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**New advances in Amyotrophic Lateral Sclerosis genetics:
towards gene therapy opportunities for familial and young
cases**

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Authors Contribution

François Muratet and Elisa Teyssou participated to the acquisition of the data. Maria-Del-Mar Amador and Séverine Boillée critically revised the manuscript for important intellectual content. Stéphanie Millecamps designed and supervised the study and wrote the paper. All authors gave final approval of the version to be submitted.

Abstract

Due to novel gene therapy opportunities, genetic screening is no longer restricted to familial cases of ALS (FALS) cases but also applies to the sporadic populations (SALS). Screening of four main genes (*C9orf72*, *SOD1*, *TARDBP* and *FUS*) identified the causes in 15% of Amyotrophic Lateral Sclerosis (ALS) patients (two third of the familial cases and 8% of the sporadic ones) but their respective contribution to ALS phenotype varies according the age of disease onset. The genetic overlap between ALS and other diseases is expanding and includes frontotemporal dementia, Paget's Disease of Bone, myopathy for adult cases, HSP and CMT for young cases highlighting the importance of retrieving the exhaustive familial history for each individual with ALS. Incomplete disease penetrance, diversity of the possible phenotypes, as well as the lack of confidence concerning the pathogenicity of most identified variants and/or possible oligogenic inheritance are burdens of ALS genetic counseling to be delivered to patients and at risk individuals. The multitude of rare ALS genetic causes identified seems to converge to similar cellular pathways leading to inappropriate response to stress emphasising new potential therapeutic options for the disease.

Key words :

Motor neuron diseases, frontotemporal dementia, inclusion body myopathy, Paget's disease of bone, pathogenic mutation

Abbreviations

ALS, Amyotrophic Lateral Sclerosis; CMT, Charcot-Marie-Tooth disease; DDR, DNA damage response; ERAD, Endoplasmic-reticulum-associated protein degradation;

FALS, familial ALS; FTD, FrontoTemporal Dementia; HSP, Hereditary Spastic Paraplegia; IBMPFD, Inclusion body myopathy with early-onset Paget disease and/or frontotemporal dementia; PDB, Paget's disease of bone; SALS, sporadic ALS; SMA, spinal muscular atrophy; VUS, variant of unknown significance; WES, whole exome sequencing; WGS, whole genome sequencing.

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Highlights

- Despite recent genetic discoveries, some FALS remain genetically not elucidated
- ALS genetic overlap with other diseases should be consider in familial history
- Due to novel gene therapy opportunities, genetic screening applies also to SALS
- Rare ALS genes recently identified have been implicated in 3 main cellular pathways
- Increasing number of VUS routinely identified require appropriate screening tools

1. Introduction

In contrast to the frequent sporadic occurrence of Amyotrophic Lateral Sclerosis disease (in 90% of cases, SALS) thought as caused by a combination of various life insults on a predisposing genetic background, the rare causes of familial ALS cases (FALS, which concern less than 10% of the cases) are better elucidated. Nonetheless, mutation discovery in these FALS has considerably improved the comprehension of ALS genetic occurrence but also paved various cellular pathways leading to motor neuron death. In populations of European origin, the 4 major ALS causing genes are *C9orf72*, *SOD1*, *TARDBP* and *FUS* and concern two third of FALS [1, 2]. For these genes it is possible to establish genotype-phenotype correlations [2]. Other genes have been recently identified using high throughput sequencing strategies in isolated families. However, applied to genetic diagnosis for an individual patient, this type of approaches identifies a large number of rare variants which afterwards need to be filtered, selected, with, few guaranty of definite pathogenicity. Although potentially overwhelming, the great number of ALS-related genes, each evocating diverse hypotheses for the disease, allows precious discoveries, and could highlight specific cellular pathways as potential targets for future therapeutic perspectives. In this paper we will detail the recent advances in ALS genetics, the growing genetic overlap with other diseases and the novel resulting hypotheses aiming to explain the pathophysiology of these neurodegenerative diseases.

2. *C9orf72*, *SOD1*, *TARDBP* and *FUS* : the four main genes causing ALS

SOD1 encoding superoxide dismutase 1, was the first identified gene in FALS [3, 4]. The transmission is usually autosomal dominant but rare cases of recessive transmission and *de novo* occurrence have also been reported [5]. There are more than 200 *SOD1* mutations reported to date, mostly being missense, leading to amino acid substitutions

detected along the entire protein sequence. These mutations concern ~10% of French FALS and 3% of French SALS. A gain of toxic function is the favored hypothesis however the exact mechanisms leading to neurodegeneration are not fully elucidated. Nevertheless, as the mutant protein accumulates in patient motor neurons and is supposed to disturb essential cellular pathways (through impairment of intracellular transport, energy supply, and protein folding or recycling) the therapeutic option currently explored is to reduce the levels of SOD1, using antisense gene therapy strategies [6]. The possibility of a targeted and significant therapeutical intervention is a game changer in the field. In this context it is critical to identify, as soon as possible, eligible patients. This implies the complete analysis of this gene, including the non coding regions, as intronic mutations leading to truncated SOD1 proteins with introduction of novel amino acids could be responsible for a significant portion of French ALS patients linked to SOD1 mutation [7].

The successive discovery of *TARDBP* (transactive response DNA-binding protein 43kDa, TDP-43) and *FUS/TLS* (Fused in Sarcoma/translocated in liposarcoma) genes, both encoding proteins with RNA binding domains have increased interest for researches on cellular mechanisms devoted to RNA metabolism regulation [8]. ALS mutations in these genes are concentrated in glycine-rich regions localised in the C-terminal part of the proteins (coding by the last 6th exon of *TARDBP* and the 14th and 15th last exons of *FUS*) suggesting the importance of these domains for the disease [9]. These mainly nuclear proteins are delocalized to the cytoplasm during ALS disease. They both have intrinsically disordered sequences, also known as prion-like domains conferring aggregate-prone properties to their encoded proteins. *TARDBP* and *FUS* mutations are usually heterozygous missense mutations responsible for dominant autosomal forms of the disease. Rare families with recessive *FUS* mutation have been

described and *de novo* mutation occurrence proved in some early onset SALS [10, 11]. These FUS mutations also include insertion/deletion mutations leading to amino-acid frameshifts or intronic mutations affecting FUS splicing, resulting all in truncation of the nuclear localization signal. Patients carrying these truncated FUS have a particularly severe phenotype [12].

