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Received: 7 December 2019 | Revised: 5 March 2020 | Accepted: 13 March 2020

DOI: 10.1111/eci.13228

REVIEW

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

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Abstract

Background: More than 30 causative genes have been identified in familial and sporadic amyotrophic lateral sclerosis (ALS). The next-generation sequencing (NGS) is a powerful and groundbreaking tool to identify disease-associated variants. Despite documented advantages of NGS, its diagnostic reliability needs to be addressed in order to use this technology for specific routine diagnosis.

Material and 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: More than 200 bibliographic references were identified, of which only 14 studies matching our inclusion criteria. 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 analysed. 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.

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

KEYWORDS

amyothrophic lateral sclerosis, evidence, gene detection, next-generation sequencing, systematic review

1 | 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

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risk is 1 in 300 for men and 1 in 400 for women with disease burden increasing with age.[2-4] 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 in nature (FALS). These familial cases of FALS are most commonly inherited with a Mendelian dominant pattern of disease within complete penetrance, although families with recessive and X-linked dominant inheritance have been reported.[5]

For a long time, ALS and frontotemporal dementia (FTD) were considered to represent two distinct clinical entities, affecting the motor and the cognitive functions, but evidence from clinical, pathological and genetic studies has emphasized the multisystem nature of these diseases with overlapping symptoms and causes. Approximately 10%-15% of FTD patients display features of motor neuron disease, whereas half of ALS cases show cognitive and behavioural impairment.[6] ALS and FTD share common genetic mutations that may be present in familial but also in apparently sporadic cases. The finding of genetic mutations in sporadic disease may be explained by de novo mutations, incomplete and age-dependent penetrance, and unrecognized familial cases.[7]

Although ALS and FTD pathogenesis remains largely unknown, recent advances in gene mutations discovery 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 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,[8] 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.[9-11] Nonetheless, mutations in *TARDBP* gene, encoding for TDP-43 protein, account for only 3%-4% of FALS [12] and 1%-2% of FTD.[13] 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*,[14] *hnRNPA1, hnRNPA2B1*,[15] *TATA-box* binding protein-associated factor 15,[16] and *TIA1*,[17] 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.[15] Protein instability and aggregation propensity characterize 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.[18] 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.[19]

In fact, mutations in genes further involved in protein clearance such as *valosin-containing protein* (VCP),[20] *ubiquilin 2* (UBQLN2),[21] *TANK-binding kinase 1 (TBK1)*,[22] *sequestosome 1 (SQSTM1)*,[23] *optineurin (OPTN)*,[24] 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.[25]

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.[26] *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[27] and inhibit nuclear import of TDP-43.[28]

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)*).[29]

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.

Genetic testing is widespread used in clinical practice to determine the causative gene mutation of a symptomatic patient with a family history of ALS. Despite the major advances in our knowledge of ALS genetics over the last decade, such clinical testing fails to identify the causative mutation in about one third of cases, most likely because the underlying gene has not yet been discovered and therefore is not included in the tested panel. In clinical practice, genetic testing is 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. Sanger sequencing remains the most cost-effective sequencing method to detect mutations of a single gene. This technique is ideal for monogenic disorders with clear clinical indication and/or known mutation hot spots.

In contrast to Sanger sequencing, next-generation sequencing (NGS) is a high throughput method for characterizing nucleotide sequences of disease-associated gene. There are a number of different NGS platforms using different sequencing technologies, but one commonality between them in these platforms is that the sequence of millions of small fragments of DNA,[30] and the speed of execution and the amount of data output generated with NGS are exponentially greater than with Sanger sequencing. NGS can also be tailored to 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 in the clinical practice, and it is used to confirm the presence of specific mutations identified by NGS due to its higher accuracy.[31,32]

Given the relevance and the complexity of NGS, our objectives were to (a) conduct a structured systematic review of studies using the NGS to identify ALS-associated genes; (b) assess the clinical and analytical validity of NGS technology to identify ALS-associated genes; (c) evaluate the current literature to highlight the usefulness of NGS in the management of ALS patients.

2 | METHODS

2.1 | Protocol registration

The systematic review protocol was developed and registered in the PROSPERO international prospective register of systematic reviews (CRD42019125537). The reporting of this systematic review is conform to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).[33]

2.2 | 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: (a) randomized controlled trials, observational, cross-sectional or cohort studies (b) studies enrolling at least five patients with ALS; (c) studies evaluating NGS methods and considering Sanger sequencing as reference;(d) studies reporting on at least one outcome of interest; (e) studies published in English, Italian or Spanish; and (f) 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 and studies on animal models 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.

