

# Evidence for an oligogenic basis of amyotrophic lateral sclerosis

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**Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder with a substantial heritable component. In pedigrees affected by its familial form, incomplete penetrance is often observed. We hypothesized that this could be caused by a complex inheritance of risk variants in multiple genes. Therefore, we screened 111 familial ALS (FALS) patients from 97 families, and large cohorts of sporadic ALS (SALS) patients and control subjects for mutations in TAR DNA-binding protein (*TARDBP*), fused in sarcoma/translated in liposarcoma (*FUS/TLS*), superoxide dismutase-1 (*SOD1*), angiogenin (*ANG*) and chromosome 9 open reading frame 72 (*C9orf72*). Mutations were identified in 48% of FALS families, 8% of SALS patients and 0.5% of control subjects. In five of the FALS families, we identified multiple mutations in ALS-associated genes. We detected *FUS/TLS* and *TARDBP* mutations in combination with *ANG* mutations, and *C9orf72* repeat expansions with *TARDBP*, *SOD1* and *FUS/TLS* mutations. Statistical analysis demonstrated that the presence of multiple mutations in FALS is in excess of what is to be expected by chance ( $P = 1.57 \times 10^{-7}$ ). The most compelling evidence for an oligogenic basis was found in individuals with a p.N352S mutation in *TARDBP*, detected in five FALS families and three apparently SALS patients. Genealogical and haplotype analyses revealed that these individuals shared a common ancestor. We obtained DNA of 14 patients with this *TARDBP* mutation, 50% of whom had an additional mutation (*ANG*, *C9orf72* or homozygous *TARDBP*). Hereby, we provide evidence for an oligogenic aetiology of ALS. This may have important implications for the interpretation of whole exome/genome experiments designed to identify new ALS-associated genes and for genetic counselling, especially of unaffected family members.**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a complex disorder affecting upper and lower motor neurons, resulting in severe muscle weakness. The incidence of ALS is ~2–3 per 100

000 person-years, and most patients die within 5 years after onset of symptoms. In 5–10% of the cases, more than one family member is affected [familial ALS (FALS)], and several genes are known to be involved in its pathogenesis,

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**Table 1.** Mutations found in *TARDBP*, *FUS/TLS*, *SOD1*, *ANG* and *C9orf72*

Gene	Mutation	Exon	FALS Families	SALS	CON	Prediction PMut	Novel
<i>TARDBP</i>	p.A90V	3	1/97	1/1192	1/1415	Pathological	
	p.G295C	6	1/97	0/1192	0/1415	Pathological	Novel
	p.N352S	6	5/97	3/1192	0/1415	Pathological	
	p.I383V	6	1/97	1/1192	0/1415	Neutral	
	Total <i>TARDBP</i> (%)		8/97 (8.2)	5/1192 (0.4)	1/1415 (0.1)		
<i>FUS/TLS</i>	p.S115N	5	0/97	1/1192	0/970	Neutral	Novel
	p.Q210H	6	1/97	0/1192	1/970	Neutral	
	p.R487C	14	0/97	1/1192	0/970	Pathological	Novel
	p.R495X	14	0/97	1/1192	0/970	N/A	
	p.R521H	15	1/97	0/1192	0/970	Pathological	
	p.R521C	15	4/97	0/1192	0/970	Pathological	
	Total <i>FUS/TLS</i> (%)		6/97 (6.2)	3/1192 (0.3)	1/970 (0.1)		
<i>SOD1</i>	p.D90A	4	1/97	1/451	3/1894	Pathological	
	p.I99V	4	0/97	1/451	0/1894	Neutral	
	Total <i>SOD1</i> (%)		1/97 (1.0)	2/451 (0.4)	3/1894 (0.2)		
<i>ANG</i>	p.G(-10)D	2	0/97	1/941	0/1582	N/A	
	p.K17I	2	2/97	3/941	2/1582	Pathological	
	p.T80S	2	0/97	1/941	0/1582	Neutral	
	p.F100I	2	0/97	1/941	0/1582	Neutral	
	Total <i>ANG</i> (%)		2/97 (2.1)	6/941 (0.6)	2/1582 (0.1)		
<i>C9orf72</i>	Long repeat (%)	N/A	35/97 (36)	87/1422 (6.1)	0/748 (0.0)	N/A	
	Total (%)		48	8	0.5		

FALS, familial amyotrophic lateral sclerosis; SALS, sporadic amyotrophic lateral sclerosis; CON, control subject; N/A, not applicable. Mutations in *TARDBP*, *FUS/TLS*, *SOD1*, *ANG* and *C9orf72* were present in 48% of the FALS families (51% of the 111 FALS patients), 8% of the SALS patients and 0.5% of the control subjects.

including TAR DNA-binding protein (*TARDBP*), fused in sarcoma/translated in liposarcoma (*FUS/TLS*), superoxide dismutase-1 (*SOD1*) and angiogenin (*ANG*) (1,2). Recently, a pathogenic hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9orf72*) has also been identified (3,4). This repeat expansion was present in up to 62% of the cases with both FALS and frontotemporal dementia (FTD), 46% of the cases with FALS and 21% of the cases with sporadic ALS (SALS) (3,4). Therefore, it is currently the most important genetic risk factor for ALS.