TARDBP encodes the nuclear TDP-43 protein, which is a component of the cytoplasmic ubiquitinated inclusions detected in the motor neurons of most FALS and SALS patients, except those carrying a mutation in *SOD1* ou *FUS* [10, 13, 14]. TDP-43 containing inclusions are also found in cortex layers of frontotemporal dementia (FTD) patients and in muscles of inclusion body myositis inflammatory myopathies. RNA Binding domains of TDP-43 can recruit heterogeneous ribonuclear proteins. TDP-43 inhibits transcription, regulates exon skipping process during splicing and is involved in RNA granule transport. FUS is involved in several cancers (including liposarcoma and osteosarcoma) due to chromosomal translocations leading to its association with transcription factors in fusion proteins. It is strongly involved in transcription initiation, splicing regulation and in mRNA granule formation after cellular stress.

The genetic discovery of *C9orf72* (chromosome 9 open reading frame 72) as the most frequent cause of ALS associated or not to FTD was a breakthrough in the field and strongly linked the two diseases genetically and neuropathologically. The mutation is an expansion of an hexamers (G4C2) repeat sequence (of several hundreds of repeats in patients versus less than 23 to 30 repeats in controls). These long GC rich regions are difficult to amplify and the expansion is thus detected using repeat-primed PCR, an alternative methodology capable of amplifying the repeats as the succession of multiple peaks [15, 16]. The presence of the repeat was shown to suppress the expression of *C9orf72* in frontal cortex, suggesting that loss of function could also contribute to the

patient phenotype [15]. For example, as *C9orf72* is involved in the formation of autophagosomes, autophagy proteolysis process could be impaired by the presence of these expansions [17]. Another toxic mechanism may be driven by aberrant splicing of long transcripts capable to sequester RNA-binding proteins in RNA foci, observed in patient neurons, leading to deregulation of alternative mRNA splicing [15]. Although the expansion is located in the non-coding region of the gene (corresponding to the promoter), it can be translated using non conventional translation process into dipeptide repeats, which accumulate in neuron cytoplasm leading to proteic toxicity [18-21]. Some of these dipeptide repeats can block nucleocytoplasmic exchanges in cellular models through indirect mechanism [22, 23].

The exact number of these large repeats can not be accurately assessed and southern blot strategy, which requires a large amount of DNA, can only detect differences of long length (difference of 50-100 repeats between 2 samples). Nevertheless, some studies have explored the potential impact of a change in the number of repeats. Across generations, it was suggested that an increase in the number of repeats could account for an anticipation in the age of onset. Differences in the repeat length across various tissues were also suggested to affect the phenotype of the neurodegenerative disease. In a clinical study, age at onset was compared between successive generations in *C9orf72* pedigrees. Disease occurred 13 years earlier in the youngest generation (at 49 years) compared to the oldest fourth generation (at 62 years) suggesting a possible progressive repeat number increase [24]. In contrast neither disease duration or severity were modified. However the authors retrieved several bias for these results including the notion that the later-born relatives of these families might recognize symptoms of the disease earlier than previous generations. Importantly, variations in repeat sizes were detected between different tissues or different regions of the CNS from the same patient.

For example the number of repeats was smaller in the cerebellum than in frontal cortex or blood. Since in FTD patients, longer repeat expansions were associated with older age at onset, this suggests that instability and repeat size increase with aging. Repeat sizes length in the cerebellum may be more stable than in other tissues, so that the repeat size in this tissue may be a good marker for disease severity, with longer sizes associated with shorter survival [25]. It was also postulated, that epigenetic silencing of mutant *C9orf72* through hypermethylation process could modulate patient phenotypes. However, *C9orf72* methylation state was not different between ALS and FTD patients and could not predict age at onset. Nevertheless in patients with FTD, *C9orf72* hypermethylation was associated with prolonged disease [26].

3. Frequency and Genotype/Phenotype comparison for the four main ALS genes in various ALS populations

We performed a retrospective analyses of a large cohort of French ALS patients (including 450 FALS index cases and 940 SALS). We showed that two thirds of French FALS patients carry a mutation in one of the major ALS-related gene (Fig. 1). Among the 150 FALS with unidentified causes, only 20 are early onset forms. Thus, the remaining genes still to be identified in FALS, concern patients with rather later disease onset. For SALS, the mutational frequency for the 4 main genes is doubled in SALS patients with onset before 40 years of age (16%) compared to older patients (8%).

C9orf72 is the major genetic cause of disease over 40 years of age, whereas *FUS* is the the major cause for the juvenile ALS and for disease occurring in younger adults [2] (Fig.1) . Trio analyses in our cohort of SALS with early onset allowed us to confirm that the *de novo* occurrence was preponderant in the *FUS*-linked SALS. For all major ALS causing genes, penetrance was incomplete. Clinically, ALS patients with *SOD1*

mutations frequently presented with lower limbs onset, predominant lower motor neuron signs and very rarely a frontal cognitive dysfunction was associated. Disease progression appears to be bimodal in *SOD1*-ALS patients, with some patients showing either rather rapid (<3 years, fast progressors) or rather slow (>7 years, slow progressors) disease course. This difference in disease course can be partly explained by mutation differences as some *SOD1* mutations, such as the G41S mutation, are associated with a particularly short survival, of around one year following the diagnosis; while others, like the N139D mutation, lead to long disease duration of more than 10 years. Nevertheless, the disease course can also be different among members of the same family carrying the same mutation, like the D83G, with disease course of several months to more than 10 years [1]. In *TARDBP* patients, disease onset appears to be predominantly in the upper limbs, frequently associated with marked bulbar dysfunction and with FTD [1]. *FUS*-related ALS phenotype is severe, with early onset and fast progression of disease leading to shorter survival [1] (Table 1). *C9orf72*-linked patients have more frequent bulbar onset, disease starts later in life, compared to *FUS* or *SOD1*, and is more frequently associated with FTD. Prognosis in terms of survival appears to be pejorative, compared to *SOD1* or *TARDBP* mutation carriers [2].

