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**The NGS technology for the identification of genes associated with the ALS. A systematic review**

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# Evaluation of next generation sequencing (NGS) technology to identify causative genes associated to the Amyotrophic Lateral Sclerosis (ALS). A systematic review.

*Valentina Pecoraro**

Contact author

Laboratorio Tossicologia

Dipartimento Interaziendale ad Attività Integrata Medicina di Laboratorio e Anatomia Patologica

Ospedale Civile S. Agostino Estense, Modena

Via Pietro Giardini 1355, 41126, Modena

Tel + 39 3961427

*Jessica Mandrioli*

Dipartimento di Neuroscienze

Ospedale Civile S. Agostino Estense, Azienda Ospedaliero Universitaria di Modena, Modena

Via Pietro Giardini 1355, 41126, Modena

Tel + 39 3961640

*Adriano Chiò*

Dipartimento di Neuroscienze "Rita Levi Montalcini", Centro SLA, Università di Torino e Azienda Ospedaliero Universitaria Città della Salute e della Scienza, Torino, Italy.

*Chiara Carone*

Laboratorio Tossicologia

Dipartimento Interaziendale ad Attività Integrata Medicina di Laboratorio e Anatomia Patologica

Ospedale Civile S. Agostino Estense, Modena

*Tommaso Trenti*

Laboratorio Tossicologia

Dipartimento Interaziendale ad Attività Integrata Medicina di Laboratorio e Anatomia Patologica

Ospedale Civile S. Agostino Estense, Modena

**Abstract**

**Background**: More than 30 causative genes have been identified in familial and sporadic Amyotrophic Lateral Sclerosis (ALS), the most frequent being represented by *C9ORF72, SOD1, TARDBP, FUS, OPTN, and VCP*. The next-generation sequencing (NGS) is a powerful and groundbreaking tool to identify disease-associated common and rare variants, and novel genetic mutations. The application of NGS, sequencing simultaneously a large number of genes, results in an acceleration of the sequencing process. Despite documented advantages of NGS, its diagnostic reliability needs to be addressed in order to use this technology for specific routine diagnosis.

**Methods:** Literature database was explored to identify studies comparing NGS and Sanger sequencing for the detection of variants causing ALS. We collected data about patients' characteristics, disease type and duration, NGS and Sanger properties.

**Results**: Our systematic search identified more than 200 bibliographic references, of which only 34 publications were eligible. After reading the full-text, we excluded 20 papers that were not meeting our inclusion criteria and we included a total of 14 studies. Only 2 out of 14 studies compared results of NGS analysis with the Sanger sequencing. Twelve studies screened causative genes associated to ALS using NGS technologies and confirmed the identified variants with Sanger sequencing. Overall, data about more 2,000 patients were analyzed. The number of genes that were investigated in each study ranged from 1 to 32, the most frequent being *FUS, OPTN, SETX* and *VCP.* NGS identified already known mutations in 21 genes, and new or rare variants in 27 genes. These variants were responsible for truncated proteins leading a loss of function.

**Conclusion:** NGS seems to be a promising tool for the diagnosis of both familial and sporadic ALS in routine clinical practice. Its advantages are represented by an increased speed and a lowest sequencing cost, but patients' counseling could be problematic due to the discovery of frequent variants of unknown significance.



**Words**: 310

**Keywords**: NGS, ALS, review, evidence, genetic analysis, gene detection, Sanger

## Background

Amyotrophic Lateral Sclerosis (ALS) is a progressive and devastating neurodegenerative disorder characterized by degeneration of motor neurons in the brain and spinal cord. It causes muscle weakness, disability, and eventually death, with a median survival of three to five years.[1]

The annual incidence rates for ALS are 2-3 per 100,000 person-years in European and US populations, while prevalence rates range between 3 and 10 per 100,000. The lifetime risk is 1 in 300 for men and 1 in 400 for women with disease burden increasing with age.[2 3 4] Most cases (90%) are classified as sporadic ALS (SALS), as they are not associated with a documented family history for the disease, while around 10% of cases are considered to be familial (FALS) - when the disease is also present in a first-degree or second-degree relative. These figures may change depending on the definition of FALS and on the methods used to assess familiarity.[5] Cases of FALS are inherited most commonly with a Mendelian dominant mode and incomplete penetrance, although families with recessive and X-linked dominant inheritance have been reported.[6]

For a long time, ALS and FTD were considered two distinct pathologies, affecting the motor and the cognitive functions, respectively, but evidence from clinical, pathological, and above all genetic studies has emphasized the multisystem nature of these diseases with overlapping symptoms and causes. Clinical and pathological examination indicate that approximately 10-15% of FTD patients display features of motor neuron disease, while around 50% of ALS cases show cognitive and behavioral impairment of which 10-15% meet diagnostic criteria for FTD.[7]

ALS and FTD share common genetic mutations that may be present in familial but also in apparently sporadic cases. These may be explained by *de novo* mutations, incomplete and age-dependent penetrance, pleiotropy (the ability of mutations in a particular gene to result in different diseases, either simultaneously or in different individuals), and unrecognized or misdiagnosed familial cases (due to inadequate documenting of family history, loss of contact among family members, reluctance to report hereditary disease, small family size, early death of at-risk individuals, non-paternity). [8]

Although ALS and FTD pathogenesis remains largely unknown, recent advances in gene mutations discover lead to significant achievements on the aetiology and mechanisms that are at the basis of this spectrum of diseases. The common denominator shared by ALS, FTD, and many different neurodegenerative diseases, such as Parkinson disease and Alzheimer disease, is the deposit and accumulation of protein aggregates leading to glial and neuronal dysfunction and eventually cell death.

In the large majority of ALS patients (97%) the main component of such aggregates is represented by TDP-43 protein[9], which is the hallmark of almost all sporadic ALS cases, and of a large part of familial or mutated ALS with some exceptions, essentially represented by the *SOD1* and *FUS* familial cases of ALS which are associated with SOD1 and FUS positive inclusions respectively. [10 11 12]

Nonetheless, mutations in *TARDBP* gene, encoding for TDP-43 protein, account for only 3-4% of familial ALS [13] and 1-2% of FTD[14]. These data suggest that TDP-43 is central to the process of the ALS-FTD spectrum, independently from *TARDBP* mutations. Indeed, mutations in other RNA regulatory genes such as *FUS, MATR3*[15], *hnRNPA1, hnRNPA2B1*[16], *TATA-box binding protein associated factor 15*[17], and *TIA1*[18], are also associated with TDP-43 proteinopathy by impairing RNA processing, likely via direct interaction with TDP-43. RNA binding proteins are also intrinsically aggregation prone, due to the so-called prion-domain present in many RNA binding proteins[19]. Protein instability and aggregation propensity characterizes also *SOD1* associated pathology, merging the many different genetic forms of these diseases. This intrinsic instability in ALS proteins requires the cell preservation of protein homeostasis, with removal of non-functional and misfolded protein[20]. To this extent, in addition to defects in RNA metabolism, impaired protein quality control (and genes involved in it) is thought to be a major contributor to ALS pathogenesis [21].