FALS can be inherited in an autosomal dominant, autosomal recessive or X-linked manner and families with low disease penetrance have frequently been described (5). Both genetic and environmental factors are known to contribute to SALS (genetic component 0.61, range 0.38–0.78) (6). It must be noted, however, that FALS might be underrepresented in ALS patients, for instance, because of inadequate recording of family history, early death due to other causes, misdiagnosis, small family sizes and illegitimacy (5).

In many of our FALS families, we observed apparent autosomal dominant inheritance patterns with evidence for incomplete penetrance. We hypothesized that this phenomenon might be due to the oligogenic inheritance of ALS. This hypothesis is supported by reports of patients with mutations in more than one ALS-associated gene (7–13). In search of evidence for oligogenic inheritance, we, therefore, sequenced *TARDBP*, *FUS/TLS*, *SOD1*, *ANG* and *C9orf72* in a cohort of 97 FALS families, as well as in large cohorts of SALS patients and control subjects.

## RESULTS

*TARDBP*, *FUS/TLS*, *SOD1*, *ANG* and *C9orf72* mutations were found in 48% of the FALS families, 8% of the SALS patients and

0.5% of the control subjects. An overview of the identified mutations is provided in Table 1 and in the Supplementary Material (Results) and has also been deposited to the ALSod database (<http://alsod.iop.kcl.ac.uk>, last accessed date on 1 June, 2012) (14,15). *C9orf72* repeat expansions were most frequently encountered, followed by mutations in *TARDBP*, *FUS/TLS*, *ANG* and *SOD1*. In 5 of 97 families, we identified mutations in more than one ALS-associated gene (Table 2). Subsequently, we performed a binomial test, which demonstrated that the frequency of families with multiple mutations is higher than expected on the basis of chance ( $P = 1.57 \times 10^{-7}$ ). An overview of all pedigrees with multiple mutations is provided in Figures 1, 2A and 3.

A p.N352S mutation in *TARDBP* was identified in three apparently SALS patients and five FALS families; their pedigrees are shown in Figures 1 and 2. Genealogical analysis showed that one SALS patient with this mutation (VIII:1) was related to two FALS families and formed a complex consanguineous pedigree (Pedigree 1, Fig. 1). The other two SALS patients with a p.N352S mutation (VII:1 and VIII:1) were found to be related to a different FALS family (Pedigree 4, Fig. 2C). Haplotype analysis revealed that all patients with p.N352S mutations in *TARDBP* shared a haplotype surrounding the mutation, which suggests a common ancestor (Supplementary Material, Fig. S1).

In Pedigree 1 (Fig. 1), a recombination event (not spanning *TARDBP*) caused the formation of a second haplotype (Supplementary Material, Table S1). In this pedigree, we identified five patients with a heterozygous p.N352S mutation, including one SALS patient (VIII:1). Two FALS patients also harboured a *C9orf72* repeat expansion (VIII:5 and VIII:6). The *TARDBP* mutation was transmitted via their unaffected 72-year-old mother (VII:4) and the *C9orf72* repeat expansion via their unaffected 75-year-old father (VII:3). Furthermore, we detected one patient from a consanguineous marriage with a homozygous p.N352S mutation (VII:10).

**Table 2.** Clinical information on families identified with mutations in multiple ALS-associated genes

PED	ID	Gene 1	Mutation 1	Haplotype	Gene 2	Mutation 2	Gender	Age at onset (y)	Site of onset	Duration (m)
PED1 <sup>a</sup>	VII:10 <sup>b</sup>	<i>TARDBP</i>	p.N352S	1 + 2	<i>TARDBP</i>	p.N352S	F	58	Cervical	>62
	VIII:5	<i>TARDBP</i>	p.N352S	2	<i>C9orf72</i>	Long repeat	M	42	Cervical	>91
	VIII:6	<i>TARDBP</i>	p.N352S	2	<i>C9orf72</i>	Long repeat	F	47	Cervical	>15
	VIII:8	<i>TARDBP</i>	p.N352S	1	N/A	N/A	M	59	Cervical	86 <sup>c</sup>
	VIII:12	<i>TARDBP</i>	p.N352S	1	N/A	N/A	F	64	Cervical	66 <sup>c</sup>
PED2 <sup>d</sup>	III:1	<i>TARDBP</i>	p.N352S	1	<i>ANG</i>	p.K171	M	61	Cervical	8 <sup>c</sup>
	III:2 <sup>e</sup>	No	No	No	<i>ANG</i>	p.K171	M	74	Cervical	37 <sup>c</sup>
	III:5	<i>TARDBP</i>	p.N352S	1	<i>ANG</i>	p.K171	F	73	Lumbosacral	47 <sup>c</sup>
	IV:5	<i>TARDBP</i>	p.N352S	1	<i>ANG</i>	p.K171	M	54	Cervical	52 <sup>c</sup>
PED5 <sup>f</sup>	III:6	<i>SOD1</i>	p.D90A	N/A	<i>C9orf72</i>	Long repeat	F	51	Cervical	77 <sup>c</sup>
PED6 <sup>g</sup>	III:5	<i>FUS/TLS</i>	p.R521C	N/A	<i>ANG</i>	p.K171	F	53	Lumbosacral	>24
PED7 <sup>h</sup>	III:5	<i>FUS/TLS</i>	p.Q210H	N/A	<i>C9orf72</i>	Long repeat	M	58	Lumbosacral	25 <sup>c</sup>

PED, pedigree; ID, identifier in pedigree; y, years; m, months. This table displays all families that were detected with mutations in multiple genes. Stutter amplifications of patients with repeat expansions in *C9orf72* are shown in Supplementary Material, Figure S3.