4. The multitude of rare ALS genes identified so far are involved in 3 main cellular pathways

Since the discovery of these 4 main ALS genetic causes, many other ALS rare genes have been identified, and this is mainly based on progress in sequencing approaches with the development of whole exome/genome (WES/WGS) sequencing analysis. The large number of these scattered rare genes can be grouped in three cellular pathways

including DNA/mRNA metabolism regulation, protein collapse and intracellular trafficking on cytoskeleton network (Table 2).

4.1. Nucleic acid homeostasis

4.1.1. RNA metabolism regulation and toxic RNA species

The discovery of TDP-43 and FUS as the main genetic ALS causes raised attention on their partner proteins and on other proteins with similar binding properties for RNA species. Mutations were identified in a group of proteins with such properties including ANG, ATXN2, EWSR1, GLE1, hnRNPA1, hnRNPA2B1, MATR3, SS18L1, TAF15. These mutations are all transmitted in an autosomal dominant mode of inheritance in ALS, and are mainly point mutations, except for the expansions of intermediate length in the polyglutamine (polyQ) repeat sequence in *ATXN2*, encoding Ataxin 2. Longer expansions in this same gene are known to cause another neurodegenerative disease: Spinocerebellar Ataxia type 2. Intermediate length expansions in *ATXN2* are considered to be a susceptibility allele, rather than a monogenic cause of ALS.

Most of these proteins share (i) properties to shuttle between nucleus and cytoplasm, (ii) agregating properties through a prion like domain and (iii) their participation to the formation of stress granules (which transiently protect mRNA from degradation upon cellular stress conditions). Thus one possible deleterious process involved in ALS might be their depletion from the nucleus leading to impairment of RNA processing.

Analysis of our cohort of 450 French FALS revealed the presence of expanded *ATXN2* repeat length (of more than 30 repates) in 13 patients (2.9% of total FALS) and the presence of 3 *ANG* variants in 5 of them. No other rare variant in any other ALS genes was found in *ATXN2* repeat carriers using this repeat cut off in contrats to 4 of the patients with *ANG* variants: they also carried mutations in *FUS*, (n=2 patients), *C9orf72* (n=1) or *MATR3* (n=1). Thus we concluded that *ANG* could be, at best, a susceptibility

allele for ALS rather than a causing gene [1, 2]. Among the 150 families with no mutation in any major ALS genes, a single variant in *EWSR1*, *GLE1*, *MATR3*, *SSI8L1* and *TAF15* was identified, each in a different FALS (each corresponding to 0.7% of FALS with no mutation in any ALS gene). However the pathogenicity of these variants (1 in frame deletion, 1 intronic splice site mutation, 3 missense mutations) remained to be ascertained.

4.1.2. DNA damage response (DDR)

DNA is daily subjected to multiple insults (for example through mitochondrial oxidations) leading to lesions (breaks, insertion/deletion mismatches or crosslinking with proteins) which requires to be recognized, excised and repaired by appropriate glycosylases/endonucleases using homologous recombination or non-homologous end-joining. This process requires the rapid activation of sensors, mediators and repair proteins into complexes on the chromatin. Compared to proliferating cells, post-mitotic neurons are particularly vulnerable to DNA damage due to their high metabolic rate and fewer alternatives for repair. Increased DNA repair activity was detected in ALS patient cortex and spinal cord indicating increased DNA damage [27]. Several ALS genes encode proteins with specific functions in this pathway.

FUS/TLS, as well as EWS (Ewing sarcoma) and TAF15 (TATA box-binding protein-associated factor 68 kDa) belong to the TET family proteins required for efficient double-strand break repair. FUS is crucial in DDR induction, allowing the early recruitment of specific DDR signaling proteins at double-strand break sites (DSBs) and is critical for the accumulation of DNA repair complex components including HDAC1 (histone deacetylase 1) at these sites [28]. Valosin-containing protein (VCP)/segregase (p97) is also involved in the repair of DSBs: after ubiquitination and in cooperation with ATXN3 it removes RNF8 conjugates from sites of DNA damage and promotes their

proper association with 53BP1, BRCA1 and RAD51 for proper repair [29]. NIMA-related kinase 1 (NEK1), which is mutated in familial and sporadic ALS [30, 31], is involved in early DDR [32]. Interestingly a large study led to identify new risk loci for ALS picked up *C21orf2* as a new gene associated with ALS risk [33]. *C21orf2* interacts with NEK1 and is also involved in DDR [34]. Similar to FUS that plays a dual role on DNA and on RNA, SETX, encoding senataxin, has nuclear localization/sorting signals as well as DNA/RNA binding domains. It is recruited at DNA DSBs, when they occur in transcriptionally active loci and resolves the RNA/DNA hybrid structures (R-loops) to regulate transcriptional termination [35]. SETX mutations cause ALS4, a rare autosomal dominant form of juvenile amyotrophic ALS with slow disease progression and a normal life span [36] and another neurodegenerative disease with juvenile onset, Autosomal Recessive Spinocerebellar Ataxia with Axonal Neuropathy (SCAN2, also called AOA2, ataxia-ocular apraxia 2) [37].

