nitrocellulose membrane (Amersham Protran 0.45µm - GE Healthcare) in the Perkin Elmer Lightning Blotter Transfer System.

- Transfer Buffer: 49mM Tris; 39mM Glycine; 20% Methanol; 0.04% SDS.
- Filter papers used for stacking: Whitman Chromatography Paper 3mm.

Membrane Blocking and Antibody Incubation

Membranes are incubated with 5% Bio-Rad Blotting-grade Blocker in TBS-T (0.1% Tween Tris-Buffered Saline) for one hour at room temperature or overnight at 4˚C, for blocking available protein binding sites in the membrane. They are subsequently incubated with the primary antibody for two hours at room temperature, washed three times with the TBS-T buffer, and incubated for one hour with secondary antibody. The VAPB monoclonal antibody used is described in Mitne-Neto et al., 2011. The anti-Flag monoclonal antibody was purchased from Sigma-Aldrich (F3165). The anti-mouse-HRP antibody was purchased from Cell Signaling (#7076S). To reveal membranes, these are incubated for 5 minutes with the ECL-Prime reagent (Amersham - GE Healthcare), whose green light chemiluminescence is detected by the Hyperfilm ECL (Amersham - GE Healthcare). The film is then washed in Carestream Kodak autoradiography GBX Developer (Sigma-Aldrich), subsequently in water, and fixed by the Carestream Kodak autoradiography GBX Fixer (Sigma-Aldrich).

VAPB cloning, expression and purification (*coloca aqui os créditos pro pessoal do lab do luis netto?*)

VAPB cloning in the pET15b vector

The previously obtained pFlag-CMV2-VAPB plasmid was used for amplifying the coding sequence through PCR. The primers used in this reaction contained NdeI and BamHI restriction enzyme cut sites, which allowed us to digest the amplicons, and insert them into the previously cleaved vector. The following primer sequences were used (restriciton enzyme cut sites are underlined) - Forward (NdeI): ATTCGC CAT ATGGCGAAGGTGGAGC; reverse (BamHI): T GGATCC CTA CAA GGCAATCTTCCCA.. After coding sequence amplification, the PCR product was purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Life Sciences), and digested with NdeI and BamHI enzymes (New England Biolabs) at 37˚C for one hour. The pET15b plasmid was digested using these same conditions. pET15b and VAPB coding sequences were ligated using the T7 ligase enzyme (Promega) at a PCR product:vector ratio of 3:1. The newly synthesized vector was then dialyzed using MF-Millipore dialysis membranes for 30 minutes. After all these steps, the final product was ready for bacterial transformation.

Bacteria Transformation through Electroporation

After plasmid preparation, these are first inserted into DH5α bacteria for amplification, and then in an expression *E. coli* strain (in this case, BL21 Codon-Plus (DE3) RIPL). The transformation of electrocompetent bacteria is described below.

100µl of bacteria was added to 5µl of dialyzed plasmid, and this mixture was inserted into an electroporation cuvette. The bacteria were then shocked with 2.5kV and 25uF capacitance. Transformed bacteria were then incubated for 1h in liquid LB medium (described below), and then spread in a petri dish containing LB-agar culture medium. They were incubated at 37˚C overnight, and colonies were then picked for sequencing. Those containing the correct VAPB coding sequences were chosen for expression tests.

Bacteria Culture Media

Liquid LB medium: 0.5% Yeast Extract, 0.5% NaCl.

LB-agar medium: 1% Triptone, 0.5% Yeast Extract, 2% agar.

5X Salt Solution for M9 Minimal Medium: NH_4Cl (5g); Na_2HPO_4 (30g); KH_2PO_4 (15g); NaCl (2.5g), diluted in 1L of MilliQ H_2O. Autoclave.

M9 Minimal Medium: 200ml of the 5X salt solution; 1ml of $MgSO_4$ 1M, 10µl of $CaCl_2$ 1M, and 500µl of 80% glicerol.

VAPB Expression in BL21 Codon-Plus (DE3) RIPL in M9 Minimal Medium

A pre-inoculates of bacteria were grown overnight at 37˚C in 50ml of liquid LB medium containing ampicillin and chloramphenicol, with a 200rpm agitation. On the following day, the pre-inoculate is diluted centrifuged, and bacteria as resuspended in 1L of M9 culture medium. The optical density of the culture is then measured at 600nm. When between 0.6 and 1, 1ml of IPTG 1M is added to the culture, which is then incubated at 20˚C overnight, at a 200rpm agitation. After expression, the bacteria were centrifuged and stored at -20˚C or used for protein extraction.