<sup>a</sup>The pedigree of this family is shown in Figure 1.

<sup>b</sup>Patient VII:10 has a homozygous mutation.

<sup>c</sup>Patient is deceased.

<sup>d</sup>The pedigree of this family is shown in Figure 2A.

<sup>e</sup>Patient III:2 presented with parkinsonism; after 5 years, he developed progressive weakness of the arms and legs with atrophy, fasciculations and hyperreflexia. He also demonstrated symptoms characteristic of FTD.

<sup>f</sup>The pedigree of this family is shown in Figure 3A.

<sup>g</sup>The pedigree of this family is shown in Figure 3B.

<sup>h</sup>The pedigree of this family is shown in Figure 3C.

In Pedigree 2 (Fig. 2A), another family is displayed with a p.N352S mutation. In this pedigree, a mutation in *ANG* (p.K171) co-segregated with ALS (16). While four patients had both *ANG* and *TARDBP* mutations, one patient (III:2) had an *ANG* mutation without a *TARDBP* mutation. This patient had a somewhat different phenotype compared with his affected family members, as he presented with parkinsonism and also developed FTD and ALS (16). DNA of patient III:3 was unavailable for testing. His children, aged between 44 and 58 years and currently without neurological complaints, did, however, have *ANG* and/or *TARDBP* mutations (IV:1 to IV:4). In total, we identified 14 patients with p.N352S mutations, 7 of whom (50%) had multiple mutations in ALS-associated genes.

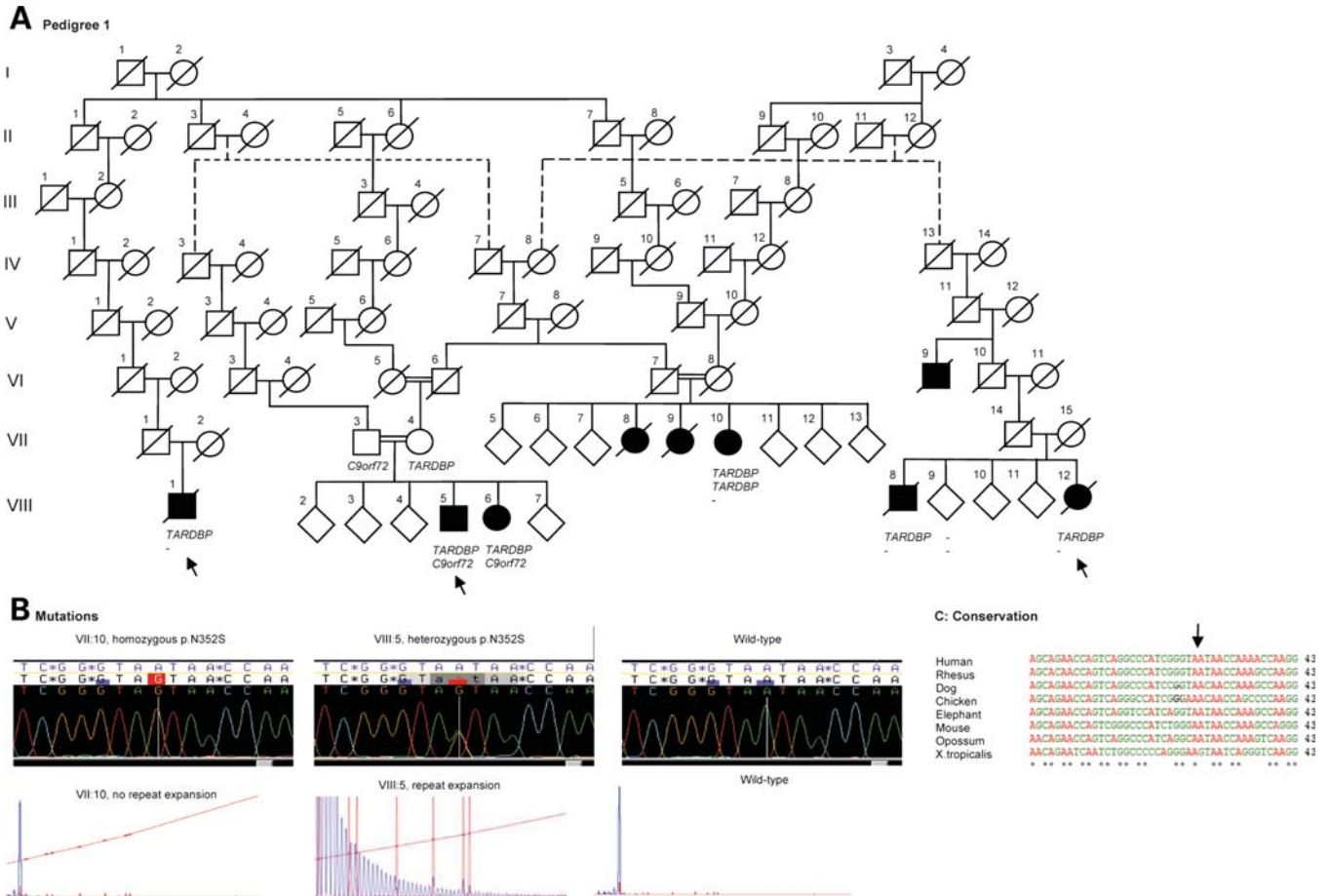
In addition, we identified a *C9orf72* repeat expansion in one patient (III:6) known to have an heterozygous p.D90A *SOD1* mutation (Pedigree 5) (17). Analysis of her pedigree revealed an autosomal dominant pattern of inheritance, as shown previously (Fig. 3A) (17). Genealogical data showed that her family originated from a small village in the Netherlands (genealogical data were available until the 18th century). A p.K171 mutation in *ANG* was also identified in a patient (III:5) with a p.R521C *FUS/TLS* mutation (Pedigree 6). DNA testing in 10 non-affected family members of patient III:5 failed to detect other subjects with mutations in *FUS/TLS* or *ANG* (Fig. 3B). Finally, another *C9orf72* repeat expansion was present in a patient (III:5) with a p.Q210H *FUS/TLS* mutation (Pedigree 7, Fig. 3C). The p.Q210H mutation, however, was also detected in one of our control subjects (Table 1) (18).