2.3 | Search methods for identification of studies

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 S1). 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.

2.4 | 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 (ie 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.[34]

2.5 | 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 was recorded: (a) type of study design (ie cross-sectional, cohort); (b) characteristics of study (authors, year, setting, objective, eligibility criteria); (c) characteristics of participants (ie sample size, age of onset, gender, disease

duration, site of onset); (d) characteristics of NGS technologies; (e) investigated outcomes as defined above.

2.6 | Quality assessment in individual studies

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").

2.7 | 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.

3 | RESULTS

3.1 | Study selection

The search strategy identified a total of 488 bibliographic references. 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 S2). Finally, 14 papers[35-48] met the inclusion criteria and were included in our evaluation (Figure 1). Of the selected papers, only 2 studies[39,48] 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.

3.2 | 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 1366 SALS. Five studies were conducted in Europe, two in Canada, two in Japan, two in Korea and one study in China, Israel and India. 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, with these data being unspecified for the remaining 60.5% of patients. Seven studies[36,40,41,43-45,47] analysed also healthy samples as control. Characteristics of included studies are summarized in Table 1.

3.3 | 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 (such as age, gender, ethnicity) 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).

3.4 | Identification of gene mutations associated with ALS

The number of genes analysed in each included study ranged from 1 to 32. The most commonly evaluated genes were *FUS, OPTN, SETX, VCP* considered in 11 studies, *ANG,*