These disease mechanisms recapitulate well also ALS and FTD caused by the *C9orf72* GGGGCC hexanucleotide repeat expansions, which represents the most common genetic cause of both diseases, explaining 25% of familial FTD and up to 88% of familial patients with both ALS and FTD [22]. *C9orf72* associated diseases are characterized by TDP-43 pathology with the accumulation of repeat-containing RNA transcribed from *C9orf72* repeat expansions, which combine with various RNA-binding proteins and, in this way can impair their function. Moreover, *C9orf72* repeat expansions produce several aggregation-prone proteins of repeating dipeptides (DPR) that alter SGs dynamics[23] and inhibit nuclear import of TDP-43[24].

In fact, mutations in genes further involved in protein clearance such as *valosin-containing protein* (VCP)[25] , *ubiquilin 2* (UBQLN2) [26] , *TANK-binding kinase 1 (TBK1)* [27], *sequestosome 1 (SQSTM1)*[28], *optineurin (OPTN)* [29], can impair protein degradation and contribute to toxic accumulation of compounds that, in turn, can inhibit protein degradation and sequester RNA and other proteins required for proper cellular function [30].

In addition, genetic studies also showed two other important pathways that participate to ALS disease: cellular trafficking and cytoskeletal integrity (mutations were identified in the genes coding for *Profilin 1 (PFN1), Tubulin alpha 4A protein (TUBA4A), Annexin A11 (ANXA1)* and *Kinesin heavy chain isoform 5A (KIF5A)* and mitochondrial functionality and transport (mutations were identified in the genes coding for *SOD1* and *Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (CHCHD10)*) [31].

Despite the outstanding importance that genetics has acquired in ALS, the complexity of this disease leaves some outstanding questions still unsolved regarding the molecular mechanisms that drive disease presentation towards ALS or FTD specific phenotype from a heterogeneous genetic background (genetic heterogeneity) and the onset of different diseases from the same gene mutation (pleiotropy) on which epigenetic factors or modifiers can act to influence disease presentation.

On this background, currently in clinical practice genetic testing is widespread used to determine the causative gene mutation of a symptomatic patient with a family history of ALS. In spite of this, about 30-40% of individuals with fALS will not have a positive test result since FALS may be caused by a gene that has

not yet been discovered or included in the tested panel. In clinical practice, genetic testing are focused either on the research of mutations of *SOD1, TARDBP, FUS* genes or to establish the presence of a hexanucleotide repeat expansion in the c*9orf72* gene. The most appropriate sequencing method to detect mutations of a single gene rather than of a limited number of genes is the Sanger method. This technique is ideal for monogenic disorders with clear clinical indication and/or known mutation hot spots; however, the selection of candidate gene(s) for sequencing is extremely difficult when inherited disorders exhibit genetic and clinical heterogeneity (as it is for ALS) with consequent high cost and anxiety for the patient's family.

Next-generation sequencing (NGS) is a high throughput analytical method for characterizing nucleotide sequences of disease-associated gene. Massive parallel sequencing of multiple DNA fragments allows genetic sequence to be determined. There are a number of different NGS platforms using different sequencing technologies, but all NGS platforms perform sequencing of millions of small fragments of DNA.[32] All technologies share the same workflow including the template preparation, sequencing, and imaging and data analysis.[33] Respect to Sanger sequencing, both the speed of execution and the amount of data output generated with NGS are exponentially greater. NGS can sequence whole genome, a selected subset of target genes or exome only. The accuracy of NGS for the whole genome and for the exome is 92% up to 95%. Sanger sequencing remains the gold standard, and it is used to confirm the presence of specific mutations identified by NGS in clinical setting, due to its higher accuracy (>99.99%).[34 35] The NGS technology allows identifying multiple genetic aberrations such as single or multiple nucleotide variants, small and large insertions or deletions, and it may be appropriate to identify disease-associated genes, to describe polymorphisms and to characterize numerous types of diseases.

We evaluated the current literature to assess the clinical and analytical validity and usefulness of NGS technology to identify ALS associated genes.

**Methods**

The systematic review protocol was developed and registered with the PROSPERO database ( CRD42019125537). For the reporting of the results, we followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).[36]

*Criteria for considering studies for this review*

We searched for studies evaluating NGS for detection of genes associated with ALS. We selected for inclusion primary studies complying with our inclusion and exclusion criteria defined as: (i) randomized controlled trials, observational, cross sectional or cohort studies (ii) studies enrolling at least five patients with ALS; (iii) studies evaluating NGS methods and considering Sanger sequencing as reference (iv) studies reporting on at least one outcome of interest (v) studies published in English, Italian or Spanish (vi) full text articles. Studies that did not replicate or confirm NGS results with the Sanger reference method, controlled

studies including less than five patients, case series and case-report, studies available as abstract only, letter and editorial publications, studies on animal models and in vitro studies were excluded. In case of a study enrolling patients affected by ALS and FTD, we considered only data concerning ALS patients. If it was not possible to get data about ALS patients only, the study was excluded.

*Search strategy*

In order to identify all primary studies, we searched the following electronic databases: Pubmed, Embase, Scopus and Cochrane Central Register of Controlled Trials (CENTRAL). Search strategy adopted was similar across the databases and it was developed using key words including "next-generation sequencing", "high-throughput nucleotide sequencing", "amyotrophic lateral sclerosis". The search strategy was developed for Pubmed and adapted for all databases (**Table I in** Supplemental material). We also examined the reference list of potentially eligible studies and contacted studies' authors if necessary. We limited the search to studies in humans and published in English, French, Italian, or Spanish. The literature search was conducted by one investigator on February 2019.