## DISCUSSION

In this study, we investigated an oligogenic model for ALS, by screening *TARDBP*, *FUS/TLS*, *SOD1*, *ANG* and *C9orf72* for

mutations in large cohorts of FALS patients, SALS patients and control subjects. In 5 of the 97 FALS families, we identified multiple mutations in ALS-associated genes. Statistical analysis demonstrated that the frequency of families with multiple mutations is higher than one might expect on the basis of chance ( $P = 1.57 \times 10^{-7}$ ). The most compelling evidence for an oligogenic basis of ALS was found in individuals with a p.N352S mutation in *TARDBP*. This mutation was detected in five FALS families and three apparently SALS patients. Genealogical analysis suggested that these patients had a common ancestor, something that was supported by the identification of a shared haplotype. In total, DNA was available from 14 patients with the p.N352S mutation, and we were able to detect a second mutation in 50% of them (*ANG*, *C9orf72* or homozygous *TARDBP*).

To date, mutations in *TARDBP* are considered to cause ALS in an autosomal dominant manner (19,20). The p.N352S mutation is predicted to be pathogenic and has not been detected in over 13 000 control subjects (Supplementary Material, Table S2). A heterozygous variant was originally reported in a German family (21). It has also been detected in one FALS patient and one SALS patient of Japanese origin (22,23). The previously published pedigrees showed incomplete penetrance: the German family showed a four generation pedigree with three affected individuals in two generations with a total of four unaffected obligate carriers of the p.N352S mutation (21). The Japanese pedigree consisted of two unaffected parents with four children of which two developed ALS. None of the offspring of the affected individuals were affected at time of publication (22). Our p.N352S pedigrees also demonstrated reduced penetrance, a finding substantiated by the detection of p.N352S in apparently sporadic cases (derived from a common ancestor). In short, our findings suggest that a second mutation might be necessary for the development of ALS.

