Value of systematic genetic screening of patients with amyotrophic lateral sclerosis

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ABSTRACT
Objective The clinical utility of routine genetic sequencing in amyotrophic lateral sclerosis (ALS) is uncertain. Our aim was to determine whether routine targeted sequencing of 44 ALS-relevant genes would have a significant impact on disease subclassification and clinical care.

Methods We performed targeted sequencing of a 44-gene panel in a prospective case series of 100 patients with ALS recruited consecutively from the Sheffield Motor Neuron Disorders Clinic, UK. All participants were diagnosed with ALS by a specialist Consultant Neurologist. 7100 patients had familial ALS, but the majority were apparently sporadic cases.

Results 21% of patients with ALS carried a confirmed pathogenic or likely pathogenic mutation, of whom 93% had no family history of ALS. 15% met the inclusion criteria for a current ALS genetic-therapy trial. 521 patients with a pathogenic mutation had an additional variant of uncertain significance (VUS). An additional 21% of patients with ALS carried a VUS in an ALS-associated gene. Overall, 13% of patients carried more than one genetic variant (pathogenic or VUS). Patients with ALS carrying two variants developed disease at a significantly earlier age compared with patients with a single variant (median age of onset=56 vs 60 years, p=0.0074).

Conclusions Routine screening for ALS-associated pathogenic mutations in a specialised ALS referral clinic will impact clinical care in 21% of cases. An additional 21% of patients have variants in the ALS gene panel currently of unconfirmed significance after removing non-specific or predicted benign variants. Overall, variants within known ALS-linked genes are of potential clinical importance in 42% of patients.

INTRODUCTION
Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterised by progressive injury and cell death of upper and lower motor neurons in the motor cortex, brainstem and spinal cord. This leads to progressive failure of the neuromuscular system with death, usually from respiratory failure, within 2–5 years of symptom onset in most cases. Up to 50% of cases also show mild cognitive impairment, with approximately 5% progressing to clinically recognised fronto-temporal dementia (FTD). While the majority of ALS cases are considered sporadic (sALS), 5%–10% have been shown to be familial, usually with autosomal dominant inheritance, and the genetic cause of approximately 60%–70% of familial ALS (fALS) cases has now been identified. The most common genetic cause of ALS is due to expansion of a GGGGCC (G4C2) hexanucleotide repeat in the first intron of the C9orf72 gene. This expansion has a frequency of 43% in fALS and 7% in sALS cases in our UK cohort, which is comparable with worldwide figures of 39.3% for fALS and 7.0% for sALS. Mutations in SOD1, TARDBP and FUS, the next most common genetic causes of ALS, have also been reported in both patients with fALS and those with sALS. Therefore, it is clear that apparently sporadic cases can also carry potentially pathogenic variants in known ALS genes. In a recent study which screened 17 ALS-related genes, 27.8% of apparently sporadic cases carried a potentially pathogenic or rare variant in a known ALS gene. In addition, it was noted that 3.8% of patients also carried multiple variants, with these cases having a significantly earlier age of onset. Another recent report from an Australian sporadic ALS cohort found that one-third of patients carried a variant of interest and 7% carried two or more variants, which again was correlated with an earlier age of onset. It has previously been reported that ALS is a six-step process, with genes, environment and time (in the form of ageing) contributing to disease development. It was proposed that individuals with a genetic variant would require fewer steps than those without such variants. Using data from an ALS registry in Italy, this proved to be the case, with individuals carrying C9orf72, TARDBP or SOD1 mutations showing a three-step, four-step and two-step process. Currently, only cases with a familial history of ALS, dementia or with a young age of disease onset tend to be routinely offered genetic screening in a clinical setting, at least in the UK. However, with the advent of therapies targeting specific genetic forms of the disease associated with SOD1 or C9orf72 mutations (Biogen sponsored clinical trials...
patients were not prioritised for genetic screening by clinicians. The diagnosis of ALS was made by an experienced neuromuscular neurologist (PJS, CJM, TMJ, CH) following appropriate investigations to exclude alternative diagnoses, and detailed clinical and demographic features were recorded for all patients.