*Outcomes measures*

The outcome of interest was the identification of known or new mutations of ALS associated gene. We considered, also, the clinical validity defined in terms of diagnostic accuracy measurements (i.e. sensibility and specificity), the analytical validity defined in terms of concordance between NGS and Sanger results, and the clinical utility defined as the ability of the NGS to improve the clinical outcomes.[37]

*Study selection and data collection*

Two researchers (VP and CC) independently screened titles and abstracts retrieved through the database searches and selected the studies for inclusion according to eligibility criteria. Disagreements were resolved by consensus. From each of the included studies, one author extracted the data in an extraction form, and the second author checked data. The following information were recorded: (i) type of study design (i.e. cross sectional, cohort); (ii) characteristics of study (authors, year, setting, objective, eligibility criteria); (iii) characteristics of participants (i.e. sample size, age of onset, gender, disease duration, site of onset); (iv) characteristics of NGS technologies; (v) investigated outcomes as defined above.

*Quality assessment*

Two researchers independently assessed the methodological quality of the included studies. We adapted the NIH Quality assessment tool of the National Institute of Health for Observational cohort and cross-sectional studies (http://www.nhlbi.nih.gov/health-pro/guidelines/in-develop/cardiovascular-risk-reduction/tools/cohort ) in an ad hoc checklist to evaluate the methodological quality of the included

studies. This checklist includes the following questions: (1) Was the research question or objective in this paper clearly stated? (2) Was the study population clearly specified and defined? (3) Were all the subjects selected or recruited from the same or similar populations (including the same time period)? (4) Were the cases consecutive? (5) Were inclusion and exclusion criteria for being in the study pre-specified and applied uniformly to all participants? (6) Were the measured outcomes clearly defined, valid, reliable, and implemented consistently across all study participants? (7) Was the intervention clearly described? (8)Was there use of concurrent controls? (9) Were the outcome assessors blinded to the exposure status of participants? (10) Were the statistical methods well described? (11) Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure and outcome(s)? (12) Were the results well described? Possible answers included "yes", "no", "partially", or "not reported". Each study was rated for an overall quality as either good (almost 8 "yes"), fair (3 "no" and 3 "not reported" or "partially"), or poor (4 or more "no" and 4 or more "not reported" or "partially").

*Data Summary*

All studies were examined in detail. For overall included studies, we reported the summary of results focusing on epidemiological and descriptive characteristics, including those with a potential for bias. Completeness of reporting for the main outcomes was described. No meta-analyses were performed due to the high heterogeneity of the studies included.

## Results

*Study selection*

The search strategy identified a total of 488 articles. Of these, 268 records remained after removing duplicates, and 234 papers were excluded based on title and abstract. The remaining 34 publications were retrieved for full evaluation. After reading the full-text, we excluded 20 out of 34 studies clearly not meeting our inclusion criteria (**Table II in** Supplemental material). Finally, 14 papers [38-51]met the inclusion criteria and were included in our evaluation (**Figure 1**). Of the selected papers, only 2 [42 51] aimed to evaluate the primary research question of determining whether NGS is more accurate than Sanger sequencing to identify pathological mutations of ALS associated genes. Details of included studies were outlined in **Table 1**.

*Characteristics of included studies*

All included publications were cohort studies. Twelve studies screened causative genes associated to ALS using NGS technologies and confirmed the identified variants with Sanger sequencing; only two studies compared results from NGS to Sanger sequencing. Overall, 2,339 patients were included of which 252 were FALS and 1,366 SALS. At baseline, age of onset ranged from 18 to 87 years, and site of onset was bulbar for

245 (10.5%) patients, spinal for 675 (29%) patients. Characteristics of included studies are summarized in **Table 1**.

*Quality assessment*

The overall methodological quality of included studies was classified as good only in four studies, fair in seven studies and poor in three studies. All studies clearly defined the research question, 13 out of 14 studies (92.8%) described clearly the inclusion and exclusion criteria and the intervention. Six studies (42.8%) reported partially the characteristics of the population enrolled, eight studies (57.1%) defined clearly the outcome measures, and 11 studies (78.6%) described well the statistical methods, but only two studies considered the potential confounding factors in their analyses. Two studies did not describe the source of their population, and only 6 studies (42.8%) described adequately the results. There were no studies with blinded outcome assessors. None of the examined studies described whether the patients were consecutive or not (**Figure 2**).

*Identification of gene mutations associated with ALS*

The number of genes analyzed in each included study ranged from 1 to 32. The most commonly evaluated genes were *FUS, OPTN, SETX, VCP* considered in 11 studies, *ANG, FIG4, SOD1, UBQLN2, TARDBP* and VAPB considered in 10 studies, *CHMP2B, DAO, DCTN1, PFN1* considered in 9 studies.

Only in two studies [42 51]all genes were sequenced with both NGS and Sanger methods and then the results were compared. In the first study[51], evaluating 8 genes, neither NGS nor Sanger revealed mutations in patients evaluated. In the second study[42], NGS technology identified 51 new or rare variants in 18 different genes, instead, Sanger sequencing identified 16 known mutations in 4 genes associated with ALS, and these mutations were identified also with NGS. Furthermore, authors reported that NGS detected potentially pathogenic mutations in 45.5% of fALS and 5.4% of sALS, and identified variants of unknown significance in 30% and rare potentially deleterious variants in 73% of ALS patients, while Sanger sequencing revealed mutations in about 23.8% and 3.8% of familial and sporadic cases, respectively.

Twelve studies analyzed genes to identify potential mutations related to ALS only by NGS technologies while Sanger sequencing confirmed the causative variants previously identified by NGS.

Only two out of 14 studies reported the false positive rate for NGS. In the first study[38] there were no false positive results, in the second one[47] the false positive rate was 26.4%.

Finally, 5 studies [38 40 42 46 47] evaluated the oligogenic features of the disease and reported that 37 out 1,880 patients (2%) harbored 2 or more potentially pathological mutations.

*Evaluation of the clinical utility*

In 13 studies[38-50], NGS allowed to identify already known mutations in 21 genes, and new or rare variants in 27 genes (**Table 2**). Identified variants were nonsense or missense mutations leading to a frameshift mutation resulting in a truncated protein and a loss of protein function. Genes associated with ALS were involved in multiple cellular functions, and harboring mutations interfered with normal cell physiology with the following main pathogenic mechanisms involved in ALS: disruption of RNA metabolism and translational biology (*C9ORF72, TARDBP, FUS, MATR3, HNRNPA1, HNRNPA2/B1, EWSR1, TAF15, ANG*), aberrant regulation of protein quality control (*UBQLN2, VCP, OPTN, VAPB, TBK1, SQSTM1*), cytoskeletal defects and trafficking abnormalities (*PFN1, TUBA4A*), and mitochondrial dysfunction and oxidative stress (*SOD1, CHCHD10*)[52] .