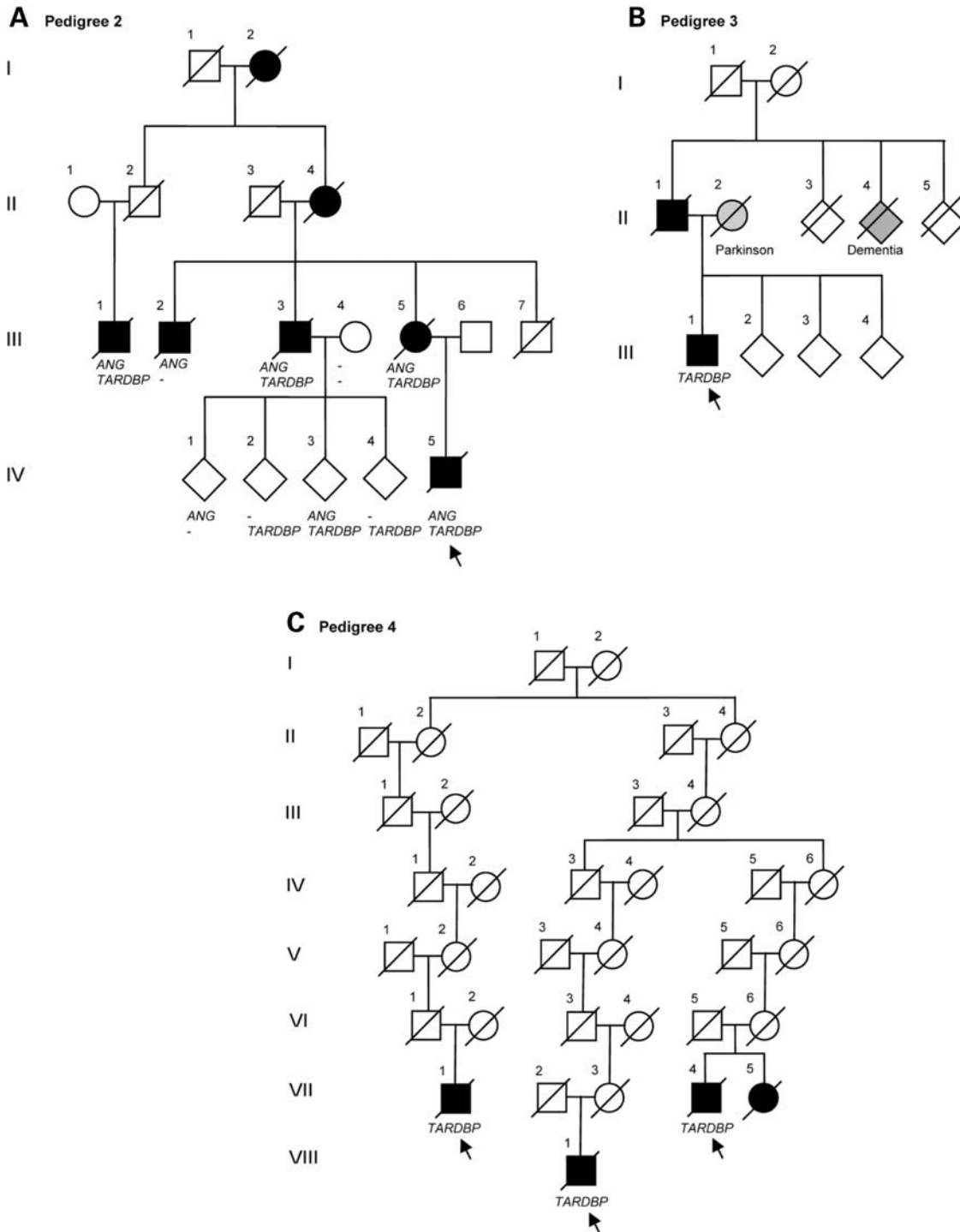


**Figure 1.** Pedigree 1 with *TARDBP* (p.N352S) mutation. (A) Screened individuals with a *TARDBP* mutation (p.N352S) are marked with 'TARDBP'. Individuals with *C9orf72* repeat expansions are marked with 'C9orf72', and individuals without these mutations are marked with '-'. (B) We show the homozygous p.N352S mutation without a *C9orf72* repeat expansion of patient VII:10. In addition, we display the heterozygous p.N352S mutation and the *C9orf72* repeat expansion of patient VIII:5. (C) Conservation of amino acid residues across species was generated using ClustalW2 online tool, <http://www.ebi.ac.uk/Tools/msa/clustalw2/>. (last accessed date on 1 June, 2012)

We have identified five FALS families with mutations in multiple ALS-associated genes. We found *FUS/TLS* and *TARDBP* mutations in combination with *ANG* mutations and *C9orf72* repeat expansions with *TARDBP*, *SOD1* and *FUS/TLS* mutations. The nature of the second mutations provides further evidence for an oligogenic aetiology of ALS. For instance, *C9orf72* repeat expansions have been reported in families with high penetrance, but also in families with many unaffected carriers (3,4). This could imply that additional genetic factors contribute to ALS pathogenesis in some *C9orf72* families. Moreover, this also suggests that genetic modifiers exist that determine why some individuals with *C9orf72* repeat expansions develop ALS, whereas others develop FTD or a combination of ALS and FTD. An oligogenic aetiology, combined with reduced ascertainment and lower life expectancy, could also explain why lower frequencies of ALS are observed in admixed populations (24,25). While in Europe, ancestral origins are shared, and a variety of rare 'at-risk' genes is inherited, the much wider variety and different combinations of 'at-risk' genes in admixed populations could result in lower frequencies of ALS, as discussed elsewhere (24).

The identification of mutations in *ANG* is compatible with an oligogenic aetiology as well. Although *ANG* mutations were initially thought to be directly pathogenic, later studies also demonstrated *ANG* mutations in control subjects (p.K17I, p.M-21I, p.P-4S and p.I46V) (26–30). The p.K17I mutation, however, does affect protein function. For instance, motor neurons transfected with p.K17I, lack the neuroprotective activity against hypoxic exposure shown by wild-type *ANG* (31). Moreover, wild-type *ANG* induces the formation of human umbilical vein endothelial cell tubes, whereas p.K17I does not, indicating a complete loss of its angiogenic activity (32). Furthermore, p.K17I demonstrates 5% of the ribonucleolytic activity of wild-type *ANG* (32). *ANG* mutations have also been shown to affect neurite extension/pathfinding and survival of motor neurons (33,34).