4.2. Proteostasis homeostasis

A deregulation of proteolysis leading to protein collapse was evidenced by neuropathological findings in ALS post-mortem tissues. Indeed, apart from the characteristic motor neuron loss, there are protein inclusions typically observed in patient motor neurons, either stained with antibodies against the Cystatin C lysosomal protein, called Bunina bodies, or ubiquitinated, called the skein-like inclusions, immunopositive for TDP-43. These TDP-43 inclusions are typically detected in all ALS cases except those with SOD1 or FUS mutations (with their own ubiquitinated mutant SOD1 or FUS deposits). These protein conglomerates observed at disease end stage could result from the overflow / clog of cellular degradation pathways during disease course. Misfolded and/or ubiquitinated proteins are usually eliminated by the proteasome (in charge of short-lived proteins) or by the autophagosome/lysosome

pathway clearing damaged organelles, long lived proteins or protein aggregates. ALS mutations were detected in several proteins implicated in various steps of these pathways either (i) able to capture ubiquitinated substrates to address them to the proteasome system and/or to the autophagosome pathway (such as sequestosome 1/p62 (SQSTM1), ubiquilin 2 (UBQLN2), valosin-containing protein (VCP) and optineurin (OPTN)); (ii) with enzymatic activities regulating autophagosome/lysosome such as TANK-binding kinase 1 (TBK1), which phosphorylates OPTN and p62 to facilitate their lysosomal transfer; (iii) involved in the maturation of autophagosomes required for stress granule clearance (VCP) or mRNA transport (ANXA11); (iv) or required for the formation of multivesicular bodies such as the charged multivesicular body protein 2b (CHMP2B) involved in the sorting of integral membrane proteins. Some of these proteolysis actors also take part in the protein degradation response triggered by cellular stress (heat shock protein response) such as UBQLN2 and DNAJC7. Like SOD1, several of these proteolysis participants (including UBQLN2, p62, TBK1, VCP) require to be dimerised or multimerised before being active. Thus, the abnormal oligomerization between wild-type and mutant forms of these actors could contribute to the pathogenic ALS process.

Whether gain or loss of function of these “proteolysors” is at work in the neurodegenerative process is a source of debate. Ubiquilin-2 and p62 are components of the ubiquitin inclusions and accumulate in degenerating motor neurons in patients carrying a mutation in the corresponding genes or even without mutations in these genes. For UBQLN2 and SQSTM1 mutation carriers the primary event triggering the neurodegenerative process could thus be the aggregation of the mutant protein. In contrast, a loss of function mechanism seems the consequence of *OPTN* mutations, including non-sense/deletion identified at the homozygous state in families with

consanguinity [38]. Similarly, non-sense/ frameshift mutations in *TBKI*, detected at the heterozygous state and leading to haploinsufficiency, evoke a loss of function of this kinase in the degenerating process [39].

In our cohort of 200 FALS with no mutation in any major ALS genes, single variants in *CHMP2B*, *DNAJC7*, *OPTN*, *SQSTM1* and *VCP* were identified, each in a different FALS. The intronic variant we found in *OPTN* was at the homozygous state and predicted to impact splicing, confirming that the recessive transmission of loss of function mutation in *OPTN* can cause ALS. The mutation we found in *VCP* (R155L) affects a hot spot residue also concerned by other variations (R155H, R155P, R155C) reported in cases with ALS and/or Inclusion Body Myopathy, Paget's disease of bone (PDB), and/or Frontotemporal Dementia (IBMPFD) [40], a multisystem proteinopathy with degenerative process affecting muscle, bone and/or central nervous system. In our series, mutations in *TBKI* were more frequent and concerned 5 FALS among patients with no mutation in major ALS genes (corresponding to a frequency of 2.5% in this subgroup of patients): among them one non-sense mutation segregated in three members of the same family with predominant upper motor neuron signs. ALS patients with *TBKI* mutations were also reported to have more frequent extrapyramidal symptoms and early memory loss was reported in cases with FTD [41].

UBQLN2 and *ERLIN2* mutations cause ALS+/-FTD and SPG18 recessive hereditary spastic paraplegia, HSP, respectively. Our results in French FALS also showed that they can be responsible of a particular form of motor neuron disease, starting early in life (before 25y) with a spastic paraplegia phenotype (severe upper motoneurone disease restricted to the lower limbs) and secondary evolving, later in life, to rapid progressive severe ALS phenotype [42, 43]. The P506A *UBQLN2* mutation, responsible for this phenotype, affects, like most of the reported *UBQLN2* mutations, one of the proline

residue of the PXX tandem repeat domain of the protein. The dominant X-linked transmission for *UBQLN2* mutations assumes males transmit to all their daughters. However the strikingly reduced disease penetrance in females that we observed for this mutation was unexpected, and constitutes important information for genetic counseling [43]. *ERLIN2* mutations causing ALS segregated with the disease in a dominant (V168M) or recessive (D300V) manner, showing that *ERLIN2* mutation inheritance appears to be, within the motor neuron disease spectrum, more complex than previously reported [42]. Both *UBQLN2* and *Erlin2* proteins share involvement in the endoplasmic reticulum-associated protein degradation (ERAD) pathway, which underline the pivotal role of this pathway used for protein degradation in this disease spectrum. The endoplasmic reticulum (ER) chaperone, *SIGMAR1*, translocates from the ER to the plasma membrane or to mitochondrial-associated membranes and plays an important role in the regulation of ion channels. A recessive mutation in *SIGMAR1* was found in a large consanguineous family with juvenile ALS [44]. Cells expressing this mutant form were less resistant to apoptosis induced by ER stress. Variants in the 3'UTR region of this gene have also been reported in a pedigree with FTD, motor neuron disease and TDP-43-positive neuropathology [45].