**Illumina targeted panel sequencing**

A panel of 44 ALS, motor system and FTD-linked genes (figure 1A) was screened to diagnostic standards using targeted next-generation sequencing by the UKAS-accredited Sheffield Diagnostic Genetics Service laboratory, as part of the AMBRoSIA project. The panel was approved for familial ALS with and without FTD by the UK Genetic Testing Network steering group. DNA for the panel was captured using SureSelectXT (Design ID: 0836801) automated library preparation and libraries were sequenced on an Illumina HiSeq 2500 in rapid run mode at $2 \times 10^7$ bP. A mean coverage of at least 100x was obtained (online supplemental figure S1).

**C9ORF72 expansion testing**

Hexanucleotide repeat expansions (G4C2) in C9ORF72 were tested by flanking PCR and, if required, fluorescent repeat primed PCR (RP-PCR) (online supplemental table S1). Fragment size analysis was performed in GeneMapper (V.3.5). Expansions were reported as normal if $<30$ repeats were detected, and all expansions of $>30$ repeats plus the classical sawtooth pattern were reported as potentially pathogenic.

**ATXN2 expansion testing**

ATXN2 repeat expansions (CAG) were tested by standard PCR with one fluorescently labelled primer (online supplemental table S2), followed by analysis using GeneMapper (V.3.5). ATXN2 repeats were reported as normal between 14 and 28; $35$ repeats and above were reported as consistent with spinocerebellar ataxia type 2 (SCA2) and repeat lengths of 29–34, which have been associated with an increased risk of ALS, were reported as intermediate repeat lengths.

**Sequencing and variant analysis**

Sequencing data were analysed using a clinically validated bioinformatics pipeline. Samples were checked for contamination (online supplemental figure S2). Reads were aligned with bwa19 (V.0.7.15) to a bespoke version of the human reference (hg19). Indels were realigned with GATK20 (V.3.7). Variants were called with GATK Haplotype Caller (V.3.7) and decomposed and normalised with vt21 (V.0.5) and uploaded to Fabric Genomics Opal (V.6.1.8). Variants with a quality score $<1500$ were excluded from further analysis. Protein coding variants, only, were retained.

The pathogenicity of a variant was determined using a multifaceted approach, including manual review in Opal, population frequency and in silico software algorithms, as well as the presence or absence of variants in our clinical reporting pipeline and the ALS literature.

Fabric Genomics Opal (V.6.1.8) provides population frequency from the 100,000 Genomes Project, Exome Variant Server of NHLBI GO Exome Sequencing Project, ExAC and gnomAD. We chose to report on overall population frequency using gnomAD, the largest dataset available.22

In silico analysis used the Omicia Score, which combines software algorithms MutationTaster,23 Polymorphism Phenotyping v2 (PolyPhen2),24 Sorting Intolerant from Tolerant (SIFT)25 and phyloto26—placental, primate and vertebrate. Additional
Neurodegeneration

The ClinVar database (2 January 2019) was also used to determine if the variant had been previously assessed. Variants previously described as pathogenic or likely pathogenic in Opal, the genetics service clinical pipeline, or the literature (based ClinVar) were labelled as such. Variants with a lesser likelihood of pathogenicity were labelled as benign and excluded from reporting if

The algorithms used were VAASS Variant Prioritization (VVP) and Combined Annotation Dependent Depletion (CADD). Classification of variant effects was based on transcripts detailed in online supplemental table S3. Potential splicing disruption was predicted using Neural Network Splice (NNSplice), GeneSplicer²⁹ and MaxEntScan.³⁰