*Distribution of c9orf72 hexanucleotide repeats*

Seven studies [40 42 44 46 47 48 49] analyzed the *c9orf72* GGGGCC repeat expansion using methods other than NGS, since NGS is challenging to detect GC-rich long regions. Two studies[42 46] found 123 (8.9%) patients carrying a pathological expansion of c*9orf72*. In two studies[40 44] the number of repeats was within the normal range. Three studies [47 48 49] did not detect hexanucleotide repeat expansion of *c9orf72* among the patients who were analyzed.

**Discussion**

The advent of NGS technology has revolutionized the way to study genetic diseases, allowing investigating a large number of genes or gene fragments in a very short time, with the ability to identify new or rare mutations. This technology has brought to the detection of an over-growing number of variants of unknown significance, making genetic counseling and patients management more complicated with further studies needed to verify genes role in ALS pathogenesis.

This literature review provides a picture about the most common genes analyzed in patients affected by familial and sporadic ALS, focusing on the possible application of NGS sequencing in clinical practice. Among the studies included in this review, five evaluated the oligogenic nature of ALS, highlighting that some patients harbored pathogenic variants in more than one ALS associated genes. This aspect is more prone to be studied by NGS with respect to Sanger sequencing, and it could contribute to explain the considerable phenotypic variability among ALS patients. On the other hand, one of the limitations of NGS is its inability to detect the hexanucleotide expansion of *c9orf72* gene, the most frequent mutation in both FALS and SALS that is analyzed separately by improved PCR based methods.

Taken together, the potential to detect a high number of mutations/variants without complete understanding of their pathological significance, the increasing information about the complexity of ALS genetics and the growing number of genetic test requests especially among at-risk subjects (relatives of an ALS patients), require a multidisciplinary team, including a neurologist, a geneticist, and a psychologist, with

expertise in the field to give adequate information and support to the patient and his/her family, in an effort to translate the knowledge of ALS genetic architecture into clinically useful information.

In fact, ALS heritability is characterized by oligogenic inheritance (a single mutation is likely not to be sufficient to cause disease despite significantly increasing risk), allelic heterogeneity, pleiotropy (especially for *C9orf72, ATXN2, TBK1, FUS, C21orf2, NEK1, MATR3, CHCHD10, VCP, hnRNPA1* and *hnRNPA2B1*) and age-dependent penetrance that make difficult the counseling of patients with genetic risk variants and their family members [8] .

On the other hand, if offering genetic testing to FALS patients is largely accepted by the clinical and scientific community, recent recommendations have suggested that genetic counseling should be offered routinely to all ALS patients.[53] Currently, genetic testing for major ALS-related genes is required to access therapeutic trials for ALS patients and any further information on the genetic factors possibly underlying ALS development is of importance. In this context it has also been proposed that genetic testing should be performed in a shorter time than it takes with classic Sanger sequencing, so that the patient could benefit from the test results. [54]

More debated is the approach to predictive testing, but the possibility of future drug therapy trials for at-risk mutation carriers should be taken into account. Moreover, genetic testing may directly benefit those undergoing it by empowering and helping them in life decisions, and lifestyle, health and procreation choices. Additionally, many individuals consider the anxiety of living with the unknown as worse than knowing whether or not to be at genetic risk. [54 55]

In this context, individuals undergoing a genetic test should be informed about the method of execution of the test and about the limitations associated with genetic test including that:

(i) a negative result of the test does not exclude the possibility of having one or more other (untested or still unknown) genetic variants contributing to the disease development;

(ii) the result of the test may not be informative in case of variants of uncertain significance;

(iii) in front of a positive gene test, the risk for patient's family members is no longer limited to the risk of developing a single condition but more than one (e.g. *C9orf72* expansion is associated to ALS, FTD, parkinsonism, and psychiatric disorders); however, the detection of a genetic variant does not necessarily imply an inevitable development of the disease in family members since almost all genes associated to ALS have a reduced penetrance.

From a technical point of view, NGS, compared to the gold standard method of sequencing, Sanger method, allows to simultaneously study either the whole genome or the whole exome (the coding portion of the genome) of several individuals in the same session of work, and besides the detection of a high number of mutations, including rare mutations in multiple patients at the same time, has the advantage of reducing time and costs.

NGS technology generates a huge amount of information that requires appropriate bio-informatic knowledge in order to analyze data accurately, and to produce interpretable results.

In the execution of the genetic analysis it is necessary to take into account the influence of some qualitative parameters, such as: (i) the preparation of the template according to different operating protocols, (ii) the commercial availability of sequencing platforms, (iii) the design of the gene panel that allows a good compromise between the level of accuracy to be achieved and the coverage of the genome; (iv) the possible sources of error arising from the sequencing itself.

Therefore, it would be optimal to establish standard working procedures for NGS in order to guarantee reproducibility, transparency and standardization, favoring the correct interpretation of the results in the clinical context. In fact, the main problem remains the interpretation of the results that derive from NGS, especially the evaluation of the possible pathogenicity of novel or rare variants that this technology allows to detect.

**Conclusions**

NGS seems is a promising technology for the diagnosis of both familial and sporadic ALS, but the uncertainty concerning the interpretation of the results restricts its use in daily clinical practice. Nevertheless, the high number of genes associated with ALS has widened the spectrum of the disease and of the biological pathways that may contribute to motor neuron degeneration showing that the disease is probably more heterogeneous than once appreciated. Knowing the genetic profiles associated with ALS is essential to the better understanding of the disease and to identify new molecular and cellular pathways that can be potential markers and targets for new therapeutic interventions. currently, NGS is a fascinating technology in the field of research, and in the next future, genetic testing will probably become important for the development of personalized genetic profiles, which combined with other related information, could bring towards a precision medicine for ALS patients.