4.3. Cytoskeleton defects

Large myelinated motor neuron axons spreading far away from the cell body are composed of the extensive cross-linked network of three structural components: actin microfilaments, intermediate filaments and microtubules. Electron microscopy studies revealed that swellings occur in the initial segment of motor axons in ALS patients, and that these swellings contain vesicles, lysosomes, mitochondria and intermediate filament proteins, including neurofilaments (heteropolymers of light with either medium or high neurofilament subunits) or peripherin. These changes are highly suggestive of

axonal transport blockade, which might arise through defects of the cytoskeleton organization impairing motor protein attachment, altered kinase activities destabilizing motor-cargo sliding and/or mitochondrial energetic breakdown [46]. Identification of mutations in a subset of patients with ALS were reported in genes participating to the actin, tubulin, and intermediate filament network as well as in motor proteins driving cargoes in anterograde and retrograde directions (Table 2).

4.3.1. ALS mutations in components of the cytoskeleton

Abnormal phosphorylation of neurofilament proteins may constitute a susceptibility factor for ALS with codon deletions/insertions in the phosphorylation repeat domain of *NEFH* (which encodes NFH) and a *PRPH* (encoding peripherin) frameshift mutation reported in some SALS [47-49]. Mutations in *PFN1* (profilin 1), an important regulator of actin polymerization, and playing an essential role in the formation and the disassembly of stress granules, were discovered in large families with ALS [50]. Mutations in tubulin Alpha 4A (*TUBA4A*), a component of microtubule network were identified after burden analysis on a large cohort of unrelated FALS. Even if the conclusion drawn was that presence of these *TUBA4A* variants was insufficient to trigger ALS by themselves, they highlighted the role of the microtubule network in the disease process [51].

4.3.2. ALS mutations in motor proteins

A point mutation in *DCTN1* resulted in a particular form of inherited, slowly progressive autosomal dominant motor neuron disease with vocal cord paralysis onset [52, 53]. Various mutations in this gene were suggested to be susceptibility factors for ALS [53]. This dynein subunit 1 (also known as P150Glued) belongs to the cytoplasmic dynein complex, which drives the retrograde microtubule-based transport of vesicles including autophagosomes.

Kinesins are molecular motors that drive cargoes along the microtubule network, mainly in anterograde direction. KIF5A is one of the 3 heavy-chain isoforms of KIF5. After homo or heterodimerization through their central coiled coil domain, these KIF5 heavy chains associate with kinesin light chains and adaptors to transport various vesicles and organelles (mitochondria, RNA granules, NFs, VAPB cargo). Missense mutations located in the N-terminal domain of KIF5A are responsible for SPG10 and Charcot-Marie-Tooth type 2 (CMT2) axonopathies. They affect microtubule binding and ATP hydrolysis. Mutations responsible for ALS are loss of function (LOF) mutations impairing splicing of exon 27 and leading to the deletion of the normal C-terminal part of the protein preventing cargo binding. Patients with these LOF mutations have early onset ALS (median at 46y) and long disease duration of over 10 years [54]. Association with FTD was also recently reported in a large pedigree with an intronic *KIF5A* mutation leading to exon 27 skipping, frame shifted and extended C-terminal peptide [55]. For this pedigree a p.F115C *MATR3* mutation was initially described [56] but was not confirmed by segregation analysis in the recent generation. Thus ALS linked to *MATR3* mutations could be rarer than reported. Of note a KIF5A cargo, VAPB, is also mutated in rare forms of atypical motor neuron disease presenting like a late-onset spinal muscular atrophy (SMA) or severe ALS in the same pedigree [57]. VAPB prevents the accumulation of misfolded proteins in the endoplasmic reticulum.

4.3.3. ALS mutations in cargo-associated proteins

ALS2 truncating mutations were identified in recessive forms of juvenile ALS, juvenile primary lateral sclerosis (JPLS) and infantile ascending hereditary spastic paralysis (IAHSP) [58-60]. Truncating mutations in exon 3 (N terminus) were suggested to be responsible for a more severe phenotype (meaning ALS), compared with truncating mutations in the C-terminus (leading to JPLS), suggesting that a longer form of ALS2

protein could preserve some of these functions. ALS2 is a guanine nucleotide exchange factor for the small GTPase RAB5 which plays a role in early endosome formation and trafficking [61].

Homozygous or compound heterozygous mutations in the *SPG11* gene were identified in several autosomal recessive disorders, including juvenile ALS with slow progression [62] as well as hereditary spastic paraplegia with thin corpus callosum, cognitive impairment and ocular abnormalities [63] or axonal Charcot-Marie-Tooth disease type 2X [64]. SPG11 encodes spatacsin, (spasticity with thin or atrophied corpus callosum syndrome protein), which maintains cytoskeleton stability and regulates synaptic vesicle transport. The genetic overlap observed between various motor neuron diseases suggests that defects in spatacsin can damage upper and/or lower motor neurons.

The first association of ALS with a mitochondrial protein was in 2014, with the French discovery of *CHCHD10* encoding a coiled-coil helix coiled-coil helix protein of the intermembrane space [65]. Muscular biopsy of these patients showed abnormal mitochondria morphology, impaired electron transfer chain and mitochondrial DNA deletion. Only one member of the first family with the S59L mutation had a pure ALS phenotype, the other members presented FTD with ALS, cerebellar ataxia or myopathy. The R15L mutation was recurrently identified in European FALS with no FTD (including one in our French cohort), the G66V variant was responsible for multiple phenotypes including CMT2 [66] whereas the pathogenicity of P34S variant remains controversial considering its frequency in controls [67]. Mutant mitochondria are structurally damaged in this disease form [65].