Protein Extraction and VAPB Purification

Bacteria pellets were resuspended in 20ml of the following buffer: 50mM Sodium Phosphate, pH = 7.4;, NaCl 300mM, and β-Mercaptoethanol 10mM (**Buffer A**), and vortexes until complete resuspension. This solution

was then sonicated (Branson Digital Sonifier equipment) at 40% amplitude in 20s pulses and 1min intervals for cell lysis. This extract is centrifuged at 15000rpm for 30min at 4˚C for soluble fraction separation. The supernatant is then filtered and is ready for the protein purification steps.

- Step I: Ni column preparation. A 1ml Ni column (Ni-NTA Superflow Cartridges - Qiagen) is washed with 10ml of MilliQ H_2O, and loaded with 2ml of a $NiSO_4$ 100mM solution, and the nickel remains as stationary phase inside the column. It is then washed again with 10ml of MilliQ H_2O, for excess nickel removal, and subsequently equilibrated with Buffer A. Mobile phases are pumped using a peristaltic pump.
- Step 2: The protein extract is carried through the column, and all the fraction of non-retained proteins is collected. This is where the VAPB protein will be located - it initially does not bind to the column. This step is used to remove VAPB fragments from our sample. The remaining proteins are eluted with the **Buffer B** (50mM Sodium Phosphate, pH = 7.4, 300mM NaCl, Imidazole 500mM). The column is then again washed with 10ml of MilliQ H_2O.
- Step 3: Triton X-100 is added to both buffers A and B, and to the non-retained protein fraction containing VAPB, to a final concentration of 1.5%. This will allow VAPB binding to the chromatographic stationary phase. The column was then equilibrated with the new buffer A containing Triton X-100. The non-retained protein fraction is then injected once again in the column, and this time, the VAPB protein will bind to the stationary phase.
- Step 4: The loaded column is added to an Äkta FPLC System (GE Healthcare), previously washed with H_2O and loaded with buffers A and B. 10ml washes are performed, with increasing concentrations of buffer B: 0% (only buffer A), 10%, and 100%. Fractions of eluted proteins are collected throughout all steps, and VAPB presence and purity as later evaluated by SDS-Page and Western Blot.

Final LC-MS/MS run parameters for the VAPA / VAPB / VAPC Targeted Assay

Chromatographic parameters

Equipment: NanoAcquity UPLC System (Waters)

Column: HSS T3 1.8µM (75µm x 150mm)

Mobile phases: (A) 2% dimethyl sulfoxide (DMSO) / 0.1% Formic Acid / H_2O

(B) 5% DMSO / 0.1% Formic Acid / Acetonitrile (ACN)

> Column Flow: 400µl/min. Run Time: 45 minutes. Run Gradient: Start: 10% of (B); 3 minutes: 10% of (B); 30 minutes: 32.5% of (B); 35 minutes: 80% of (B); 40 minutes: 80% of (B); 41 minutes: 10% of (B).

Q-Exactive Parameters:

Tune file settings:

Scan Parameters: Microscans: 4; Scan range: 140-1000 m/z; Polarity: positive.

NSI Source:

- Spray Voltage: 2.1kV. Capillary temperature: 250˚C. SLens RF Level: 55.

Method file settings: two modes were activated - a Full Scan (MS), followed by PRM.

Full Scan Settings (Full MS - SIM)

- Run time: 0-45 minutes. Polarity: positive. Resolution: 35000. AGC Target: $2x10^5$. MaxIT: 50. Scan Range: 400-1600 m/z.

PRM Settings (PRM)

- Default charge: 2. MS^2: Resolution: 17500. AGC Target: $5x10^4$. MaxIT: 200 m/z. Isolation window: 2m/z. Fixed first mass: 150 m/z. Spectrum data: centroid.

PRM Inclusion List - contains mass-to-charge ratios searched when in PRM mode, the charges for each peptide, the collision energy used for each one, and the peptide sequence. Peptide YCV(…) is alkylated; therefore, the mass of the carbamidomethylation was added to the total peptide mass.

Mass [m/z]	CS [z]	Polarity	(N)CE	Peptide
809.44596	2	Positive	17	HEQILVLDPPTDLK
1,156.55813	2	Positive	28	FMVQTIFAPPNTSDMEAVWK
824.4079	2	Positive	28	VAHSDKPGSTSTASFR
824.94887	2	Positive	22	VEQVLSLEPQHELK
1,161.01798	2	Positive	28	FMVQSMFAPTDTSDMEAVWK
785.92539	2	Positive	28	TVQSNSPISALAPTGK
736.03929	3	Positive	20	YC[+57.0]VRPNSGIIDAGASINVSGR
1,408.61278	2	Positive	35	WTADEEDSAEQQPHFSISPNWEGR

Table S3.1: PRM Inclusion List, containing the m/z for mass filtering, charge state of each peptide, collision energy and peptide sequence.

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