**MEETING REPORT**

**Focus on the Heterogeneity of Amyotrophic Lateral Sclerosis**

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Abstract

# Introduction

The clinical manifestations of amyotrophic lateral sclerosis (ALS) are variable with respect to age and site of onset, disease progression, relative upper versus lower motor neuron involvement, and the occurrence of cognitive and behavioural change. This remains the case in those families with known disease-causing variants, suggesting that additional disease-modifying factors exist. While cognitive impairment (FTD) and extrapyramidal signs are frequent comorbidities of ALS and are associated with a more severe disease (8–10), ALS is less frequent and, when present, is less severe in patients with diabetes, cardiac disease and/or hyperlipidemia (11,12).

Disease heterogeneity is likely underpinned by the presence of different pathogenic mechanisms. These include: (1) Alterations in nucleocytoplasmic transport of RNA molecules and RNA-binding proteins; (2) Altered RNA metabolism; (3) Impaired proteostasis with accumulation of aggregating proteins (TDP-43, FUS, SOD1, DPRs); (4) Impaired DNA repair; (5) Mitochondrial dysfunction and oxidative stress; (6) Oligodendrocyte dysfunction and degeneration; (7) Neuroinflammation; (8) Defective axonal transport; (9) Defective vesicular transport; (10) Excitotoxicity. Here we examine the concept of disease heterogeneity through the prism of precision medicine, and explore whether it is possible to identify differing disease genotypes and phenotypes to help in the selection of more homogeneous populations to be enrolled in clinical trials. We therefore assess the availability of biomarkers that might contribute to the identification of differing geno-phenotypes, and explore whether genotypic and phenotypic differences in animal models may help provide a better definition of the heterogeneity of ALS in humans.

# Heterogeneity and comorbidity

It is now well recognized that there are considerable differences in the worldwide distribution of ALS. The incidence of ALS is known to differ not only between different continents but also countries within the same continent (2). Although these differences can be explained in part by the accuracy of case ascertainment and the life expectancy of the target population, other factors are also implicated. The mortality of the disease also varies with the ancestral origin of the affected population (3) and environmental factors are also likely to be associated with ALS at least within some cohorts, such as those in the military (4) or professional soccer players (5). A history of repeated traumatic events and sustained physical exercise has been associated with earlier onset of symptoms in affected individuals as shown in professional soccer players (14).

It also the case that ALS and other neurodegenerative diseases have common risk factors and pathogenic mechanisms, and genetic susceptibility combined with shared environmental risk factors may explain the presence of comorbidities. Further insights on the comorbidity of ALS help to verify the effects of the association on the pathophysiology, clinical characteristics and outcome of the disease. For example, there is increasing epidemiologic evidence of an association between ALS and a wider spectrum of neurodegenerative and neuropsychiatric disorders both in patients and among family members. Possible mechanisms that might underpin the association of ALS with other neurodegenerative diseases include changes in synaptic integrity and in glial function. Astrogliosis is linked to synapse function via the tripartite synapse, but astrocytes also control the availability of gliotransmitters and adenosine. Astrocyte activation, via overexpression of adenosine kinase (ADK), can induce a deficiency in the homeostatic tone of adenosine (6). Such a mechanism could help to explain the observation that neuropsychiatric symptoms including schizophrenia, obsessive-compulsive disorder, autism, and alcoholism, occur more frequently in ALS kindreds than in controls (7).

Genetic background is a key determinant of the ALS phenotype and, perhaps, of the response to treatment. The lifetime risk of ALS is higher in men than in women (15). Families of SOD1 ALS patients show a marked disease heterogeneity, with variation in age of onset and severity, suggesting that there are genetic modifiers of disease presentation (16). Compared to sporadic ALS, familial ALS can be associated with a younger age at onset of the disease (17). Correlations between genetic variants and different clinical profiles in ALS, such as age at onset, disease duration, and site of onset, have been defined (18). However, carrying a disease mutation does not inevitably lead to ALS due to incomplete penetrance and modifying factors. Many ALS-associated genes are also implicated in other conditions, including FTD and cerebellar disease (19). The genetic heterogeneity of ALS is documented by the high number of disease-associated variants (20) and there is increasing evidence that affected individuals can carry multiple disease-associated variants with additive or synergistic effects (21). The variability of the genetic background is associated with differing components of the complex pathogenic mechanisms of the disease (22). In this context, ALS has been envisaged as a multistep process (23) and the disease can be the result of the cumulative effects of differing causative factors, including the genetic load, the physiological aging process, and the action of environmental exposure (24) that precede the self-perpetuating phase of the disease leading to death. Identification of these steps could lead to preventive and therapeutic avenues and, in this context, the genetic stratification of patients may help the development of a personalized medicine approach for ALS. For example, results from a preliminary study show that treatment with lithium increases survival only in patients homozygous for the rs12608932 SNP within *UNC13A* gene (25). Moreover, with the emerging and promising efficacy of gene therapy, genetic stratification may become an indispensable strategy for preventing and treating ALS. However, the role of all these factors is still ill-defined because ALS genetic susceptibility is still incompletely assessed.