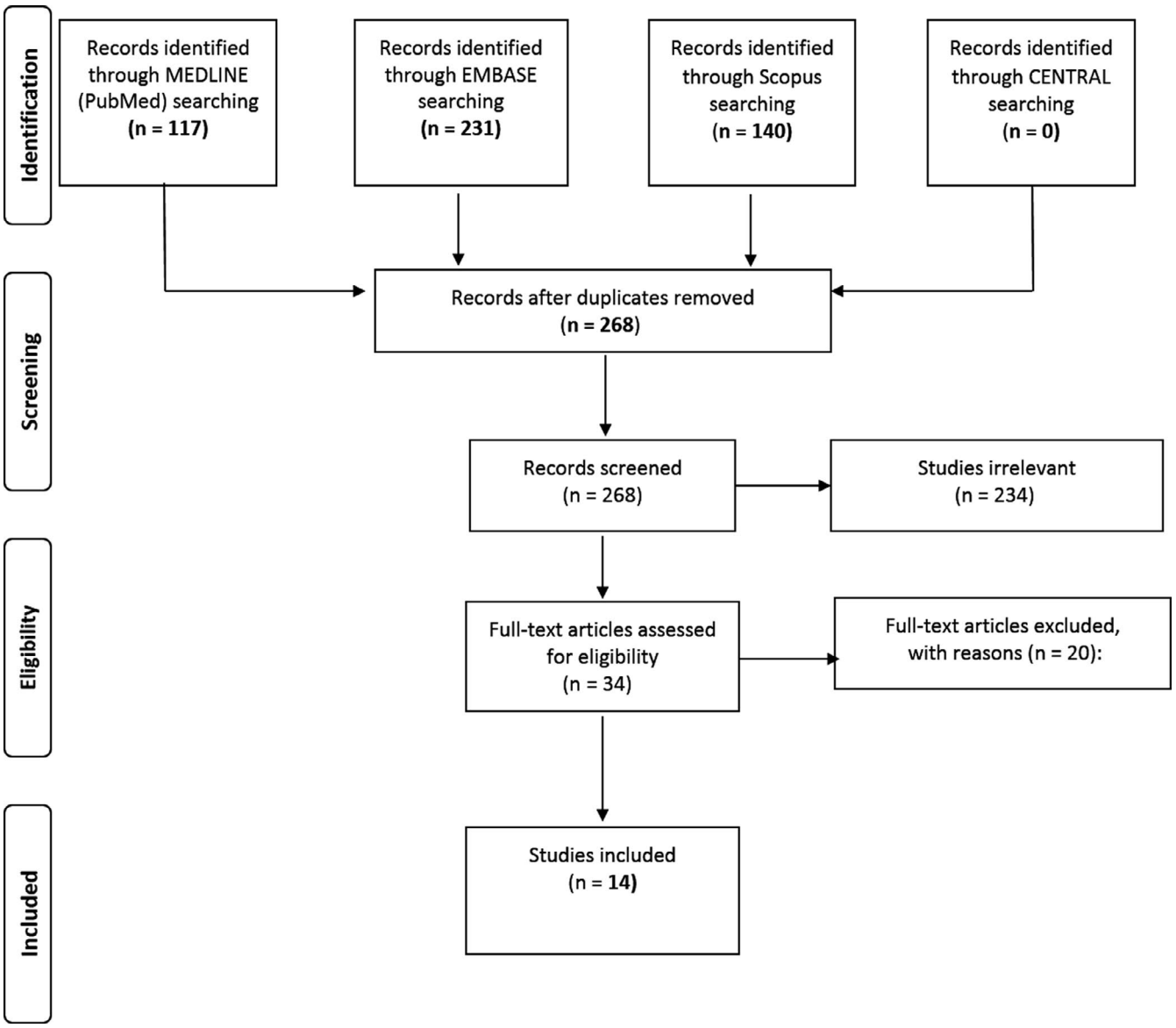

FIGURE 1 Flow diagram search

FIG4, SOD1, UBQLN2, TARDBP and VAPB considered in 10 studies, *CHMP2B, DAO, DCTN1, PFN1* considered in 9 studies. This scenario was observed in previous survey.[49]

Only in two studies[39,48] all genes were sequenced with both NGS and Sanger methods and then the results were compared. In the first study,[48] evaluating 8 genes, neither NGS nor Sanger revealed mutations in patients evaluated. In the second study,[39] NGS technology identified 51 new or rare variants in 18 different genes; instead, Sanger sequencing identified 16 known mutations in 4 genes (*SOD1, TARDBP, FUS, MATR3*) 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 analysed 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,[35] there were no false positive results; in the second one,[44] the false positive rate was 26.4%. Finally, five studies [35,37,39,43,44] evaluated the oligogenic features of the disease and reported that 37 out 1,880 patients (2%) harboured 2 or more potentially pathological mutations.

3.5 | Evaluation of the clinical utility

In 13 studies,[35-47] NGS allowed to identify already known mutations in 21 genes, and new or rare variants in 27 genes (Figure 3). Identified variants were nonsense or missense

TABLE 1 Characteristics of included studies evaluating NGS technologies applied to molecular diagnosis of ALS

Author	No. patients included			Age of onset (mean ± SD or median (range))	Site of onset	Mean disease duration (year) from diagnosis	Type of NGS	Genes analysed by NGS
	Total	No. fALS	No. sALS					
Farhan 2016[35]	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[36]	379	9	Nr	59.5 ± 12.2	Bulbar n = 102 Spinal n = 277	35.8 ± 26.1	Illumina NextSeq500	*OPTN*
Kim 2016[37]	152	4	148	55.7 ± 5.8	Bulbar n = 49 Spinal n = 99		HiSeq 2000	*ALS2, ANG, DAO, FIG4, FUS, GRN, MAPTOPTN, SETX, SIGMAR1, SOD1, SPG11, SQSTM1, TAF15, RARDBP, UBQLN2, VAPB, VCP*
Kim 2017[38]	129	Nr	Nr	55.8 ± 10	Bulbar n = 30 Spinal n = 97 Respiratory n = 1 Axial n = 1		Illumina MiSeq or NextSeq500	*TBK1*
Lamp 2018[39]	296	45	210	61.3 (18-87)	Bulbar n = 62 Spinal n = 205 Spinobulbar n = 1 Respiratory n = 1 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[40]	247	83	164	Nr	Nr	Nr	Illumuna HiSeq2000/2500	*MATR3*

(Continues)

TABLE 1 (Continued)

Author	No. patients included			Age of onset (mean ± SD or median (range))	Site of onset	Mean disease duration (year) from diagnosis	Type of NGS	Genes analysed by NGS
	Total	No. fALS	No. sALS					
Liu 2014[41]	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[42]	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[43]	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[44]	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[45]	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[46]	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

(Continues)

TABLE 1 (Continued)

Author	No. patients included			Age of onset (mean ± SD or median (range))	Site of onset	Mean disease duration (year) from diagnosis	Type of NGS	Genes analysed by NGS
	Total	No. fALS	No. sALS					
Tripolszki 2017[47]	28	Nr	Nr	Nr	Nr	Nr	Roche	FUS, SETX, c9orf72
Turk 2017[48]	43	Nr	Nr	66 (28-78)	Nr	24 months (3-153 months)	IonTorrent	*CAPZA1, CAPZB, CCDC53, FAM21C, KIAA1033, KIAA0196, VCP, WASH1*

mutations leading to a frameshift mutation resulting in a truncated protein and a loss of protein function.