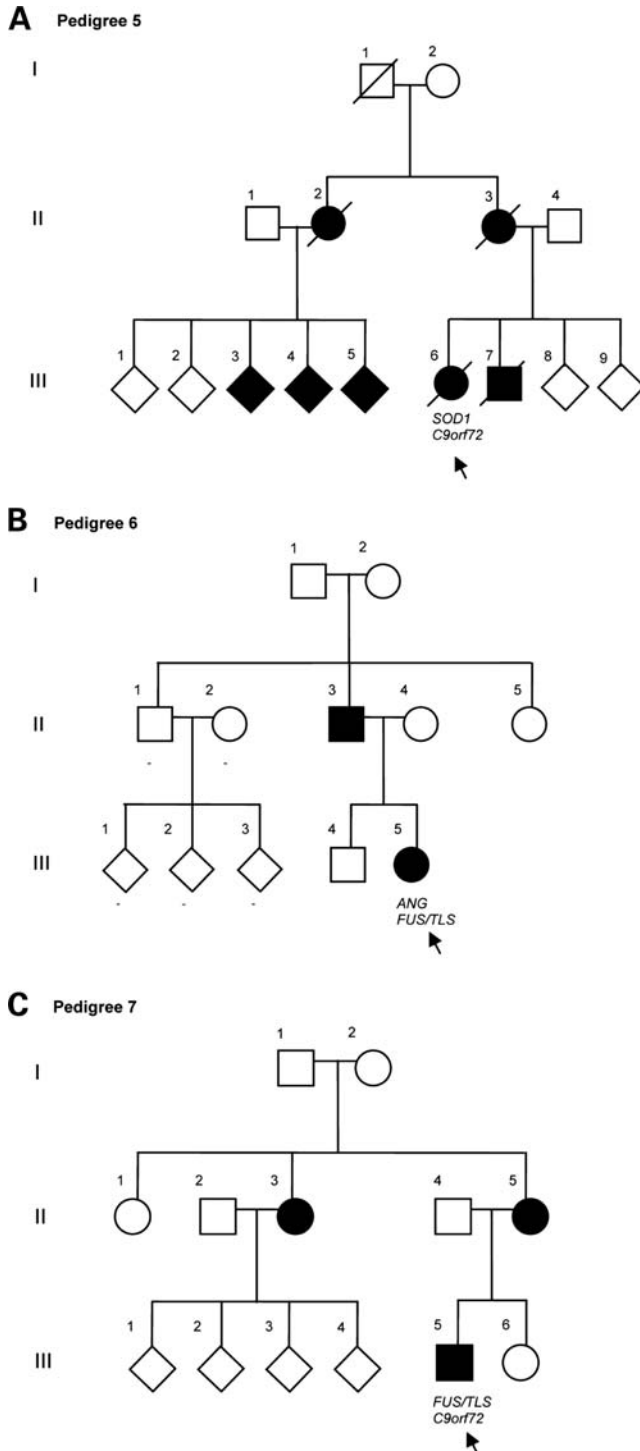
Importantly, a large international collaborative study (6471 ALS patients and 7668 control subjects from 15 centres) has recently revealed that *ANG* mutations are a risk factor for ALS, conferring a substantial risk (odds ratio, OR = 9.2) (30). Interestingly, this study also showed that *ANG* mutations are a risk factor for Parkinson's disease (OR = 6.7) (30). Hence, we argue that *ANG* variants may not be directly



**Figure 2.** Pedigrees 2, 3 and 4 with *TARDBP* (p.N352S) mutations. (A) Patients III:1, III:5, III:3 and IV:5 have both *TARDBP* (p.N352S) and *ANG* (p.K17I) mutations. For simplicity, just part of the pedigree is shown. Screened individuals who did not have a *TARDBP* or *ANG* mutation are marked with '-'. (B) Pedigree of an additional FALS patient with a *TARDBP* (p.N352S) mutation. (C) Pedigree of two SALS patients with p.N352S mutations in *TARDBP*, which were distantly related to a FALS patient.

pathogenic, but that they confer a risk for ALS and Parkinson's disease. This hypothesis is strengthened by the detection of *ANG* variants in ALS families with incomplete penetrance and mutations in other ALS-associated genes, as shown by our present study. The combination of a genetic risk factor with a

large effect (such as an *ANG* mutation), and another mutation with incomplete penetrance, fits the oligogenic disease model well. That *ANG* mutations also confer risk for Parkinson's disease suggests that multiple genetic factors influence phenotypic characteristics.



**Figure 3.** Pedigrees 5, 6 and 7 with multiple mutations. (A) Patient III:6 has both a *SOD1* (p.D90A) mutation and a *C9orf72* repeat expansion. DNA of other family members was not available for testing. (B) Patient III:5 has both an *ANG* (p.K17I) mutation and a *FUS/TLS* (p.R521C) mutation. For simplicity, just part of the pedigree is shown. (C) Patient III:5 has both a *FUS/TLS* (p.Q210H) mutation and a *C9orf72* repeat expansion. DNA of other family members was not available for testing.

Another double mutation was seen in Pedigree 5, where the heterozygous p.D90A *SOD1* mutation was observed in combination with a *C9orf72* repeat expansion. The p.D90A

mutation is known to cause ALS in both an autosomal dominant and an autosomal recessive manner. In Scandinavia, p.D90A is a relatively common polymorphism in the general population (2.5%) and only homozygous individuals develop ALS. Outside Scandinavia, p.D90A seems to cause ALS in an autosomal dominant fashion (it must be noted that autosomal dominant p.D90A pedigrees are rare) (1,17,35–37). This could be attributed to a genetic modifier on the Scandinavian haplotype, but an alternative explanation might be that the p.D90A haplotype outside Scandinavia co-segregates with a second (pathogenic) mutation (5), as in Pedigree 5.

We also detected a p.K17I *ANG* mutation in a patient with a p.R521C *FUS/TLS* mutation, the most frequent *FUS/TLS* mutation (9,11,18,38–49). In another FALS family, we detected a *C9orf72* repeat expansion and a *FUS/TLS* mutation of unknown pathogenicity (p.Q210H), which we also detected in one control subject (0.1%) (18). *FUS/TLS* mutations are considered to cause ALS in an autosomal dominant manner. However, incomplete penetrance is frequently observed, and one of the pedigrees that led to the discovery of *FUS/TLS* demonstrated an autosomal recessive pattern as well (38). The co-occurrence of *ANG* mutations and *C9orf72* repeat expansions in families with *FUS/TLS* mutations suggests a complex inheritance of ALS.

