Phenotypic heterogeneity in amyotrophic lateral sclerosis type 8 and modifying mechanisms of neurodegeneration

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In his accounts "On longevity and shortness of life", Aristotle considered how diseased states could be interchangeably associated with long and short lifespans. He believed that the presence of opposite elements and the environment were the sole determinants of this variability. Nowadays, also struck by the same phenomenon observed by the Greek philosopher, human geneticists are still trying to define the etiological basis of the phenotypic plasticity in neurodegenerative disorders. Among such diseases, amyotrophic lateral sclerosis (ALS) stands out as a highly heterogeneous condition. Patients affected by ALS commonly start manifesting symptoms such as weakness in the upper or lower limbs, difficulty in climbing stairs, fasciculations and loss of muscular mass. As the disease progresses, patients become wheelchair-bound and bulbar signs such as dysarthria and dysphagia become more pronounced. Death occurs on average after 3 years of onset, mainly due to respiratory failure (Renton et al., 2014). However, this canonical course is frequently variable, with a myriad of phenotypic alterations (Swinnen and Robberecht, 2014). Here, we describe different aspects of amyotrophic lateral sclerosis type 8 (ALS8) clinical variability, both in terms of clinical manifestations and in rate of disease progression. Then, we outline our recent work on ALS8 patients (Oliveira et al., 2020), in which we tried to address the molecular underpinnings of clinical progression variation in the patients we studied. We were able to rule out well-described genetic modifiers, such as *EPHA4* and *UNC13A*, and potential copy number variation alterations. Interestingly, both cell death rates and energetic metabolism appeared to be different among the severe ALS8, mild ALS8 and controls, suggesting an attenuation of pathological process in the less affected patients. Whole transcriptomic analysis of induced pluripotent stem cells (iPSCs)-derived motor neurons pointed that both "mild patients" presented 43 upregulated and 66 downregulated genes, when compared to controls and the "severe" group. Interestingly, most of the identified genes were associated with protein synthesis and protein targeting to endoplasmic reticulum (ER). Expression of protein translation markers' pMTOR, 4EBP1 and RPS6 were found to be high in the mild ALS8 individuals, when compared to both controls and the severe group. To sum up, our data point that mitigating factors are most likely preventing neurodegeneration in ALS8 through maintenance of protein synthesis. Further studies, assessing the relationship among these potential genetic modifiers and the pathophysiology in ALS8, are fundamental. They might shed light on venues for treatment of this devastating disease.

ALS8: First reported in Brazil, ALS8 is an autosomal dominant form of ALS that mainly presents spinal onset phenotypes (Nishimura et al., 2004a). As a result, patients often develop weakness, fasciculation, muscular atrophy and tremor. Since its initial description, in a seminal paper reporting seven related families, a remarkable clinical variability was noted. Patients were classified as "typical ALS8" or "atypical ALS8", showing a rapid progression and differing from each other by the presence of tremor in the "atypical group". Additionally, other affected patients from the same kindred were classified as carrying late-onset spinal muscular atrophy (Nishimura et al., 2004b). Linkage analysis, followed by Sanger sequencing of candidate genes, identified in 20q13 a single mutation (C>T at exon 2) at the *Vesicle Associated Protein B* gene (*VAPB*; c.166 C>T). This pathogenic variant changed proline to serine at amino acid 56 of VAPB protein, and segregated with the three main phenotypic groups (Nishimura et al., 2004b). Further studies identified *VAPB* P56S variant in ALS patients from the USA, Germany and China, ruling out a potential founder effect (Funke et al., 2010; Di et al., 2016; Guber et al., 2018). More recently, other ALS families have been described with different *VAPB* mutations, namely T46I and P56H (Sun et al., 2017). Interestingly, all variants are located at VAPB's Major Sperm Protein domain. This region is implicated in multiple physiological roles, being responsible for interacting with other protein partners, among them PTPIP51, FAF1, ATF6 and others (Baron et al., 2014; Gomez-Suaga et al., 2019). A novel V234I mutation in *VAPB* was reported in one ALS patient who also carries a pathogenic repeat expansion in C9orf72 (chromosome 9 open reading frame 72), another ALS causative gene (van Blitterswijk et al., 2012), but the pathogenic potential of that VAPB mutation has not been assessed.