5. Overlap with other diseases

Other diseases affecting motor neurons spanning from the upper motor neuron (such as HSP) to the lower motor neuron disease (such as CMT2) and including frontal motor

cortex disease (FTD) share several genetic candidates with ALS (Fig. 2-3). FTD is diagnosed in ~15% of ALS patients [68, 69] and ~15% of patients with FTD secondarily develop ALS [70, 71], although some degree of frontal cognitive dysfunction is thought to be present in a larger proportion of ALS patients. *C9orf72*, *TARDBP*, *FUS*, and the cytoskeleton-associated proteins *MAPT*, *CHMP2B* and *CHCHD10* are common genes for this overlapping disease spectrum. As mentioned above, HSP and CMT2 share, with ALS, mutations in *SPG11* and *KIF5A* and HSP and ALS can be both caused by mutation in *UBQLN2* and *ERLIN2*. Distal myopathies, characterized notably by the presence of rimmed vacuoles (thought to be autophagic vacuoles due to lysosomal defects) on muscles biopsies after Gomori trichrome staining, share, with ALS, at least *VCP*, *SQSTM1* and *MATR3* genetic causes. *VCP*, *SQSTM1*, *hnRNPA1* and *hnRNPA2B1* mutations were also discovered in the IBMPFD multisystem proteinopathies. In this complex disease spectrum, patients can have one, two or more affected systems. A clinical study of 145 patients with *VCP* mutations showed the following resulting disease proportion: 90% myopathy, 50% PDB, 30% FTD and 9% ALS with overlap of the different symptoms [72]. Thus, it can be relevant for clinicians to consider also these related diseases as relevant familial history for ALS patients.

SQSTM1 mutations, also identified in IBMPFD, have to be interpreted with caution. This gene was initially reported as responsible for PDB, with high occurrence frequency in European populations (3% of people older than 55 years). Several *SQSTM1* variants initially reported in ALS patients are actually quite frequent in control populations with their sequenced genome available today, and very limited cosegregation data have been described for *SQSTM1* mutations in ALS families [73]. We identified more *SQSTM1* mutations in SALS (3/80) [74] than in FALS cases (1/380). Even if:

- incomplete penetrance could explain *SQSTM1* mutation preponderance in SALS cases and
- the more frequent p62 inclusions we observed in motor neurons of these mutated SALS argued in favor of the pathogenicity of these mutations, we cannot rule out the possibility that a *SQSTM1* mutation alone is not sufficient to trigger ALS but requires oligogenic inheritance.

A digenic *TIA1/SQSTM1* inheritance was already reported in three families with distal myopathy [75] and a similar association of *SQSTM1* with other gene(s) could be required to trigger ALS.

6. The interpretation of variant pathogenicity has become crucial

The development of next generation sequencing technologies offers the possibility to analyze increasing numbers of genes in clinical genetic diagnosis. Given the large number of variants identified by such strategies, it has become crucial to assess the pathogenicity of each variant identified, in order to deliver the most accurate genetic counseling to patients and relatives. The guidelines proposed by the American College of Medical genetics and Genomics use different categories to classify variants in pathogenic mutation (very strong, strong, moderate and supporting categories) and benign variant (stand alone, strong and supporting categories) [76]. Another classification proposed to subcategorize variants in 5 groups from pathogenic or likely pathogenic (class 5 and 4) to likely benign and benign (class 2 and 1) separated by the variant of unknown significance (VUS, class 3) group [77]. Both classifications have been used in Italian ALS populations and led to similar results [78, 79]: after screening the main ALS genes, two third of the familial cases are genetically elucidated (~70%). After screening for ALS, FTD and rare genetic causes of other motor neuron diseases in the remaining (~30%) patients, 5% of the cases (1.7% of the initial population) can be

firmly explained by the discovery of a pathogenic variant (class 4 or 5), 45% of them (15% of the initial population) are uncertain (with a VUS, a heterozygous variant in susceptibility genes, or a heterozygous variant in recessive genes) and half of them remained unexplained (with no or benign variant in rare genes). These results suggest that additional genetic causes are missing to complete the genetic puzzle in ALS.

Despite these guidelines, several specific points require further attention in ALS cases:

- incomplete penetrance can sometimes hide a familial transmission of the disease and is reported for most ALS genes reported to date
- the list of genes to study in ALS is difficult to define in part because of the genetic overlap with other diseases;
- some variants responsible for ALS can be found in the general population and the proposed 0.005% for threshold frequency is not consensual;
- tools for large scale reproducible and reliable results are still missing to perform functional studies and address the damaging impact of the growing lists of identified VUS variants.

7. Conclusion

In summary, the individual contribution of the multitude of rare genes to the etiology of ALS is relatively small. However, each of these discoveries has contributed to our current understanding of the causes of ALS and their underlying pathophysiological consequences. Protein aggregation is observed for several ALS causing genes. As most ALS cases (including those with no mutation in *TARDBP* gene) accumulate TDP-43 inclusions, multiple genetic causes can lead ultimately to a common degenerative pathway. The progress in ALS genetic underlines the genetic overlap between adult onset ALS, FTD, PDB and myopathy and, for juvenile or early onset ALS, with HSP (*SPG11*, *ALS2*, *KIF5A*) and CMT2 (*KIF5A*). Moreover a disease continuum starting by

HSP and evolving to severe ALS can also be observed (*UBQLN2*, *ERLIN2*) (Fig. 3). Genetic discoveries also highlighted several pathways: mRNA regulation (*TARDBP*, *FUS*, *VCP*, *MATR3*, *HNRNPA1*, *PFN1*), DNA damage repair (*FUS*, *MATR3*, *VCP*) proteolysis (*C9orf72*, *OPTN*, *SQSTM1*, *TBK1*, *UBQLN2*, *VCP*) and intracellular trafficking (*PFN1*, *TUBA4A*), critical for motor neuron survival. Of note, some gene candidates could be classified in more than one single cellular process as, for example *ANXA11* encoding Annexin A11 involved in transport of RNA granules on lysosomes for long distance trafficking along cytoskeleton (i.e. linking RNA metabolism and proteolysis components). First gene therapy clinical trials using antisense strategies against *SOD1* have started. Other genetic therapy approaches for *FUS* and *C9orf72* mutated patients are expected to arise soon. Strategies to restore misfolded protein clearance and dissolve protein aggregates could be useful to prevent TDP-43 mis-accumulations. Other pharmacological molecules able to regulate cellular stress response could become valuable in the future. In the meantime, further research aiming to identify the undiscovered ALS causative genes is needed.