# Biomarkers

Neurofilament Light Chain (NFL)

Levels of plasma and CSF NFL and, to a lesser extent, phosphorylated neurofilament heavy chain (NFH) have been shown to be elevated specifically in ALS patients compared to Alzheimer disease’s patients (27). Interestingly, in the natural history of ALS, serum NFL levels are elevated as far back as one year preceding the earliest clinical symptoms or signs of disease (28). In addition, they are higher in fast versus slow progressing ALS patients (29). Using a novel tissue-enhanced biofluid mass spectrometry technique to study the plasma proteome in ALS with peripheral blood mononuclear cells (PBMC) as tissue calibrator (TMTcalibrator™), a panel of protein biomarkers have been identified in fast and slow progressing ALS patients that partially overlap with the fast and slow progressing transgenic SOD1G93A mice at a pre-symptomatic and symptomatic stage (30,31). They include innate immunity, acute phase response, immunosenescence and metabolism, suggesting immunomodulation as an early therapeutic intervention.

Peripheral Blood mononuclear cells (PBMCs)

A peculiar clinical characteristic of ALS is the wide distribution in age of onset, which is probably caused by different combinations of genetic and environmental factors. Analysis of PBMC from ALS patients with extreme ages of onset, ≤ 55 and ≥75 years of age, identified a panel of protein biomarkers differentially expressed as potential modifying factors in the development of the disease (32). Most of these markers are associated with the maintenance of protein homeostasis and may suggest that a difference in the ability to upregulate protective proteins underlies the differential susceptibility to ALS, which supports the possibility that boosting the protein quality control system might be an effective therapeutic approach. In particular, it has been observed that patients with early disease onset have low PPIA levels in PBMCs. PPIA, a foldase and a molecular chaperone (33,34), binds TDP-43 in the low-complexity domain and regulates its function (35). The absence of PPIA in the SOD1 mouse model induces TDP-43 pathology and exacerbates mutant SOD1 aggregation, accelerating disease progression, suggesting that PPIA is a disease modifier (ref). Interestingly, some of these biomarkers were also identified in extracellular vesicles (EVs) from plasma of ALS patients and seem predictive of fast and slow disease progression, suggesting a potential use as prognostic biomarkers (V. Bonetto and M. Basso, unpublished data). EVs are potentially an attractive source of biomarkers. They are released continuously into biofluids, their cargo reflects the physiological state of their parent cells and may affect neighbouring and/or long-distant recipient cells. For example, astrocytes expressing mutant SOD1 secrete EVs carrying mutant SOD1 that induces the death of wild-type motor neurons (36). Despite great advances in the field of EVs, there is lack of validated procedures to isolate highly pure and intact EVs (37). A novel method of EV isolation based on nickel-binding beads (NBI) has been discussed (38). NBI has several advantages that render it suitable for a clinical setting. It is rapid, cost-effective and isolates/purifies highly stable EVs free of co-isolated protein aggregates, a major issue for EVs from blood samples.

MicroRNA

MicroRNAs (miRNAs) are small non-coding RNA molecules that play an important role as epigenetic regulators of gene expression, acting at the post-transcriptional level. They may participate in pathogenic cascades and/or mirror cellular adaptation to insults influencing the susceptibility to develop ALS and likely its heterogeneity. Unlike other classes of RNA, miRNAs are remarkably stable since they are protein-bound or entrapped in exosomes and therefore can be easily measured in many biological fluids including plasma, serum, CSF, muscle and saliva. Several studies have identified numerous dysregulated miRNA profiles in the blood of ALS patients in comparison with healthy controls. Interestingly, it was reported that a specific subset of miRNAs which was reduced in the serum of patients with familial and sporadic ALS was also reduced in presymptomatic ALS mutation carriers even 10-20 years before any clinical manifestation (26). These findings, if replicated, may be of fundamental importance to identify a predictive profile of the disease in early asymptomatic stages in order to possibly apply preventive therapies. However, the high variability of miRNAs expression and the poor overlapping of the results among studies make difficult, today, to correlate a specific miRNA signature with a specific ALS clinical presentation and progression in patients with sporadic ALS. Thus, in order to use these biomarkers for the stratification of ALS patients and as prognostic indicators and therapeutic targets, more validation studies in large cohorts of patients are required.