**Tables and Figures**

Table 1: Characteristics of included studies

Table 2: Studies identifying known or new ALS mutations

Figure 1: Flow diagram search

Figure 2: Quality assessment

**Supplemental material**

Table I: Search Strategies developed for literature databases
Table II: Summary of excluded studies

**Key points**

- ALS is a genetic disease in which a lot of genes contribute to the disease development, and some variants contribute to increase the risk of onset in each patient.
- The current literature describes the main genes involved in familial and sporadic ALS, highlighting that a single mutation does not inevitably lead to ALS, and that many ALS-associated genes are also implicated in other neurological conditions.
- NGS is a promising technology to study ALS associated genes, and it also allows to identify rare and novel mutations.
- The identification of variants with unknown significance by NGS sequencing complicates genetic counseling for ALS patients and warrants a multidisciplinary approach with expertise in the field for patients and at-risk individuals approaching genetic test.

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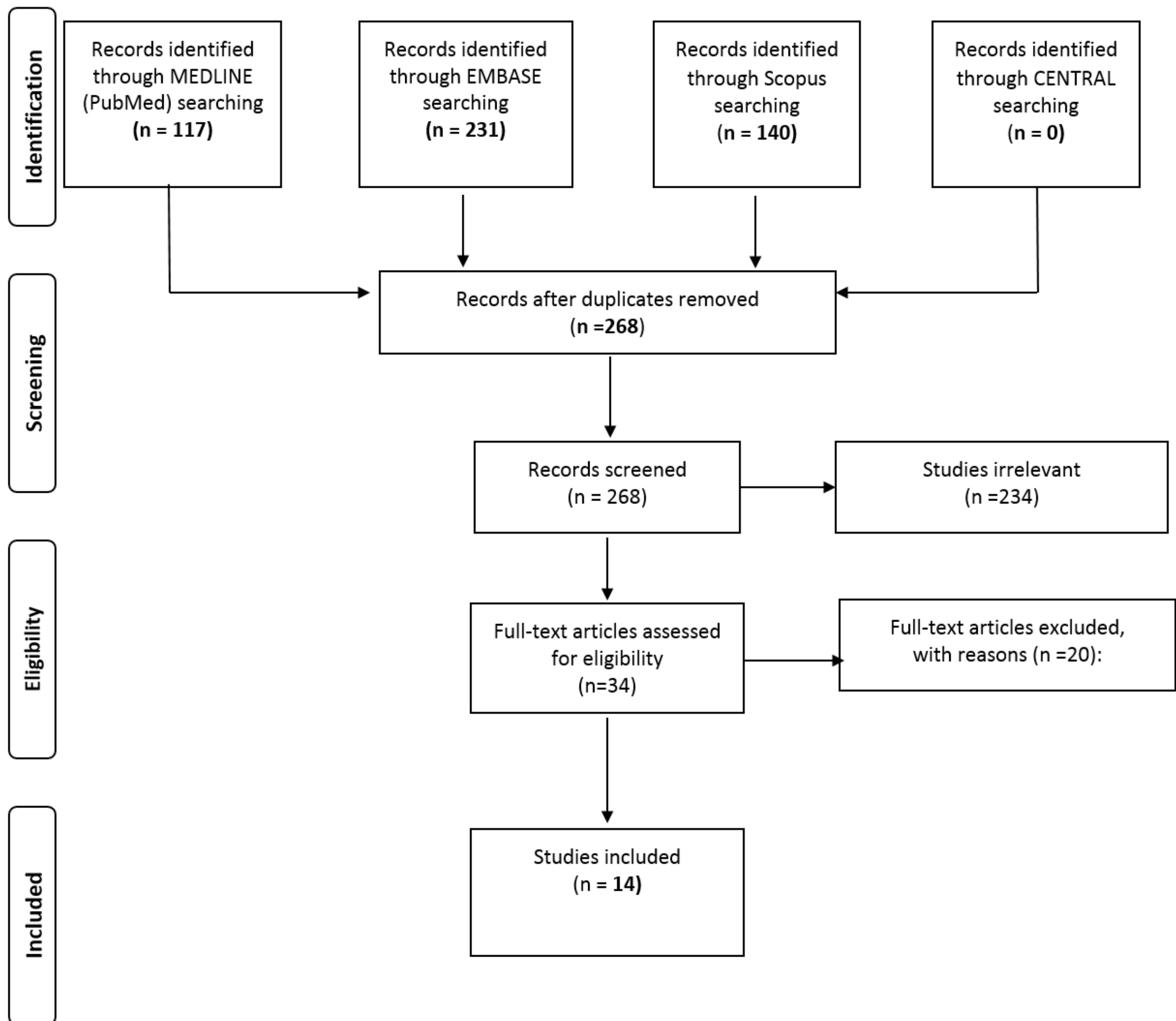
Figure 1