Recent experimental evidence has demonstrated VAPB is located at ER contact sites (Guber et al., 2018; Gomez-Suaga et al., 2019). Such ER junctions play crucial roles in tethering this organelle with mitochondria and endosomes, whereby it regulates Ca^{2+} transport, lipid metabolism, fission/fusion dynamics in mitochondria, autofagosome biogenesis and other physiological processes. However, the relative importance of such pathways for ALS8 onset and progression is not known.

ER – mitochondria junctions have been particularly studied in the context of neurodegenerative processes. The interaction of VAPB and PTPIP51, located in such specific sites, has been demonstrated to be determinant for synaptic stability in cultured hippocampal neurons (Gomez-Suaga et al., 2019). Failure in synapsis is a common pathway for multiple neurodegenerative processes, not only in ALS, but also Parkinson's disease and Alzheimer's disease. Therefore, it has been speculated that VAPB, through its interactions at ER contact sites, might be associated with common mechanisms of disease. Its overexpression in SOD1 mice, for instance, has been found to decrease neuropathology in this animal model (Mitne-Neto et al., 2011; Kim et al., 2016).

Using the Drosophila model of human VAPB-induced neurotoxicity, Sanhueza et al. (2015) identified potent modifiers of ALS8-mediated defects, involving a diverse set of biological functions such as proteolysis and vesicular trafficking, as well as endocytic trafficking and genes controlling proliferation and apoptosis. Guber et al. (2018) investigated a potential association of nucleocytoplasmic transport defects and ALS8, using patient's fibroblasts. The authors observed, apart from typical endoplasmic reticulum stress, augmented cytoplasmic Ran protein retention in affected individuals' cells. Although this hypothesis is interesting, as nuclear transport interruption is a hallmark finding in ageing and other neurodegenerative disorders, further analyses are still needed to consolidate alterations in this pathway with ALS8.

ALS8 patients present a great variability in age of onset. In some individuals, the first symptoms are observed in their 30's, while others remain asymptomatic until their late 60's. Progression of clinical manifestations also varies greatly, with some patients surviving with only mild alterations for

as long as 40 years, and others having a more typical ALS clinical course of < 5 years (Nishimura et al., 2004b). This finding suggests that mitigating factors might play a role in ALS8 progression. The search for genetic modifiers underlying ALS severity is of great interest since it might pave the way for novel therapies. In this scenario, the investigation of familial cases may be more informative, once patients carrying the same pathogenic mutation share more similar environmental influences and genetic background.

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

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

Aiming to further evaluate the mechanism underlying the observed discordant clinical progressions (Oliveira et al., 2020), iPSCs technology was used for obtaining motor neurons from each ALS8 patient (**Figure 1**), and functional studies were performed (Oliveira et al., 2020). Considering that ALS8 is probably caused by VAPB haploinsufficiency (Mitne-Neto et al., 2011), we first sought to investigate whether a higher expression of this gene in the iPSC-derived motor neurons