The most frequent variants are T341C, c.280G > A and c.380T > C for SOD1 gene, G1484A for DCTN1 gene, c.562A > G for RNF19A gene, T472G for SEXT gene, G1108A, G2083A, T2656C, A3037G for SPG11 and G510A for VAPB gene. Furthermore, Morgan et al[43] reported a large number of patients with mutations in NEFH gene (Table 2).

3.6 | Distribution of c9orf72 hexanucleotide repeats

Seven studies [37,39,41,43-46] analysed the *c9orf72* GGGGCC repeated expansion using methods other than NGS, since NGS is challenging to detect GC-rich long regions. Two studies [39,43] found 123 (8.9%) patients carrying a pathological expansion of c*9orf72*. In two studies,[37,41] the number of repeats was within the normal range. Three studies[44-46] did not detect hexanucleotide repeat expansion of *c9orf72* among the patients who were analysed.

4 | 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 counselling and patients' management more complicated with further studies needed to verify genes role in ALS pathogenesis.

In the worldwide, the origin of difference in ALS incidence is a matter of debate. Older age, male sex, family history of ALS have all been established as risk factors, but also environmental risk factors (such as exposure to heavy metals, pesticides, head trauma, electromagnetic field, high BMI and nutritional state, BMAA and even physical activity) and genetic factors (more than 20 different genes have been implicated in FALS and SALS, like SOD1, TARDBP, FUS, OPTN, VCP, UBQLN2, c9orf72, TBK1) contributing to the onset of the disease.[50] Since 2014, seven novel genes associated with ALS (MATR3, CHCHD10, TBK1, TUBA4A, NEK1, C21orf2, and CCNF) have been identified by genome-wide association studies, whole genome studies, or exome sequencing technologies. The precise disease mechanisms attributed to these novel genes are unclear, but they code for proteins associated with one or more molecular pathways involved in ALS (dysfunction in global protein homoeostasis resulting from abnormal protein aggregation or a defect in the protein clearance pathway, mitochondrial dysfunction,