Figure 1  Clinical screening of all patients with ALS identifies pathogenic, likely pathogenic and variants of uncertain significance (VUS). (A) The 44 genes covered by the clinical ALS panel (42 profiled by next-generation sequencing and 2 by PCR). Those genes in which pathogenic (red), likely pathogenic variants (orange) or VUS (blue) were identified after filtering. No pathogenic variants or VUS were found in the genes depicted in the white boxes. Background colour of the box represents the most severe variant found in that gene. (B) Schematic of our variant analysis and filtering process. (C) Counts of reportable (red) and variants of unknown significance in ALS genes in the Sheffield AMBRoSIA cohort. (D) Five clinically reportable variants in SOD1. (E) 4 Variants of unknown significance were discovered in SPG11. Interestingly, 3 of these cluster in the C-terminal domain. Benign and likely benign mutations are conversely distributed throughout the protein. Protein domain figures created using ProteinPaint (https://pecan.stjude.cloud/proteinpaint).
they had one of the following properties: (1) intronic or synonymous variants with no predicted effect on splice sites; (2) an allele frequency >0.001 according to gnomAD;22; (3) previously reported as benign in ClinVar; (4) a CADD score <15.27 Variants of uncertain significance (VUS) were determined as those that did not fit the aforementioned criteria for pathogenic/likely pathogenic or benign. CADD score is not available for indels and therefore indels were denoted as VUS if minor allele frequency (MAF) <0.001 according to gnomAD;22 0.001 and non-Strand (FS) >200, ReadPosRankSum <−20 (n=43) and if the following QC criteria: Quality Depth (QD) <2, Fisher ProjectMine sequencing consortium), leaving 11 indels (online supplemental table S4 for details of the indels). Three of these patients had reported a family history of ALS. Identification of C9ORF72 was in 10 patients with ALS (10% of the cohort). Three of the patients with a clinically reportable pathogenic mutation had apparently sporadic disease.

As expected, the most frequently identified pathogenic mutation was in C9ORF72 in 10 patients with ALS (10% of the cohort). Three of these patients had reported a family history of ALS. Identification of C9ORF72 and SOD1 mutations is particularly important because these genes are associated with ongoing genetic-therapy trials (Biogen BIIB067 and BIIB078).

We identified two patients with sporadic ALS with clinically reportable changes in FIG4: p.Ile41Thr and p.Thr689AsnfsTer12 (table 2). The p.Ile41Thr mutation is reported as pathogenic in ClinVar, with no conflicts. The p.Thr689AsnfsTer12 variant has been reported twice before and only in sporadic ALS, suggesting variable penetrance. A frameshift mutation is likely to lead to haploinsufficiency (online supplemental figure S5). FIG4 variants are thought to disrupt local folding of the protein leading to a reduction in stability which inhibits the normal function of the protein in lysosomal trafficking.33

Four additional mutations in three patients were identified which were classified as clinically reportable (table 2). A single patient with sALS carried a p.Pro777Leu NEFH mutation and a p.Thr293Ile ALS2 mutation. The p.Thr293Ile change was heterozygous, whereas SOD2 mutations are usually considered to be autosomal recessive.34 As a result, the clinical significance of this finding is currently uncertain. However, it is possible that the two identified changes act synergistically. A single patient harboured a p.Ala2338Val SPG11 mutation and a further single patient carried an inframe deletion within TBK1 p.Glu643del. SPG11 mutations are usually considered to be recessive and therefore the clinical significance of this finding is at present uncertain. The TBK1 change is reported in ClinVar as pathogenic. We identified one patient with an expansion of 29 CAG repeats in the ATXN2 gene which has been reported as a risk factor for ALS.35 The majority of cases in this cohort had 22 or 23 repeats (range 15–29).

Panel screening leads to identification of VUS

Thirty-four variants in 26 patients with ALS (3 familial and 23 sporadic (26%) were reported in ClinVar as of uncertain significance, or absent from ClinVar and met our criteria for predicted

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ACMG, American College of Medical Genetics and Genomics; ALS, amyotrophic lateral sclerosis; NA, not available.

RESULTS

Prospective genetic testing leads to identification of clinically reportable pathogenic mutations

We profiled a panel of 44 relevant genes (figure 1A) in 100 prospectively identified patients with ALS using Best Practice NHS pipelines. In 21 patients (21%), we identified 22 clinically reportable pathogenic and likely pathogenic variants (table 2 and figure 1B,C). Seven of the 21 patients reported a family history of ALS in a first-degree relative, but 14 patients with a clinically reportable pathogenic mutation were classified as clinically reportable (table 2). The p.Ile41Thr mutation is reported as pathogenic in ClinVar, with no conflicts. The p.Thr689AsnfsTer12 variant has been reported twice before and only in sporadic ALS, suggesting variable penetrance. A frameshift mutation is likely to lead to haploinsufficiency (online supplemental figure S5). FIG4 variants are thought to disrupt local folding of the protein leading to a reduction in stability which inhibits the normal function of the protein in lysosomal trafficking.33

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Panel screening leads to identification of VUS

Thirty-four variants in 26 patients with ALS (3 familial and 23 sporadic (26%) were reported in ClinVar as of uncertain significance, or absent from ClinVar and met our criteria for predicted
pathogenic variants (table 3). Also, 5/26 patients with a VUS had an additional pathogenic variant.