## Figure 2

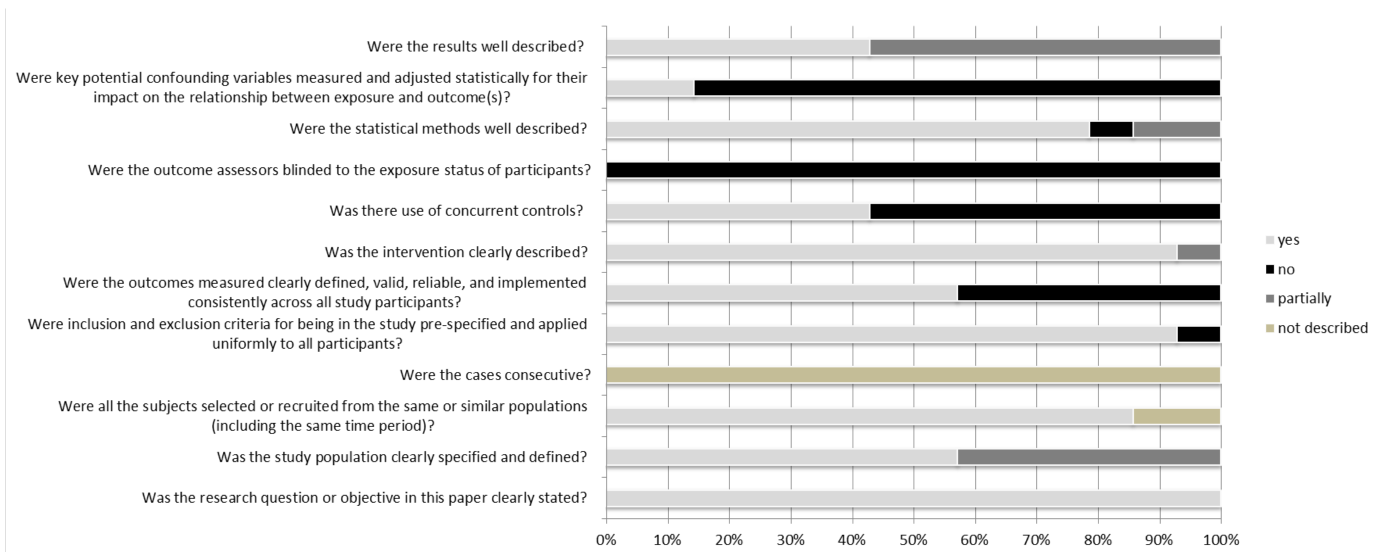


**Table 1.**Characteristics of included studies evaluating NGS technologies applied to molecular diagnosis of ALS

| Author | No. patients included | | | Ageof onset (mean ± SD or median (range)) | Siteof onset | Mean disease duration (year) from diagnosis | Type of NGS | Genes analyzed by NGS |
|---|---|---|---|---|---|---|---|---|
| | Total | No. fALS | No. sALS | | | | | |
| **Farhan 2016** | 22 | Nr | Nr | 61.9 ± 9.1 | Bulbar n=2 | Nr | ONDRISeq | *ALS2, ANG, ARHGEF28, ATXN2, CENPV, CHMP2B, DAO, DCTN1, FIG4, FUS, HNRNPA1, HNRNPA2B1, MAPT, NEFH, OPTN, PFN1, PRPH, SETX, SIGMAR1, SOD1, TARDBP, UBQLN2, UNC13A, VAPB, VCP, APOE* |
| **Goldstein 2016** | 379 | 9 | Nr | 59.5 ±12.2 | Bulbar n=102<br>Spinal n=277 | 35.8±26.1 | Illumina NextSeq500 | *OPTN* |
| **KimHJ 2016** | 152 | 4 | 148 | 55.7±5.8 | Bulbar n=49<br>Spinal n=99 | | HiSeq 2000 | *ALS2, ANG, DAO, FIG4, FUS, GRN, MAPTOPTN, SETX, SIGMAR1, SOD1, SPG11, SQSTM1, TAF15, RARDBP, UBQLN2, VAPB, VCP* |
| **KimYE2016** | 129 | Nr | Nr | 55.8 ±10 | Bulbar n=30<br>Spinal n=97<br>Respiratory n=1<br>Axial n=1 | | Illumina MiSeq or NextSeq500 | *TBK1* |
| **Lamp 2018** | 296 | 45 | 210 | 61.3 (18-87) | Bulbar n=62<br>Spinal n=205<br>Spinobulbar n=1<br>Respiratory n=1<br>Unknown n=27 | 3 | Ion Torrent | *ALS, ANG, BSCL2, CHMP2B, DCTN1, ERBB4, FIG4, FUS, GRN, HNRNPA1, MAPT, MATR3, OPTN, PFN1, PSEN1, PSEN2, SETX, SOD1, SPG11, TARDBP, UBQLN2, VAPB, VCP* |
| **Leblond 2016** | 247 | 83 | 164 | Nr | Nr | Nr | Illumuna HiSeq2000/2500 | *MATR3* |
| **Liu 2014** | 8 | 8 | 0 | Nr | Nr | Nr | Illumina HiSeq2000 | *ALS, ANG, CHMP2B, DAO, DCTN1, FIG4, FUS, OPTN, PFN1,* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | *SETX, SIGMAR1, SOD1, SPG11, SQSTM1, TARDBP, UBQLN2, VAPB, VCP* |
| **Marangi 2017** | 322 | 8 | 226 | Nr | Nr | Nr | Ion Torrent | *ANG, ATXN2, CHCHD10, CHMP2B, CHRNA4, DAO, DCTN1, EPHA4, EWSR1, FIG4, FUS, GLE1, GRN, HNRNPA1, HNRNPA2B1, MAPT3, MATR3, NIPA1, OPTN, PFN1, SETX, SIGMAR1, SOD1, SQSTM1, SS18L1, TAF15, TARDBP, TBK1, TUBA4A, UBQLN2, VAPB, VCP* |
| **Morgan 2017** | 1126 | 131 | 995 | fALS 56 (24-85), sALS 61 (25-88) | Nr | Nr | Illumina MiSeq | *ALS2, ANG,CHMP2B, DAO, DCTN1, FIG4, FUS, NEFH, OPTN, PFN1, PON1, PON2, PON3, PRPH, SETX, SOD1, SQSTM1, TARDBP, TREM2, UBQLN2,VAPB VCP, VEGF.,* |
| **Nakamura 2016** | 508 | 39 | 469 | 62.1 (IQR 53.5-68.4) | Nr | Nr | HiSeq2000 and Ion Torrent PGM | *ALS2, ANG, ATXN2, CHMP2B, DAO, DCTN1, EWSR1, FIG4, FUS, GRN, NEFH, OPTN, PFN1, PRPH, RNF19A, SETX, SIGMAR1, SOD1,SPG11, SQSTM1, TAF15, TARDBP, TFG, UBQLN2, VAPB, VCP, ZNF512B* |
| **Narain 2018** | 154 | 5 | 149 | Nr | Nr | Nr | Illumina MiSeq | *ANG, CHMP2B, DAO, DCTN1, ELP3, ERBB4, FIG4, FUS, LUM, MATR3, OPTN, PFN1, PON1, PON2, PON3, PRPH, SETX, SOD1, SPAST, SQDTM1, TAF15, TARDBP, UBQLN2, VAPB, VCP* |
| **Nishiyama 2017** | 51 | 51 | 0 | Nr | Nr | Nr | Illumina MiSeq | ALS2, ANG, ATXN2, CHMP2B, DAO, DCTN1, FIG4, FUS, NEFH, OPTN, PFN1, PRPH, SETX, SIGMAR1, SOD1, SPG11, TAF15, TARDBP, UBQLN2, VAPB, VCP |
| **Tripolszki 2017** | 28 | Nr | Nr | Nr | Nr | Nr | Roche | FUS, SETX, c9orf72 |
| **Turk 2017** | 43 | Nr | Nr | 66 (28-78) | Nr | 24 months | IonTorrent | *CAPZA1, CAPZB, CCDC53,* |

| | | | |
|---|---|---|---|
| | | (3-153 months) | *FAM21C, KIAA1033, KIAA0196, VCP, WASH1* |