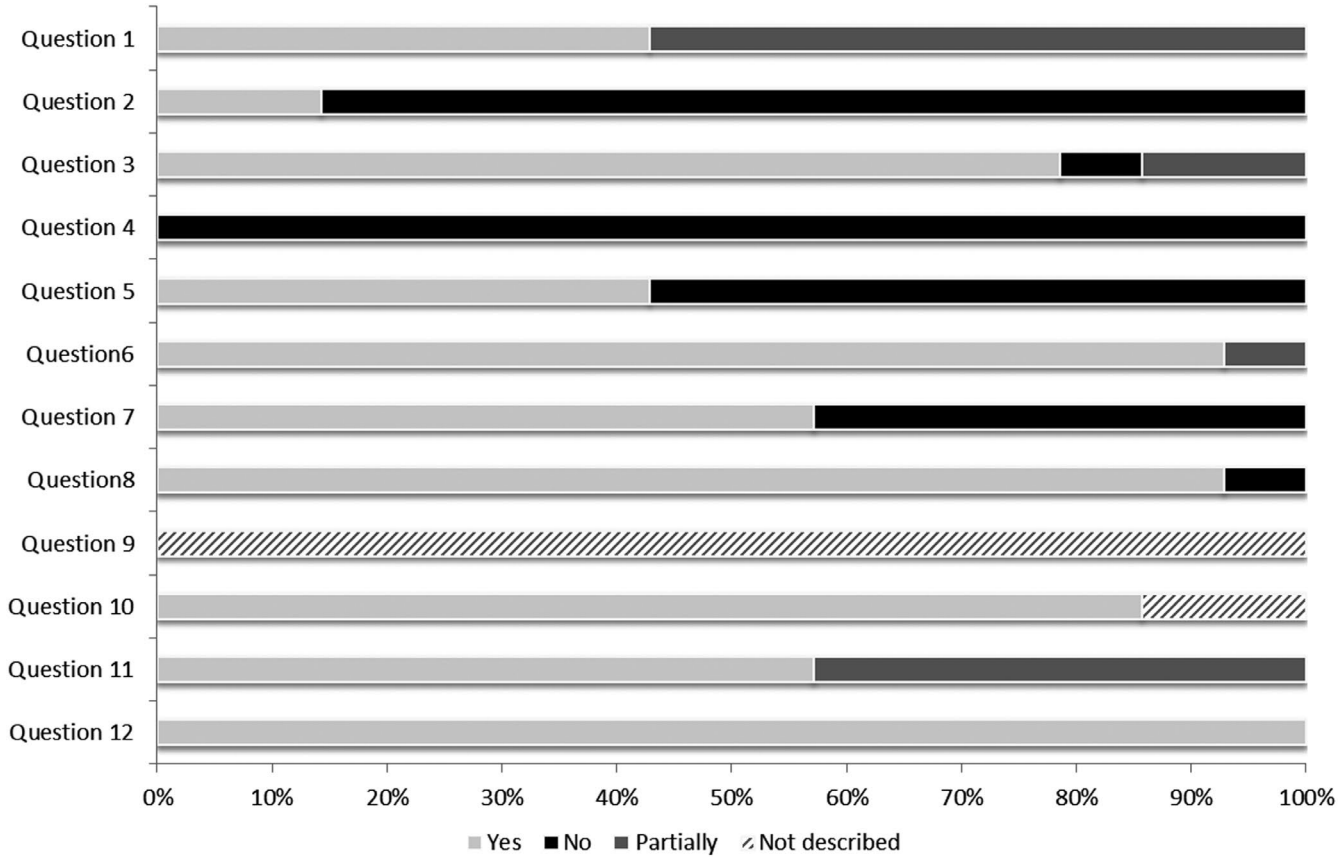

FIGURE 2 Quality assessment. Question 1: Was the research question or objective in this paper clearly stated? Question 2: Was the study population clearly specified and defined? Question 3: Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Question 4: Were the cases consecutive? Question 5: Were inclusion and exclusion criteria for being in the study pre-specified and applied uniformly to all participants? Question 6: Were the outcomes measured clearly defined, valid, reliable, and implemented consistently across all study participants? Question 7: Was the intervention clearly described? Question 8: Was there use of concurrent controls? Question 9: Were the outcome assessors blinded to the exposure status of participants? Question 10: Were the statistical methods well described? Question 11: Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure and outcome(s)? Question 12: Were the results well described?