Without pedigree information or population-matched controls, it is difficult to conclusively demonstrate pathogenicity. ALS has a polygenic rare variant architecture and therefore we filtered for rare predicted pathogenic variants which we quantified as MAF <0.001 and CADD >1527 (online supplemental figure S6). In addition, we used population-matched whole genome sequencing data from 5954 patients with ALS and 2238 controls included in Project MinE35 to check for evidence of pathogenicity based on case:control ratio (Fisher exact test p≤0.05). VUS were excluded from further analysis if they were not significantly associated with ALS in population-matched data, unless low frequency within the whole genome sequencing dataset (<10 individuals) prevented conclusive testing.

For each identified VUS, we performed a literature search to explore the case that this was indeed a newly identified ALS-risk variant. In particular, we sought evidence that a discovered VUS was present within the same functional domain as described pathogenic variants: for TBK1, NEK1 and FIG4 this included nonsense and frameshift changes because pathogenic changes in these three genes are thought to act via loss of function.33 36 Based on these criteria, we conclude that the following VUS are likely to be pathogenic: p.Met405Val and p.Gln327Glu are present in >10% of this cohort and present in Project MinE controls and those present in >10% of this cohort and present in Project MinE controls.

Indels have high rates of inaccurate sequencing. We removed all non-coding indels, those with low quality and those present in >10% of this cohort and present in Project MinE controls leaving two frameshift variants, seven inframe deletions and two splice region variants. FIG4 has a frameshift in codon 689 which has a frameshift close to the end of the protein at codon 1969 which has a very low population frequency (0.0000636—online supplemental figure S5) and SPG20 has not previously been reported in gnomAD (V.2.1.1—online supplemental figure S5). Also, 5/26 patients with a VUS had an additional pathogenic variant.
Panel genes where no changes were found
For 20 (highlighted in figure 1A) of the 44 genes screened in our panel, no mutations (pathogenic or VUS) were identified in any member of our cohort. Many of these genetic changes are individually rare and our data are consistent with this. The absence of identified VUS in patients with sporadic ALS would be consistent with a model in which these genes harbour only high-effect, highly penetrant mutations.

Clinical correlation supports pathogenicity of VUS
In the absence of experimental validation, the biological effect of genetic variation can be inferred from correlation with clinical phenotype. The number of clinically reportable variants and VUS were combined to assign a ‘mutation-load’ to 42 patients with ALS (figure 2A). Patients with ALS without discovered variants were excluded because of absent information. To determine whether mutation load was clinically relevant, we compared age of onset between patients with one or two variants. Patients with ALS carrying two variants (n=13) compared with one variant developed disease at a significantly earlier age (log-rank test, \( p=0.0074 \), median age of onset=56 years vs 60 years, figure 2B,C). It is not yet possible to assess any potential effect on disease duration because 67% of this cohort are still living. Correlation with clinical phenotype is therefore consistent with a functional effect of both clinically reportable genetic variants and VUS.

DISCUSSION
An important strength of our study is its prospective nature. We performed targeted sequencing of ALS-relevant genes in 100 prospectively identified patients with ALS attending a large ALS centre in Northern England. This identified clinically reportable genetic changes in 21% of patients of whom 15 with \( \text{C9ORF72} \) or \( \text{SOD1} \) mutations would potentially be eligible for recruitment into an ongoing genetic-therapy trial. The number of clinically actionable results is likely to increase with the anticipated development of new genetic-therapy approaches for ALS. Previous genetic studies of ALS have been largely retrospective and were therefore unable to determine the utility of genetic screening in the clinic. In contrast, the present study strongly suggests that routine genetic testing should be offered in both patients with familial ALS and those with sporadic ALS, at least in our population.
We identified 22 clinically reportable mutations and 34 VUS with potential for pathogenicity in 42 patients, including 35 patients with apparently sporadic ALS. Moreover, we identified a strong correlation between mutation load and age of onset, suggesting that the majority of the VUS may be functionally important. Evaluation of the pathogenicity of individual VUS requires further study, but comparison with previously described variants in the literature suggests that we have identified novel likely pathogenic changes in TARDBP (two variants), EWSR1, SPG11 (two changes) and NEK1 (two variants). Genetic changes were absent from 20 genes in the panel in this cohort (figure 1A).