**Table 2**: Known, new or rare mutations identified in the studies that were included in the systematic review

| Gene | Known mutations | New or rare mutations |
|---|---|---|
| ALS2 | Kim, Morgan | Kim, Nakamura, Nishiyama |
| ANG | Morgan, Narain, Nishiyama | Lamp |
| ATXN2 | - | Nakamura, Nishiyama |
| CHMP2B | Morgan | Narain |
| DAO | Narain | Nakamura, Narain |
| DCTN1 | Liu, Morgan | Lamp,Nakamura, Nishiyama |
| ERBB4 | - | Lamp, Narain |
| FIG4 | Morgan | Lamp, Nakamura |
| FUS | Morgan, Nakamura | Kim, Nakamura |
| MAPT | Kim | Kim, Lamp |
| MATR3 | Lamp | Leblond, Marangi |
| NEFH | Morgan | Nakamura,Nishiyama, |
| OPTN | Goldstein,Lamp, Morgan, Nishiyama | Narain |
| PFN1 | Morgan | - |
| PRPH | Morgan | Nakamura, Nishiyama |
| PSEN1 | - | Lamp |
| PSEN2 | - | Lamp |
| RNF19A | - | Nakamura |
| SETX | Nishiyama, | Kim, Lamp, Nakamura, Narain, Nishiyama, Triposki |
| SIGMAR1 | - | Nishiyama |
| SOD1 | Kim, Lamp, Liu, Morgan, Nakamura, Narain | - |
| SPG11 | - | Kim, Lamp, Nakamura, Nishiyama |
| SQSTM1 | Kim, Morgan | Kim, Narain |
| TAF15 | - | Kim, Nakamura, Nishiyama |
| TARDBP | Lamp, Morgan, Nakamura, Narain, Nishiyama | Kim |
| TBK1 | Kim_b | |
| TFG | - | Nakamura |
| VAPB | Morgan | Lamp |
| VCP | Morgan, Nakamura | - |
| UBQLN2 | Morgan | Kim, Lamp |
| ZNF512B | - | Nakamura |

## Supplemental material

**Table I**: Search Strategies developed for literature databases

| **PubMed** |
|---|
| (((((("High-Throughput Nucleotide Sequencing"[Mesh]) OR "High-Throughput Nucleotide Sequencing") OR "next generation sequencing") OR next generation sequencing) OR "ngs")) AND (((("Amyotrophic Lateral Sclerosis"[Mesh]) OR "Amyotrophic Lateral Sclerosis") OR Amyotrophic Lateral Sclerosis) OR ALS) |
| |
| **Emabase** |
| #1 next AND ('generation'/exp OR generation) AND ('sequencing'/exp OR sequencing)<br>#2 'next generation sequencing'<br>#3 ngs<br>#4 #1 OR #2 OR #3<br>#5 amyotrophic AND lateral AND sclerosis<br>#6 'amyotrophic lateral sclerosis'<br>#7 als<br>#8 #5 OR 6 OR #7<br>#9 #4 AND #8 |
| |
| **CENTRAL** |
| #1 MeSH descriptor: [High-Throughput Nucleotide Sequencing]<br>#2 "next generation sequencing"<br>#3 ngs<br>#4 #1 or #2 or #3<br>#5 MeSH descriptor: [Amyotrophic Lateral Sclerosis]<br>#6 'amyotrophic lateral sclerosis'<br>#7 #5 or #6<br>#8 #4 and #7 |
| |
| **Scopus** |
| (TITLE-ABS-KEY("High-Throughput Nucleotide Sequencing") OR TITLE-ABS-KEY("next generation sequencing") OR TITLE-ABS-KEY (ngs)) AND (TITLE-ABS-KEY( amyotrophic AND lateral AND sclerosis) OR TITLE-ABS-KEY(als)) |

**Table II**: Summary of excluded studies

| | Study | Reason for exclusion |
|---|---|---|
| 1 | Brohawn DG, O'Brien LC, Bennett JP, Jr.(2016) RNAseq Analyses Identify Tumor NecrosisFactor-Mediated Inflammation as a Major Abnormalityin ALS Spinal Cord. PLoS ONE 11(8): e0160520. | RNA analysis |
| 2 | Couthouis J, Raphael AR, Daneshjou R, Gitler AD (2014) Targeted Exon Capture and Sequencing in Sporadic Amyotrophic Lateral Sclerosis. PLoSGenet 10(10): e1004704. | Included only patients with known mutations |
| 3 | Farhan SM, Gendrom TF, Petrucelli L, Hegele RA, Strong MJ. OPT p.Met468Arg and ATXN2 intermediate length polyQ extension in families with c9orf72 mediated amyotrophic lateral sclerosis and frontotemporal dementia. Am J Med Genet 2018; 177B:75-85 | Case report |
| 4 | Garton FC, Benyamin B, Zhao Q, Liu Z, Gratten J, Henders AK, Zhang ZH, Edson J, Furlong S, Morgan S, Heggie S, Thorpe K, Pfluger C, Mather KA, Sachdev PS, McRae AF, Robinson MR, Shah S, Visscher PM, Mangelsdorf M, Henderson RD, Wray NR, McCombe P. Whole exome sequencing and DNA methylation analysis in a clinical amyotrophic lateral sclerosis cohort. Molecular Genetics &Genomic Medicine 2017; 5(4): 418–428 | No Sanger considered |
| 5 | Giannoccaro MP, Bartoletti Stella A, Piras S, Pession A, De Massis P, Oppi F, Stanzani-Maserati M, Pasini E, Baiardi S, Avoni P, Parchi P, Liguori R, Capellari S. Multiple variants in families with amyotrophic lateral sclerosis and frontotemporal dementia related to c9orf72 repeatexpansion: further observations on their oligogenic nature. J Neurol (2017) 264:1426–1433 | No Sanger considered |
| 6 | Johnson JO, Mandrioli J, Benatar M, Abramzon Y, Van Deerlin VM, Trojanowski JQ, Gibbs JR, Brunetti M, Gronka S, Wuu J, Ding J, McCluskey L, Martinez-Lage M, Falcone D, Hernandez D, Arepalli S, Chong S, Schymick J, Rothstein J, Landi F, Wang M, Calvo A, Mora G, Sabatelli M, Monsurrò MR, Battistini S, Salvi F, Spataro R, Sola P, Borghero G, Italsgen, Galassi G, Scholz SW, Taylor JP, Restagno G, Chiò A, Traynor BJ. Exome sequencing reveals VCP mutations as a cause of familial ALS. Neuron. 2010 December 9; 68(5): 857–864. | Case report |
| 7 | Kenna KP, McLaughlin RL, Byrne S, Elamin M, Heverin M,Kenny EM, Cormican P, Morris DW, Donaghy CG, Bradley DG, Hardiman O. Delineating the genetic heterogeneity of ALS using targeted high-throughput sequencing. J Med Genet 2013;50:776–783. | No Sanger considered |
| 8 | Krüger S, Battke F, Sprecher A,Munz M, Synofzik M, Schöls L, Gasser T, Grehl T, Prudlo J andBiskup S (2016) Rare Variants in Neurodegeneration Associated Genes Revealed by Targeted Panel Sequencing in a German ALS Cohort. Front. Mol. Neurosci. 9:92. | No Sanger considered |
| 9 | Matamala JM, Arias-Carrasco R, Sanchez C, Uhrig M, Bargsted L, Matus S, Maracaja-CoutinhoV, Abarzua S, van Zundert B, Verdugo R, Manque P, Hetz C. Genome-wide circulating microRNA expression profiling reveals potential biomarkers for amyotrophic lateral sclerosis. Neurobiology of Aging 64 (2018) 123e138 | Study on animal models |
| 10 | Mitropoulos K, Papadima EM, Xiromerisiou G, Balasopoulou A, Charalampidou K, Galani V, Zafeiri KV; Dardiotis E, Ralli S, Deretzi G, John A, Kydonopoulou K, Papadopoulou E, di Pardo A, Akcimen F, Loizedda A, Dobričić V, Novaković I, Kostić VS, Mizzi C, Peters BA, Basak N, Orrù S, Kiskinis E, Cooper DN, Gerou S, Drmanac R, Bartsakoulia M, Tsermpini E, Hadjigeorgiou GM, Ali BR, Katsila T, Patrinos GP. Genomic variants in the FTO gene are associated with | Study of gene association |