Imaging

The value of structural and functional imaging studies of the brain and spinal cord has been recently assessed in a systematic review (39). Magnetic resonance imaging (MRI) of the brain and spinal cord can be used as a biomarker to identify abnormal patterns in preclinical studies of genetically predisposed individuals (40). Along with structural imaging, a number of techniques have been used, including diffusion tensor imaging, magnetic resonance spectroscopy, tractography, and functional MRI. The most sensitive findings include abnormalities in the motor areas with a variety of patterns that reflect disease severity, progression and duration. Detection of atrophy in multimodal spinal cord MRI has been associated with shorter survival (41). MRI studies have been also performed in asymptomatic patients with ALS carriers of *C9orf72* mutation (42). In these cases, cervical cord imaging detected white matter atrophy exclusively in subjects older than 40 years, and progressive corticospinal tract fractional anisotropy reduction was identified during a 18-month follow-up.

# Cellular and animal models

Disease models for many of the recently discovered genes are currently in development and are expected to shed light on the respective disease mechanisms in different genetic subtypes of ALS. These experimental models aim to recapitulate the neuropathological and genetic heterogeneity of the disease and to investigate the molecular aspects of the pathology that might be amenable to therapeutic intervention. Rodent models are considered to mimic human disease more closely than small animal models, and can be valuable for unravelling pathogenic mechanisms and for proof-of-concept studies. A wide range of ALS mouse models are now available. Each model has its own distinct characteristics depending on the nature of the introduced mutation and on the specific changes to the gene of interest. Among the ALS mouse models so far available, those carrying mutations in *SOD1*, *TARDBP* or *FUS* genes are the most prevalent (43). Two of the most widely used types of ALS mouse models are: (1) Transgenic mice and (2) Mice with mutations in endogenous genes via gene targeting or chemical mutagenesis, that express mutant genes at physiological levels. While the majority of transgenic mice are made by randomly inserting human ALS genes into the mouse genome, which typically results in overexpression of mutant or wild type proteins, gene targeting is used to insert specific mutations into the hortologue mouse gene, with the aim of maintaining physiological levels of the mutant protein. In the first condition, mice usually exhibit a more severe phenotype and can be good models for studying the late stage of the disease and for testing a wide range of treatments aimed to ameliorate the progression of symptoms. Conversely, the mice carrying a mutation in endogenous genes usually show a mild phenotype, offering a window into the early stages of the disease even if they often fail to manifest a clear pathological profile within the average mouse lifespan. Like ALS patients, ALS mouse models also show significant heterogeneity due to a number of factors including their genetic background, sex and different mutations in same gene. Thus, transgenic SOD1 mice show disease heterogeneity due to differences in specific SOD1 mutations, the number of transgene copies or in the expression levels of the mutant SOD1 protein. However, even in the presence of the same gene mutation and similar expression of mutant gene, they show a significant variability in disease severity.

For example, the transgenic mice overexpressing the SOD1 G93A mutation that are the most widely used mouse model for ALS, show variability in the disease course despite they carry the same transgene copies and the same expression levels of SOD1 protein, and this is due to their different genetic background (44,45). These findings, in addition to emphasizing the importance of genetic background for the standardization of experimental models, suggest that there are genetic modifiers that can influence the disease presentation (44). This is consistent with the observation in ALS patients carrying the same dominant SOD1 mutation, but showing high inter-individual variability in the age of onset and disease progression rate (16,46), emphasizing the involvement of modifiers. The identification of these modifiers and their associated molecular pathways may help to discover prognostic biomarkers and to develop promising therapeutics to be translated in ALS patients. For example, a recent proteomic analysis of the PBMC from fast and slow SOD1 G93A mice identified a panel of differentially expressed proteins that partially overlap those found in the PBMC of early and late onset ALS patients. Interestingly, some of these proteins were also changed in the spinal cord of mice, indicating that PBMC can mirror the pathomechanisms in the central nervous system (32). The lower expression of chaperones and foldases in both the early onset patients and mice further confirms that a defect in the proteostasis likely contributes to an accelerated disease. In another experimental setting, such as the tissue enhanced plasma proteomic analysis, there was a partial overlap of the plasma/PBMC proteome between fast and slow progressing ALS patients and SOD1 G93A mice, which highlight an early inflammatory and acute phase response in both humans and animal models even if independent on progression speed (30). These findings provide the rationale for further translational investigation of these experimental paradigms to identify prognostic biomarkers that may be used for stratification of patients and potential therapeutic targets for an effective slowing down of the disease.