SALS is considered to be a complex disease in which multiple environmental and genetic risk factors contribute to disease susceptibility. In this study, we also provide evidence for a complex aetiology of FALS. We propose that the phenotypic variability that is frequently detected within FALS families is due to multiple genetic factors. An oligogenic aetiology of ALS has important implications for the design and interpretation of future experiments. For instance, in many studies, patients with known mutations in ALS-associated genes have been excluded for mutational screening of other genes. Based on our data, it would be interesting to include individuals with mutations as well. In addition, an oligogenic aetiology should be considered in experiments aiming at the identification of new ALS-associated genes (whole exome/genome experiments) and in genetic counselling, especially of unaffected family members.

To conclude, although great progress has recently been made in unravelling the pathogenesis of ALS, our data suggest that many genetic risk and modifying factors are yet to be identified.

## MATERIALS AND METHODS

### Subjects

Samples were collected at national referral centres for ALS in the Netherlands: University Medical Centre Utrecht, Academic Medical Centre Amsterdam and Radboud University Nijmegen Medical Centre. A total of 111 FALS patients (97 different families) were screened for non-synonymous mutations in *TARDBP*, *FUS/TLS*, *SOD1* and *ANG* and tested for *C9orf72* repeat expansions (Supplementary Material, Table S3). In addition, we screened 1192 SALS patients for mutations in *TARDBP* and *FUS/TLS*. A subgroup of these patients had already been screened for mutations in *SOD1*, *ANG* and *C9orf72*, as described elsewhere (16–18,30,50).

All ALS patients were diagnosed according to the El Escorial criteria (51,52), and to minimize selection and ascertainment biases, our patients were selected based on the Dutch Prospective Population-based ALS registry (53).

DNA was also obtained from control subjects of Dutch descent and screened for mutations in *TARDBP* ( $N = 1415$ ) and in *SOD1* ( $N = 1894$ ). Dutch control subjects had previously been screened for mutations in *FUS/TLS*, *SOD1*, *ANG* and *C9orf72* (17,18,30,50). These subjects were unrelated volunteers accompanying non-ALS patients to the Neurology Clinic, spouses of SALS patients or control subjects that participated in the Prospective Population-based study on ALS in the Netherlands (54).

All material was obtained with the approval of the Institutional Review Board, and participants provided informed consent.

### Genetic analysis

Subjects were screened for non-synonymous mutations in *TARDBP* (NM\_007375.3), *FUS/TLS* (NM\_004960, exons 5, 6, 14 and 15), *SOD1* (NM\_000454) and *ANG* (NM\_001145), as described previously (16–18,55). Briefly, coding regions were amplified by touchdown polymerase chain reaction with primers in adjacent intronic or non-coding regions. Subsequently, we used BigDye Terminator 3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and DNA Analyser 3730XL for sequencing. Data analysis was performed with PolyPhred and identified mutations were confirmed on genomic DNA (at least three times) (56). PMut was used to predict the impact of these mutations on the structure and function of the proteins (<http://mmb2.pcb.ub.es:8080/PMut/>, last accessed date on 1 June, 2012). Primers and protocols used to determine hexanucleotide repeat sizes in *C9orf72* (NM\_018325) have been described elsewhere (50).

### Genealogical analysis

In-depth genealogical analysis was performed to identify common ancestors of FALS patients. Lists of descendants from index patients were compiled, and thereafter, pedigrees were generated using civil records/registers and church records of the Dutch population.

### Haplotype analysis

To further establish evidence for common ancestry between families, we performed extended haplotype analysis on FALS families and SALS patients with the same mutation (*TARDBP*; p.N352S). For this analysis, we used six extragenic polymorphic markers flanking *TARDBP* (D1S1612, D1S503, D1S244 proximal of *TARDBP* and D1S2667, D1S2740 and D1S1597 distal of *TARDBP*, Supplementary Material, Fig. S1). Validity of the constructed haplotype was determined by segregation analysis in families and patients whose DNA was available for testing.

### Statistical analysis

In order to assess whether the observed frequency of samples with multiple mutations was in excess of what one might expect on the basis of chance, we performed a binomial test using the statistical analysis program R (CRAN; <http://www.R-project.org>, last accessed date on 1 June, 2012). The following formula was used, based on Table 1, to determine the number of families (with and without multiple mutations) and the detected mutation frequencies (in FALS families and control subjects): (pbinom ([number of families with multiple mutations = 5], [total number of families = 97], [detected mutation frequency in FALS families (0.48) multiplied by the detected mutation frequency in control subjects (0.005)], lower.tail = FALSE, log.p = FALSE)).

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

*Conflict of Interest statement.* None declared.