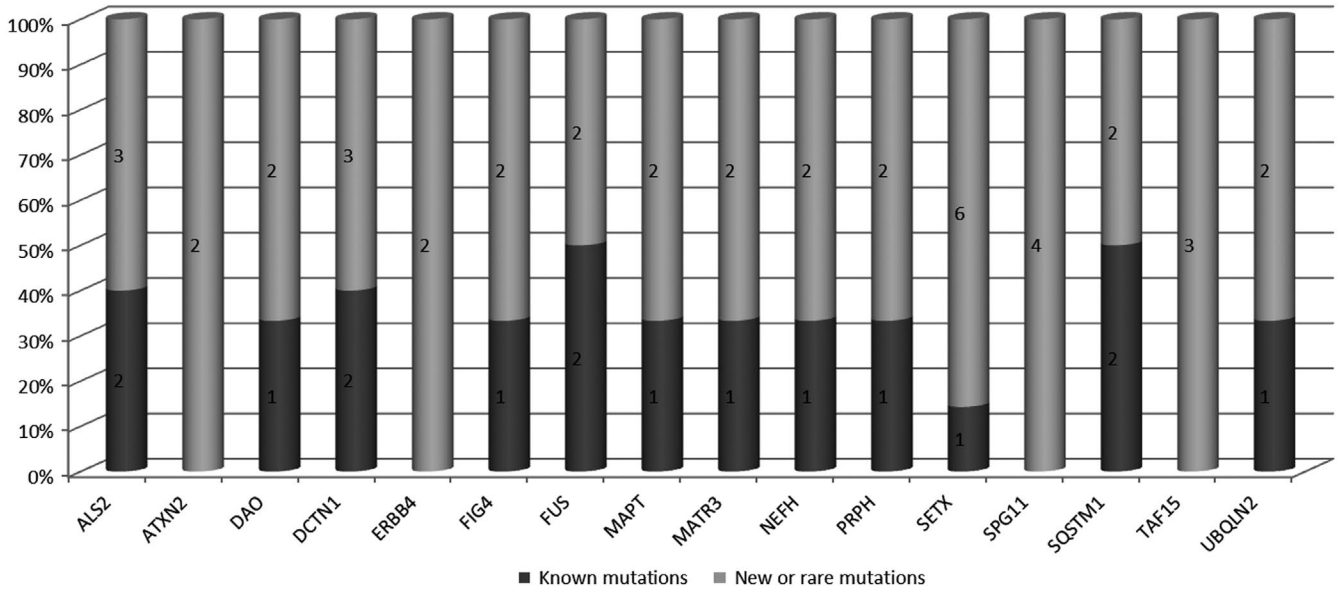

FIGURE 3 Number of known, new or rare ALS mutations identified

TABLE 2 Variants identified in each evaluated genes

Gene	Studies	N patients	Variants
C9orf72	Farhan 2016[35]; Lamp 2018[39]	16	
SOD1	Kim 2016[37]	1	p.N87S
	Lamp 2018[39]	4	G286A; C287G; G435C (2)
	Liu 2014[41]	3	p.F21C; p.G148D; C147R
	Morgan 2017[43]	16	C25G;G229T (3);A272C (3);A305G;C319T;G335A;T341C (5);A403G
	Nakamura 2016[44]	25	c.115C > G; c.140A > G (3);c.280G > A (2);c.319C > G (3);c.380T > C (3);c.425G > C;c.449T > C;c.131A > G;c.280G > A (4);c.380T > C (4); c.404G > A (2)
	Narain 2018[45]	2	G148D; H44R
ALS2	Kim 2016[37],*	1	p.Q435L
	Lamp 2018[39]	5	A35G; A1037G;C1048T;C1115G; A3958T
	Nakamura 2016[4,4,*]	1	c.62A > G
	Nishiyama 2017[46,*]	2	c.575C > T; c.1511C > T
ANG	Lamp 2018[39],*	1	A208G
	Morgan 2017[43]	1	A250G
	Narain 2018[45]	1	K41I
	Nishiyama 2017[46]	1	c.379G > A
ATXN2	Nakamura 2016[44,*]	1	c.3781A > G
	Nishiyama 2017[46,*]	3	c.2174C > T; c.2063A > G (2)
CHMP2B	Morgan 2017[43]	3	A85G (2);G206A;
	Narain 2018[45,*]	1	E45K
DAO	Nakamura 2016[44,*]	1	c.968C > T
	Narain 2018[45,*]	2	R199Q; E121K
DCTN1	Lamp 2018[39,*]	9	G1484A (6);G3146A (2);C3746T
	Liu 2014[41,*]	4	p.G59R
	Morgan 2017[43]	1	C2353T
	Nakamura 2016[44],*	6	c.497C > G; c.461G > A; c.1806C > G; c.1915C > T; c.2529G > C; c.3185G > A
	Nishiyama 2017[46],*	2	c.3782G > A; c.202A > G
ERBB4	Lamp 2018[39],*	1	G3400T
	Narain 2018[45],*	1	R103H
FIG4	Lamp 2018[39],*	3	G244A; 1032dupT;C1078T
	Morgan 2017[43]	3	T122C
	Nakamura 2016[44],*	3	c.320T > C; c.397A > G; c.1147A > C
FUS	Morgan 2017[43]	6	C1292T; G1520A;C1561T;G1562A (2);G1562T
	Nakamura 2016[44],*	7	c.1541G > C;c.1562G > A (2);c.1562G > T;c.1574C > T; c.242A > G;c.1541G > C
MAPT	Kim 2016[37],*	1	p.R5H
	Lamp 2018[39],*	3	T671G;G1013A; A2048G
MATR3	Lamp 2018[39]	1	C2521T
	Leblond 2016[40],*	3	p.V394M; c.48þ1G > T; c.-339þ2T > A
	Marangi 2017[42],*	6	p.Q66K,p.G153C (2),p.E664A,p.S707L, N787S.
NEFH	Morgan 2017[43]	897	G1387A (173);C1844T (414);2368_2370delAAG; A2414C (309)
	Nakamura 2016[44],*	2	c.559G > A; c.855C > A
	Nishiyama 2017[46],*	3	c.559G > A; c.855C > A; c.607G > C

(Continues)

TABLE 2 (Continued)