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Genes	Mean age at onset in years (range)	Onset in LL (%)	Onset in UL (%)	Bulbar Onset (%)	Disease duration	FTD occurrence (%)
<i>C9orf72</i>	58 (30-81)	30	25	40	<3 years	40
<i>SOD1</i>	51 (21-73)	80	11	9	<3 years (50%) >7 years (30%)	0
<i>TARDBP</i>	52 (27-78)	25	65	10	< 7 years	17
<i>FUS</i>	43 (25-68)	40	45	5	< 3 years	5
Other causes	60 (32-85)	40	35	25	< 7 years	17

Table 1. Clinical characteristics of FALS patients with a mutation in one of the main ALS related genes

Clinical data were analyzed from 500 French FALS members.

FTD, FrontoTemporal Dementia; LL, lower limbs; UL, upper limbs.

	Nucleic acid metabolism		Proteolysis	Cytoskeleton / trafficking
	DNA damage	RNA metabolism		
ALS2				X
ANG		X		
ANXA11		X	X	
ATXN2		X		
C9orf72		X	X	
CHCHD10				X
CHMP2B			X	
DCTN1				X
DNAJC7			X	
ERLIN2			X	
EWSR1		X		
FUS	X	X		
GLE1		X		
hnRNPA1		X		
hnRNPA2B1		X		
KIF5A				X
MAPT				X
MATR3		X		
NEK1	X	X		
NFEH				X
OPTN			X	
PFN1		X		X
PRPH				X
SETX	X	X		
SIGMAR1			X	X
SOD1		X		
SPG11				X
SQSTM1			X	
SS18L1		X		
TAF15		X		
TARDBP		X		
TBK1			X	
TIA1		X		
TUBA4A				X
UBQLN2			X	
VAPB			X	X
VCP	X	X	X	

Table 2. ALS genes are involved in 3 main cellular pathways.

ALS genes encode proteins that play roles either in nucleic acid metabolism (DNA damage response and mRNA metabolism regulation), proteolysis (including the ubiquitin proteasome system, lysosomal pathway, ERAD and heat shock response) and cytoskeleton network.

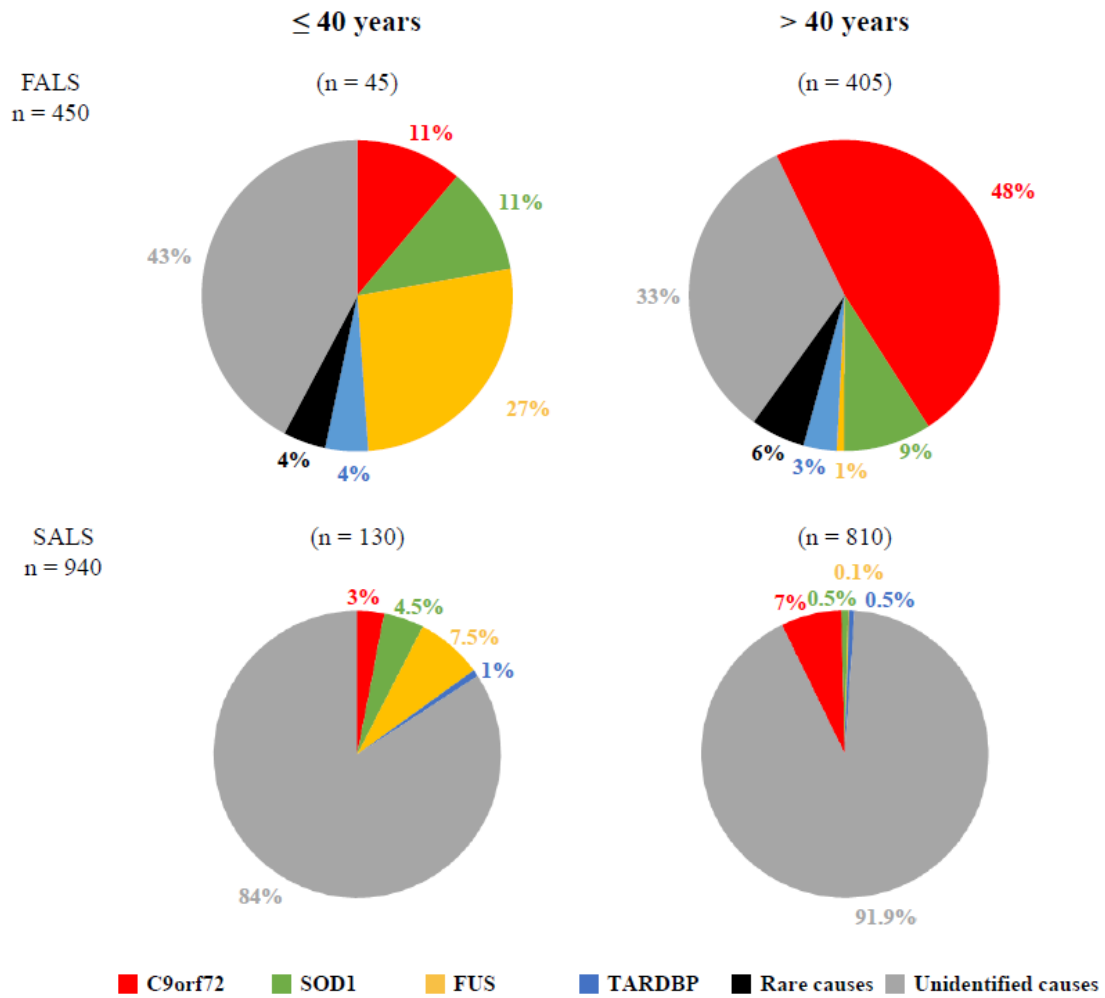


Fig. 1. Proportion of gene mutations in various ALS populations.