Phenotypic differences can be seen also when comparing mice with endogenous gene mutations such as the ENU-induced SOD1 D83G mutation. While the genetic defect in heterozygous mice produces only subtle running wheel defects, in homozygous animals it induces motor neuron loss and peripheral neuropathy (47).

Modelling TDP-43 and FUS ALS in mice has proved to be rather challenging. TDP-43 is extremely dosage-sensitive and tightly autoregulated. TDP-43 loss of function induced by silencing the *TARDBP* gene with siRNA can lead to neurodegeneration; however, in transgenic mice even slight overexpression of wild-type (WT) or mutant TDP-43 may cause multiple RNA changes and lead to non-specific toxicity, making it difficult to identify the pathogenic profile that occurs physiologically in disease (43). Recently, novel mouse lines with point mutations within endogenous Tardbp gene were generated throughout the N-ethyl-N-nitrosuera (ENU) mouse mutagenesis programme in order to dissect the molecular effect of TDP-43 loss or gain of function *in vivo* in the absence of the confounding effects of ectopically expressed transgenes. The p.M323K mutation in the C-terminal low complexity glycine rich domain (LCD) of TDP-43 causes a splicing gain of function with prevalent skipping of usually expressed exons, so called skiptic exons, associated with the development of a late progressive neuromuscular phenotype with partial motor neuron loss (48). On the contrary, the p.F210I mutation in the important RNA recognition motif 2 (RRM2), leading to a loss of function in the RNA binding activity of TDP-43, did not cause a pathological phenotype. Another example of the variable phenotype of mutant TDP-43 mice comes from mice harbouring the TDP-43 p.Q331K mutation. While the transgenic TDP-43 Q331K mice with mouse prion protein promoter show nuclear loss of TDP-43 accompanied by early signs of motor coordination impairment and motor neuron loss (49), the gene targeted knock-in TDP-43 Q331K mice show a 45% increase in nuclear TDP-43 with neither motor coordination impairment nor motor neuron loss (50). This suggests that a TDP-43 gain-of-function into the cytoplasm is likely implicated in the pathomechanism of ALS-associated TDP-43 mutations and the heterogeneity in the phenotype may depend on the expression levels of the mutant protein and/or the genetic background (mPrp vs mTardbp promoter).

In transgenic FUS mouse models, the hemizygous mice overexpressing human wild-type FUS show no evidence of motor phenotype or pathology (ref). On the contrary, homozygous transgenic mice, overexpressing 1.7 fold FUS levels compared to non-transgenic littermates, develop an aggressive phenotype: early onset tremor, progressive hind limb paralysis and death by 12 weeks, motor neuron degeneration, denervation and focal muscle atrophy (51). The surviving motor neurons show increased cytoplasmic expression of FUS with globular and skein-like FUS-positive and ubiquitin-negative inclusions with partial glial change. Another mouse model developed by inserting a human FUS mutation located in intron 13 splicing acceptor site, causes exon 14 skipping and a frameshift in exon 15. The mice develop progressive movement defects with partial denervation and 20% motor neuron loss in 18 month old mice (52). However, the pathological aggregation of FUS was not required for the disease initiation.

Ideally, access to this variety of ALS animal models will help recapitulate the heterogeneity of the neuropathological and genetic aspects of the disease, enabling the investigation of the wide array of pathomechanisms that might be amenable to therapeutic intervention.

If mouse models provide the best models for studying the systems of ALS, small animal models, such as Caenorhabditis elegans, Drosophila *melanogaster* and zebrafish, can be used to investigate certain molecular aspects related to the genetics of ALS and to screen for potential modifiers of the underlying pathogenic mechanisms and disease process. Interesting examples of the toxicity of ariginine-rich dipeptide repeat proteins (DPRs) derived from C9orf72 mutation have been shown in the *Drosophila melanogaster*. Van den Bosch and co-workers performed an RNAi screen for genetic modifiers and demonstrated that the toxicity of these DPRs is due to an indirect inhibitory effect on the nucleocytoplasmic transport through stress granule formation (53). The use of zebrafish for the screening of modifiers of the C9orf72 repeat RNA-induced motor axonopathy, identified downregulation of heterogeneous nuclear ribonucleoproteins (hn-RNPs) as a potential mechanism of toxicity (L. Van Den Bosch, unpublished data?). Interestingly, mislocalization of these proteins was also observed in fibroblasts of C9orf72 patients as well as in C9orf72 patient induced pluripotent stem cells (iPSCs) derived motor neurons. All of these experimental paradigms provide evidence for the implication of different disease processes such as nucleocytoplasmic transport factors, stress granule formation, and RNA-binding proteins as possible mechanisms responsible of the heterogeneity of the disease.