Gene	Studies	N patients	Variants
OPTN	Lamp 2018[39]	3	G701A; C910T; C1588A
	Morgan 2017[43]	55	A1670G
	Narain 2018[45],*	2	K489E; K489E
	Nishiyama 2017[46]	1	c.1433A > G
PFN1	Morgan 2017[43]	1	A350G
PRPH	Nakamura 2016[44],*	2	c.104C > T; c.773A > T
	Nishiyama 2017[46],*	1	c.100T > C
PSEN1	Lamp 2018[39],*	1	A953G
PSEN2	Lamp 2018[39],*	4	C211T; T236C;C389T; A520G
RNF19A	Nakamura 2016[44],*	6	c.562A > G (4); c.842G > A; c.259G > A
SETX	Lamp 2018[39],*	13	G59A (2);T472G (4); A2975G (2);3072_3074dup;G3229A;T4660G; C7562T; T7640C
	Morgan 2017[43]	12	T7640C
	Nakamura 2016[44],*	4	c.2374T > C (3);c.628A > G
	Narain 2018[45],*	1	L2163V
	Nishiyama 2017[46],*	5	c.4955T > G; c.1880T > C; c.7771G > A; c.4660T > G (2)
	Tripolszki 2017[47],*	1	(c.791A > G,
SIGMAR1	Nishiyama 2017[46],*	1	c.632G > A
SPG11	Lamp 2018[39],*	28	C23G; T359C; A616G;G1108A (5); T1698G (2);G2083A (4);T2656C;A3037G (6);A3265G; C3905T; G4261A; T5075G; C6625T; C7069T;T7132C
	Nakamura 2016[44],*	10	c.855A > C;c.704A > C;c.7258T > A; c.6532T > G; c.5341C > T;c.3990G > C; c.2751G > T; c.1509C > G;c.805G > A (2)
	Nishiyama 2017[46],*	3	(c.4772C > T; c.6043G > A; c.1202A > C
SQSTM1	Kim 2016[37],*	1	p.P439L
	Morgan 2017[43]	11 5	A712G; C1175T
	Narain 2018[45],*	2	G262R; P438L
TAF15	Nakamura 2016[44],*	1	c.577G > A
	Nishiyama 2017[46],*	1	c.161C > T
TARDBP	Kim 2016[37],*	2	G1144A
	Morgan 2017[43]	10	C269T (2); G859A (2); C962T; A1009G; G1043T; T1122G; A1132G (2)
	Nakamura 2016[44]	2	c.892G > A; c.1147A > G
	Narain 2018[45]	2	M337V; N267V
	Nishiyama 2017[46]	2	c.1069G > A; c.1127G > A
TBK1	Kim 2017[38]	4	c.1414delA; c.1150C > T; c.1424T > C;c.1426G > A
TFG	Nakamura 2016[44],*	1	c.1079G > A
UBQLN2	Lamp 2018[39]X*	1	C53T
	Morgan 2017[43]	1	C1490A
VAPB	Lamp 2018[39],*	2	T390G
	Morgan 2017[43]	8	G510A (7); G551A
VCP	Morgan 2017[43]	1	A340G
	Nakamura 2016[44]	1	c.463C > T
ZNF512B	Nakamura 2016*	5	c.2537G > A; c.2308C > T; c.2093C > T; c.1777C > T; c.1520G > A

*Studies identified novel variant; (number of variants identified).

altered RNA metabolism, impaired cytoskeletal integrity, altered axonal transport dynamics and DNA damage accumulation due to defective DNA repair) some of which are in common with other genes implicated in ALS, so therapeutics targeting these pathways could be useful for a broad group of patients stratified by genotype.[51] The evolution of technologies has made the whole genome sequencing more accessible and facilitates the analysis of larger patients cohorts, leading to a better understanding of the molecular defects and helping to identify rare polymorphisms in the non-coding intergenic regions of the genome and structural variants that might contribute to ALS development. Increased knowledge about the genetic profiles that protect or confer disease risk in patients with ALS will change the way clinical trials are done and to identify therapeutic targets. In this context, a recent large-scale genome-wide association study identified a common missense variant and several rare loss-of-function mutations within the microtubule motor protein-encoding gene, KIF5A, as candidate ALS risk factors, further supporting perturbations in cytoskeletal function play an important role in ALS and offering a potential target for drug development.[29] Evidence suggests that other genomic variants, such as copy-number variations (CNVs), that change gene dose rather than gene function, may exert a more pronounced effect on the onset and rate of disease progression. Morello et al[52] analysed CNVs occurring in the same patients, by using a customized exon-centred comparative genomic hybridization array (aCGH) covering a large panel of ALS-related genes. A large number of novel and known disease-associated CNVs were detected in SALS samples, and identified 24 potential candidates for genomic-based patient stratification providing a road map to facilitate genome-guided personalized diagnosis and treatments.[52]

This literature review provides a picture about the most common genes analysed 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 harboured pathogenic variants in more than one ALS-associated gene. 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. Digenic inheritance was first described twenty-five years ago in the setting of retinitis pigmentosa.[53] Though it has been speculated that this inheritance pattern is present in ALS, it is noteworthy that many of the descriptions of the "second" mutation are not definitively pathogenic. 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 analysed separately by improved PCR-based methods.[54,55]