Mutations in RNA binding proteins are significantly associated with ALS.37 Most prominently, TARDBP encodes the protein TDP-43 which is the major component of the characteristic proteinopathy in ALS. Pathogenic mutations are clustered in the C-terminal glycine-rich domain which is important for interactions with other RNA-binding proteins.18 We have identified two patients with rare and predicted deleterious mutations in TARDBP which are both located in the glycine-rich domain (table 3 and online supplemental figure S8). Both changes (p.Met322Val and p.Met405Val) are absent from 125 748 population controls in gnomAD and are predicted deleterious (CADD >15), although neither has been previously reported. FET proteins including EWSR1, FUS and TAF15 are RNA-binding proteins which have been associated with ALS. We identified several VUS within FET proteins with some evidence for pathogenicity (table 3). One patient carried a p.Met459Thr EWSR1 mutation within the RNA binding region which contains other ALS-associated mutations.

We identified four variants within SPG11 (figure 1E): one clinically reportable change plus two of the VUS. All four variants cluster in the spatacsin C-terminal domain, suggesting that this could represent a region of functional importance. Conversely, benign mutations in this gene tend to be more dispersed and none are found in the spatacsin C-terminal domain. We conclude that the two p.Ile2246Thr and p.Arg2318Cys VUS we identified are highly likely to be pathogenic.

Mutations in TBK1,33 NEK1,39 and FIG4 have conclusively been shown to be loss of function (LOF) changes. In our cohort, we identified two VUS within NEK1 which significantly disrupt the translated sequence and are therefore predicted to be highly pathogenic via a LOF mechanism (online supplemental figure S8). This included p.Gln1034Ter and c.1665+2T>C which is a splice site mutation. Both mutations are rare or absent from gnomAD and are predicted pathogenic (table 3). Similarly, we identified a clinically reportable frameshift mutation in FIG4 (p.Thr689AsnfsTer12-FIG4) and an in-frame deletion in TBK1 (p.Glu643del-TBK1) which has previously been reported in a single case in ClinVar (online supplemental figure S7). Our data add to the weight of evidence that this change is pathogenic and that mutations in TBK1 are an important contributor to the genetics of sporadic ALS.39

VUS were identified based on universal measures of pathogenicity and population frequency.22,27 However, we added an additional filtering step based on case:control ratio within a population-matched whole genome sequencing dataset.35 When combined with clinically reportable changes, the resulting VUS were associated with age of onset which suggests that these are likely to represent pathogenic changes. We confirmed previous reports that C9ORF72 expansions are frequently associated with additional likely pathogenic mutations in other ALS genes.40 In the present cohort, 40% of patients with pathogenic C9ORF72 expansions carried an additional VUS. The presence of such an additional genetic variant may be one factor influencing the penetrance of C9ORF72 mutations.

The current model of ALS is considered a multistep process in which steps constitute genetic and/or environmental exposures.12 A consistent finding in ALS genetics is that identification of a highly penetrant genetic risk factor correlates with earlier age of onset. It follows that such mutations might be associated with fewer steps and this has been supported.5 Our work suggests that, when correctly prioritised, VUS can also contribute steps leading to earlier age of onset and potentially function as a prognostic biomarker. Similar findings have been reported previously but not in a prospective cohort; moreover, the latter study was limited to a smaller number of genes. If an index case carries one or more VUS within ALS-linked genes, then screening for VUS within family members may inform risk and even age of onset counselling. In the present cohort, an additional VUS was associated with ~4 years earlier age of onset.

In conclusion, we have performed a prospective genetic study of 100 consecutive patients with ALS attending our clinic. Our results indicate that screening of known ALS genes can lead to clinically actionable results in ~21% of patients, and in a further ~21% of patients a VUS may be discovered with potential clinical implications. As future studies expand the number of verified genetic causes of ALS, these percentages are likely to increase. We developed a pipeline for prioritising VUS using whole genome case–control cohorts to predict clinical outcomes. Although this study took place in a large tertiary referral ALS centre and requires further validation in other settings, our data suggest that all patients with ALS should, with careful counseling, be offered genetic testing, especially in light of new personalised medicine treatments in development.

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REFERENCES


Neurodegeneration