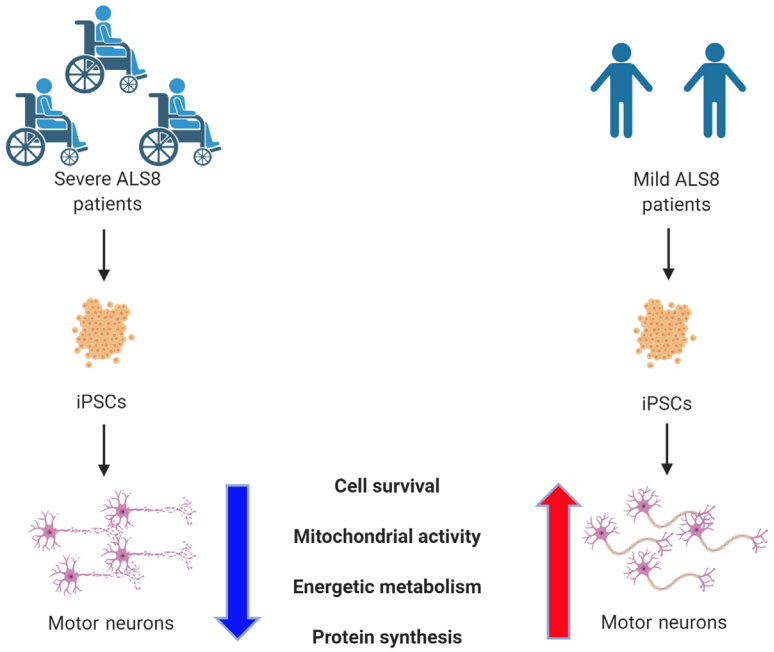

Figure 1 | Three severe and two mild ALS8 patients from the same family, all carrying the same VAPB P56S genetic mutation were studied along with three controls.
PBMCs were separately collected from these subjects, and then reprogrammed into induced pluripotent stem cells (iPSCs). iPSCs were differentiated into motor neurons, which were found to be dissimilar between the two ALS8 groups in terms of cell viability, mitochondrial activity, energetic metabolism and levels of expression of genes involved in protein synthesis; increased activity of such pathways in the motor neurons of mild ALS8 patients compared with the severe ones suggests mitigating mechanisms of neurodegeneration.

from milder patients could explain the phenotypic attenuation. Interestingly, *VAPB* was found to be equally downregulated in both affected ALS8 groups, regardless of their clinical status. Surprisingly, however, we observed that severe ALS8 patients presented higher degrees of cell death and lower oxidative metabolism than the controls and mildly affected individuals (Oliveira et al., 2020).

As mTOR signaling pathway is a key regulator of several aspects of neuron physiology, such as autophagy and protein synthesis, we set out to investigate its key components (pmTOR, 4EBP1 and RPS6). Interestingly, upon caloric restriction for 48 hours, we found increased levels of pmTOR in the mild ALS8 compared to severe ALS8 and controls. On the other hand, surprisingly, we identified a higher expression of 4EBP1 in one mild patient and RPS6 in the other, when compared to controls and the "severe" group (Oliveira et al., 2020). Such data suggested, therefore, that the mild ALS8 individuals present a high protein translation activity. The difference between 4EBP1 and RPS6 protein levels is most likely due to epistatic effects.

A whole transcriptomic analysis of the iPSC-derived motor neurons showed that severe ALS8, mild ALS8 and controls also differed among them regarding their gene expression patterns. Most interestingly, we were able to identify 43 upregulated and 66 downregulated genes in mild ALS8 motor neurons, when compared with the other experimental groups. Most of the upregulated genes, such as *RPL9*, *RPS3*, *RPS15A*, *RPL8* were associated with pathways regulating protein translation and targeting to ER (Oliveira et al., 2020). This finding further supported our western blotting assays, which suggested mitigating mechanisms based on higher protein synthesis (Oliveira et al., 2020). They were also in line with evidence from other experimental models. Through puromycin incorporation assays, Briese et al. (2020) observed impaired protein translation in mice motor neurons knocked down for *TARDBP* gene, highlighting the importance of this pathway for neuronal physiology. Interestingly, the authors also described gene expression alterations consistent with synaptic failure, and mitochondrial dysfunction (Briese et al., 2020). Analogous findings of *VAPB*-induced neurodegeneration were identified in *Drosophila*, strengthening the idea of VAPB as a regulator of protein synthesis in motor neurons (Deivasigamani et al., 2014).

Proteostasis has been pointed as a central aspect of ALS physiopathology. Spinal motor neurons, apart from their decreased capacity of triggering heat shock protein response, present a highly concentrated colloid, due to protein concentrations above their solubility levels (Yerbury et al., 2020). Because of

such characteristics, these cells appear to be more susceptible to protein homeostasis imbalances than other neural cell subtypes. Our recent work (Oliveira et al., 2020) goes in line with such observations, as it suggests that differentially expressed genes associated with translation are bypassing *VAPB* deficiency to maintain protein synthesis. This would result in greater cell viability and mitochondrial activity in mild ALS8 patients. It is not yet clear however, how ER – mitochondria contact sites and their key proteins, among them VAPB, regulate translation and coordinate it with synaptic output in motor neurons. Calcium efflux might be a good candidate-signaling mediator, as it is widely known to regulate mTOR pathway, has been associated with VAPB function and plays crucial roles in synapsis.

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

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