The hexanucleotide repeat expansion (GGGGCC) in the first intron of the long gene c9orf72 is involved in the pathogenic mechanism of FALS, to a lesser extent of SALS, and frontotemporal dementia. Wild-type alleles contain no more than 20-30 repeats, whereas affected patients could have more than hundred or thousand repeats. The c9orf72 repeat expansion accounts for a significant percentage of familial and sporadic ALS in Caucasian populations but is rare in Asian cohorts. The origin of this mutation dates back 1500 years to the Vikings contributing to its spread through their European invasion.[56,57] At present, we known that the most common genetic mutation in Caucasian population is c9orf72, accounting more than 40% of FALS and 5%-20% of SALS, with particular high prevalence in Finland.[57] In Asian population, this mutation occurs less frequently. The prevalence of c9orf72 in ALS cases is much lower in East (less than 4% in Japan) and South Asia (5.9% among FALS and 1.6% among SALS in Iran). A meta-analysis showed that in Asian populations, the most common mutations in ALS were the SOD1 mutations (FALS 30.0%, SALS 1.5%), followed by FUS (FALS 6.4%, SALS 0.9%), C9orf72 repeat expansions (FALS 2.3%, SALS 0.3%) and TARDBP mutations (FALS 1.5%, SALS 0.2%), while in European populations, the most common mutations in ALS were the C9orf72 repeat expansions (FALS 33.7%, SALS 5.1%), followed by SOD1 (FALS 14.8%, SALS 1.2%), TARDBP (FALS 4.2%, SALS 0.8%) and FUS mutations (FALS 2.8%, SALS 0.3%).[4]

Taken together, the potential to detect 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 genetic counsellor, 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 counselling of patients with genetic risk variants and their family members.[7]

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 counselling should be offered routinely to all ALS patients.[58] 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.

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.[59,60] 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.[61-63]

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 (eg *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-informatics knowledge in order to analyse 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: (a) the preparation of the template according to different operating protocols, (b) the commercial availability of sequencing platforms, (c) 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; (d) 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, favouring 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.

Further, the rapid diffusion of NGS into clinical setting is due to the decrease of sequencing costs. Actually, the entire genome of patients can be sequenced in less than one week at a cost between $5000 and $10 000.[64] The cost is reducing rapidly, in the near future, commercial producers will perform whole genome sequencing for less than $1000.[65] Several studies evaluated the economic impact of NGS only considered the equipment and consumable costs for library preparation and sequencing, underestimating the real costs. Other aspects to consider are the cost of personnel needed for samples preparation, time for storage and interpretation of a large amount of data, bioinformatic analysis, costs for training of health care professionals and counselling. Both whole genome and whole exome sequencing are usefulness for the diagnosis of disease, whole exome sequencing is less costly than whole genome and represent an appropriate subset of the genome in which to search for disease-causing variants, making results more efficient and improving the patients managements. Increasing sequencing speed and decreasing cost, make NGS more affordable, improve the diagnostic pathway contributing to personalized medicine in a major way.

5 | CONCLUSIONS

NGS 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.

CONFLICT OF INTEREST

VP, CC; TT; JM and AC declare that they do not have any conflict of interest. BT has a patent European Union patent on the clinical testing and therapeutic intervention for the hexanucleotide repeat expansion of C9orf72 issued, and a patent United States patent on the clinical testing and

therapeutic intervention for the hexanucleotide repeat expansion of C9orf72 Issued. BT has received research grants from: The Myasthenia Gravis Foundation, The Robert Packard Center for ALS Research, The ALS Association, The Italian Football Federation (FIGC), The Center for Disease Control and Prevention, The Muscular Dystrophy Association, Merck Inc, Microsoft Research. BT is part of the Intramural Research Program (IRP) at the National Institutes of Health and receives funding through the IRP.

AUTHOR CONTRIBUTIONS

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission. VP conceived and designed the study. VP and TT wrote the protocol. VP designed and implemented the search strategies. VP and CC selected studies, assessed validity, and extracted data. VP entered and analysed the data. All authors interpreted the data, prepared the full review and contributed to its revision, interpretation of results and approval.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Pecoraro V, Mandrioli J, Carone C, Chiò A, Traynor BJ, Trenti T. The NGS technology for the identification of genes associated with the ALS. A systematic review. *Eur J Clin Invest*. 2020;50:e13228. https://doi.org/10.1111/eci.13228