Human iPS Cells

A promising area of progress in modelling the heterogeneity of human ALS comes from the development and use of human induced pluripotent stem cells (iPSCs) and derived motor neurons . Many important functional disease-relevant phenotypes have already been identified in human iPSC-derived spinal motor neurons, including increased cell death, cytoskeleton disorganization, defects in nucleocytoplasmic transport, and changes in excitability in relation to their specific genotype (1). iPSC-derived motor neurons have the advantage of carrying the genetic background of the patient and expressing physiological levels of the mutant ALS genes. In particular, this may be very relevant and useful in the case of the C9orf72 gene mutation where large hexanucleotide repeat expansions cannot be fully recapitulated by over-expressing recombinant DNA of a relatively short size in animal models. Using iPSC-derived spinal motor neurons from C9orf72-mutated patients to investigate possible epigenetic modifiers of expanded repeat RNA toxicity, a high inter-patient cell line variability in C9orf72 promoter methylation, gene expression and RNA foci formation has been observed, due also to the variability in the repeat expansion length of these lines (A. Ratti, unpublished data). However, the C9orf72 repeat expansions show instability during iPSC reprogramming from both fibroblasts and PBMCs (D. Bardelli et al, in press), as also observed to occur among different patients’ tissues (van Blitterswijk M et al, Lancet Neurol. 2013 Oct;12(10):978-88; Nordin A et al, Hum Mol Genet. 2015, 24(11):3133-42) and during in vitro passages, raising some caution when using this disease cell model. This may not be the case for other ALS disease models such as TDP-43 models, as TDP-43 pathology represents the pathological hallmark in 98% of ALS cases (both familial and sporadic). Recently, Fujimori et al. developed a multiplex phenotypic profile including neurite regression, stress granules, LDH leakage, cleaved caspase-3 and abnormal phosphoTDP-43 aggregates capable of subdividing the variable phenotype severity in iPSC derived motor neurons from 32 sporadic ALS lines (54). Interestingly, they found that the variability of *in vitro* phenotype severity correlated with clinical sporadic ALS classification based on the ALS functional rating scale (ALSFRS-R). Although substantial uncertainties remain on the limits to the confounding effects of re-programming techniques, iPSC passage number, and differentiation batch (55), this work is attractive in the perspective of modelling the heterogeneity of sporadic ALS for a first step towards a personalized medicine strategy. Furthermore, since ALS is a complex multisystem and multifactorial disease, non-cell autonomous mechanisms must necessarily be considered in the modelling of ALS heterogeneity, either in co-culture systems or in *in vivo* animal models.

Although the translational value of each disease model remains unclear, since examples of successful translation to patients are still lacking, a cross-model approach, in which disease mechanisms identified in less-complex systems like cultured motor neurons are later validated in more-complex models such as animal models and patient-derived cells or samples, has a higher chance of successful translation to the clinics.

# Precision medicine in therapy development for ALS

A more accurate epidemiological analysis of different registries has allowed the development of validated models that can reliably predict outcomes even at the individual patient level (56,57). A variety of biomarkers that may be predictive not only of the development of the disease, but also of the variability in its progression, have been identified, although they still need to be validated across different cohort of patients. The broad variety of genetic mutations identified in ALS and ALS with FTD leads to an extensive development of preclinical models from cells to animals that also demonstrate heterogeneous outcomes. Even if they may raise concerns about their validity for human disease because of their incomplete or differing phenotypes, they are valuable tools to identify the pathobiological mechanisms associated to the different ALS gene mutations and to provide the proof of concept for the development of targeted therapies. In this regard, there is growing optimism with respect to gene therapy. In fact, the phase1/2 study of Tofersen, an antisense oligonucleotide (ASO) against SOD1 administered into the cerebral spinal fluid of patients with SOD1-ALS, has given promising evidence in slowing the clinical, functional and respiratory function decline in fast progressing patients. These findings prompted the advance of this clinical programme to a phase 3 clinical trial, which is currently underway, to confirm its efficacy and safety in SOD1-ALS patients and to further investigate its therapeutic potential. Demonstrating the potential of ASOs to target the other genetic drivers of disease will turn on the hope for a true prevention and blocking treatment of this devastating disease and will provide the rationale for a personalized medicine.

**Declaration of interest**

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