| | | |
|---|---|---|
| | sporadic amyotrophic lateral sclerosis in Greek patients. Human Genomics (2017) 11:30 | |
| 11 | Morgan S, Shoai M, Fratta P, Sidle K, Orrell R, Sweeney MG, Shatunov A, Sproviero W, Jones A, Al-Chalabi A, Malaspina A, Houlden H, Hardy J, Pittman A. Investigation of next-generation sequencing technologies as a diagnostic tool for amyotrophic lateral sclerosis. Neurobiology of Aging 36 (2015) 1600.e5e1600.e8 | No Sanger considered |
| 12 | Pang SY, Hsu JS, Teo KC, Li Y, Kung MHW, Cheah KSE, Chan D, Cheung KMC; Li M, Sham PC, Ho SL. Burden of rare variants in ALS genes influences survival in familialand sporadic ALS. Neurobiology of Aging 58 (2017) 238.e9e238.e15 | No Sanger considered |
| 13 | Prudencio M, Belzil VV, Batra R, Ross CA, Gendron TF, Pregent L, Murray ME, Overstreet KK, Piazza-Johnston AE, Desaro P, Bieniek KF, DeTure M, Lee WC, Biendarra SM, Davis MD, Baker MC, Perkerson RB, van Blitterswijk M, Stetler CT, Rademakers R, Link CD, Dickson DW, Boylan KB, Li H, Petrucelli L. Distinct brain transcriptome profiles in c9orf72-associated and sporadic ALS. Nat Neurosci. 2015 August ; 18(8): 1175–1182. | brain transcriptome profiles |
| 14 | Satoh J, Asahina N, Kitano S, Kino Y. A Comprehensive Profile of ChIP-Seq-Based Olig2Target Genes in Motor Neuron Progenitor Cells Suggests the Possible Involvement of Olig2 in the Pathogenesis of Amyotrophic Lateral Sclerosis. Journal of Central Nervous System Disease 2015:7: 1–14 | No gene anaysis |
| 15 | Si Y, Cuic X, Crossmand DK, Haoc J, Kazamela M, Kwona Y, Kinga PH. Muscle microRNA signatures as biomarkers of disease progression in amyotrophic lateral sclerosis. Neurobiology of Disease 114 (2018) 85–94 | Study on animal models |
| 16 | Simandi Z, Horvath A, Cuaranta-Monroy I, Sauer S, Deleuze JF, Nagy L. RXR heterodimers orchestrate transcriptional control of neurogenesis and cell fate specification. Molecular and Cellular Endocrinology (2017) 1e12 | Study on animal models |
| 17 | Wilke C, Baets J, De Bleecker JL, DeconinckT, BiskupS, Hayer SN, Züchner S, SchüleR, De JongheP, Synofzik M. Beyond ALS and FTD: the phenotypic spectrum of TBK1 mutations includes PSP-like and cerebellar phenotypes. Neurobiology of Aging 62 (2018) 244.e9e244.e13 | Case report |
| 18 | Wu J, Shen E, Shi D, Sun ZS, Cai T. Identification of a novel Cys146X mutation of SOD1 in familial amyotrophic lateral sclerosis by whole-exome sequencing. Genet Med 2012:14(9):823–826 | Case report |
| 19 | Wu C, Fan D. A Novel Missense Mutation of the DDHD1 Gene Associated with Juvenile Amyotrophic Lateral Sclerosis. Frontiers in Aging Neuroscience 2016; 8: 1-5 | Case report |
| 20 | Wu CH, Fallini C, Ticozzi N, Keagle PJ, Sapp PC, Piotrowska K, Lowe P, Koppers M, McKenna-Yasek D, Baron DM, Kost JE, Gonzalez-Perez P, Fox AD, Adams J, Taroni F, Tiloca C, Leclerc AL, Chafe SC, Mangroo D, Moore MJ, Zitzewitz JA; Xu ZS, van den Berg LH, Glass JD, Siciliano G, Cirulli ET, Goldstein DB, Salachas F, Meininger V, Rossoll W, Ratti A, Gellera C, Bosco DA, Bassell GJ, Silani V, Drory VE, Brown RH, Landers JE. Mutations in the Profilin 1 Gene Cause Familial Amyotrophic Lateral Sclerosis. Nature. 2012 August 23; 488(7412): 499–503 | Study of gene expression |