Proportion of patients with familial (FALS) or sporadic (SALS) form of ALS with early (age of onset before 40 years) or late (age of onset after 40 years) occurrence. Rare causes included *ATXN2* (>30 repeat expansions) and mutations in *VAPB*, *UBQLN2*, *PFN1*, *CHCHD10*, *TBK1* and *ERLIN2*.

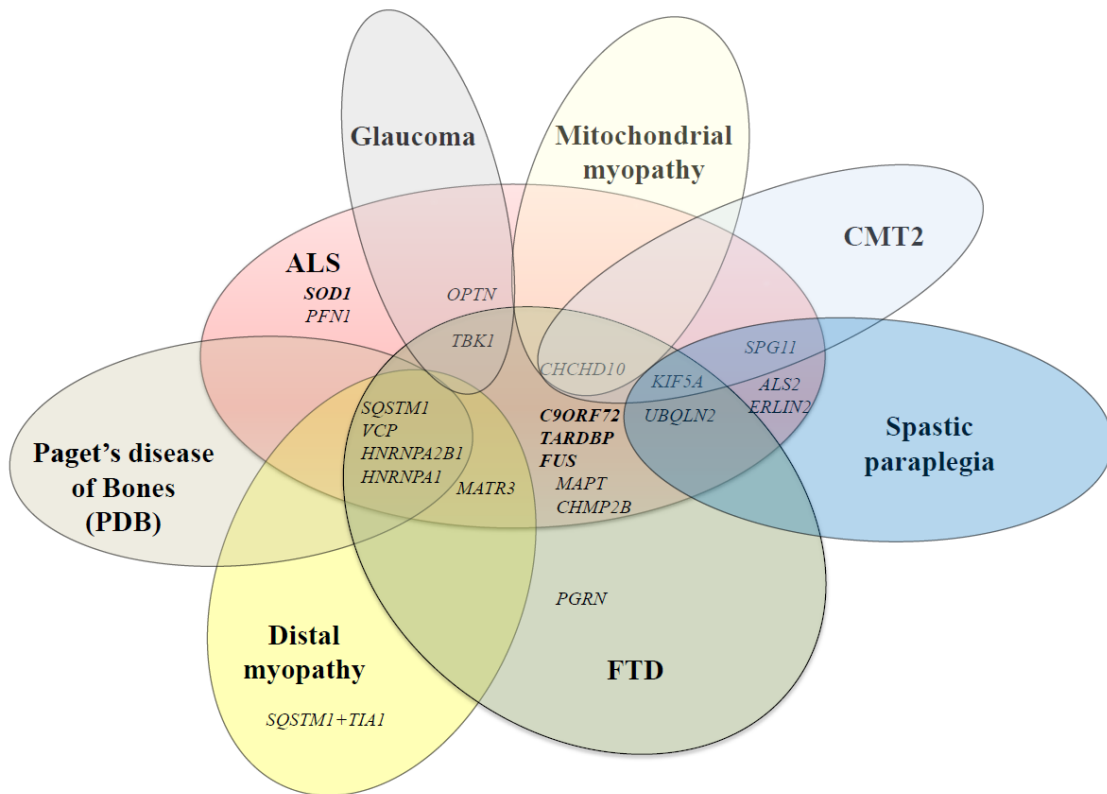


Fig. 2. The genetic overlap between ALS and other degenerative diseases

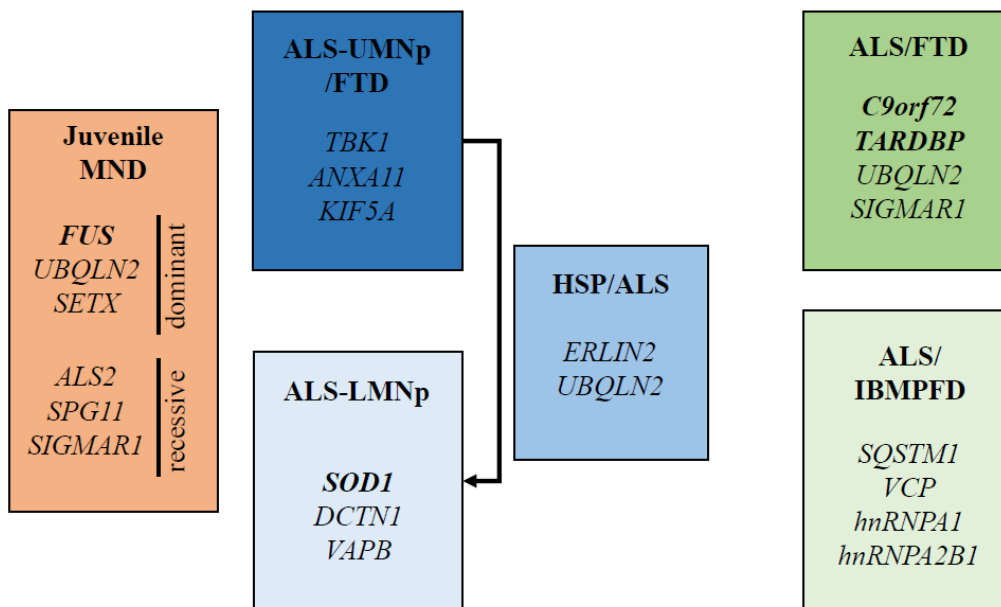


Fig. 3. Different ALS presentations with associated genes.

ALS, Amyotrophic lateral sclerosis; FTD, frontotemporal dementia; HSP, hereditary spastic paraplegia, IBMPFD, Inclusion body myopathy with early-onset Paget disease and/or frontotemporal dementia; LMNp, lower motor neuron predominance; MND, motor neuron disease; UMNp, upper motor neuron predominance. The 4 main ALS genes are in bold.