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### REFERENCES

1. Valdmanis, P.N. and Rouleau, G.A. (2008) Genetics of familial amyotrophic lateral sclerosis. *Neurology*, **70**, 144–152.
2. Van Damme, P. and Robberecht, W. (2009) Recent advances in motor neuron disease. *Curr. Opin. Neurol.*, **22**, 486–492.
3. Renton, A.E., Majounie, E., Waite, A., Simon-Sanchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L. *et al.* (2011) A hexanucleotide repeat expansion in *C9ORF72* is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*, **72**, 257–268.
4. DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J. *et al.* (2011) Expanded GGGGCC hexanucleotide repeat in noncoding region of *C9ORF72* causes chromosome 9p-linked FTD and ALS. *Neuron*, **72**, 245–256.
5. Andersen, P.M. and Al-Chalabi, A. (2011) Clinical genetics of amyotrophic lateral sclerosis: what do we really know? *Nat. Rev. Neurol.*, **7**, 603–615.
6. Al-Chalabi, A., Fang, F., Hanby, M.F., Leigh, P.N., Shaw, C.E., Ye, W. and Rijsdijk, F. (2010) An estimate of amyotrophic lateral sclerosis heritability using twin data. *J. Neurol. Neurosurg. Psychiatry*, **81**, 1324–1326.
7. Hand, C.K., Mayeux-Portas, V., Khoris, J., Briolotti, V., Clavelou, P., Camu, W. and Rouleau, G.A. (2001) Compound heterozygous D90A and D96N *SOD1* mutations in a recessive amyotrophic lateral sclerosis family. *Ann. Neurol.*, **49**, 267–271.
8. DeJesus-Hernandez, M., Kocerha, J., Finch, N., Crook, R., Baker, M., Desaro, P., Johnston, A., Rutherford, N., Wojtas, A., Kennelly, K. *et al.* (2010) De novo truncating *FUS* gene mutation as a cause of sporadic amyotrophic lateral sclerosis. *Hum. Mutat.*, **31**, E1377–E1389.
9. Chio, A., Calvo, A., Moglia, C., Ossola, I., Brunetti, M., Sbaiz, L., Lai, S.L., Abramzon, Y., Traynor, B.J. and Restagno, G. (2011) A de novo missense mutation of the *FUS* gene in a 'true' sporadic ALS case. *Neurobiol. Aging*, **32**, 553.e23–553.e26.

10. Daoud, H., Valdmanis, P.N., Gros-Louis, F., Belzil, V., Spiegelman, D., Henrion, E., Diallo, O., Desjarlais, A., Gauthier, J., Camu, W. *et al.* (2011) Resequencing of 29 candidate genes in patients with familial and sporadic amyotrophic lateral sclerosis. *Arch. Neurol.*, **68**, 587–593.
11. Millicamps, S., Salachas, F., Cazeneuve, C., Gordon, P., Bricka, B., Camuzat, A., Guillot-Noel, L., Russaouen, O., Bruneteau, G., Pradat, P.F. *et al.* (2010) SOD1, ANG, VAPB, TARDBP, and FUS mutations in familial amyotrophic lateral sclerosis: genotype-phenotype correlations. *J. Med. Genet.*, **47**, 554–560.
12. Gros-Louis, F., Andersen, P.M., Dupre, N., Urushitani, M., Dion, P., Souchon, F., D'Amour, M., Camu, W., Meininger, V., Bouchard, J.P. *et al.* (2009) Chromogranin B P413L variant as risk factor and modifier of disease onset for amyotrophic lateral sclerosis. *Proc. Natl Acad. Sci. USA*, **106**, 21777–21782.
13. Luigetti, M., Lattante, S., Zollino, M., Conte, A., Marangi, G., Del Grande, A. and Sabatelli, M. (2011) SOD1 G93D sporadic amyotrophic lateral sclerosis (SALS) patient with rapid progression and concomitant novel ANG variant. *Neurobiol. Aging*, **32**, 1924.e15–1924.e18.
14. Lill, C.M., Abel, O., Bertram, L. and Al-Chalabi, A. (2011) Keeping up with genetic discoveries in amyotrophic lateral sclerosis: the ALSod and ALSGene databases. *Amyotroph. Lateral Scler.*, **12**, 238–249.
15. Radunovic, A. and Leigh, P.N. (1999) ALSODatabase: database of SOD1 (and other) gene mutations in ALS on the Internet. European FALS Group and ALSOD Consortium. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.*, **1**, 45–49.
16. van Es, M.A., Diekstra, F.P., Veldink, J.H., Baas, F., Bourque, P.R., Schelhaas, H.J., Strengman, E., Hennekam, E.A., Lindhout, D., Ophoff, R.A. and van den Berg, L.H. (2009) A case of ALS-FTD in a large FALS pedigree with a K171 ANG mutation. *Neurology*, **72**, 287–288.
17. van Es, M.A., Dahlberg, C., Birve, A., Veldink, J.H., van den Berg, L.H. and Andersen, P.M. (2010) Large-scale SOD1 mutation screening provides evidence for genetic heterogeneity in amyotrophic lateral sclerosis. *J. Neurol. Neurosurg. Psychiatry*, **81**, 562–566.
18. Groen, E.J., van Es, M.A., van Vught, P.W., Spliet, W.G., van Engelen-Lee, J., de Visser, M., Wokke, J.H., Schelhaas, H.J., Ophoff, R.A., Fumoto, K. *et al.* (2010) FUS mutations in familial amyotrophic lateral sclerosis in the Netherlands. *Arch. Neurol.*, **67**, 224–230.
19. Lagier-Tourenne, C., Polymenidou, M. and Cleveland, D.W. (2010) TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. *Hum. Mol. Genet.*, **19**, R46–R64.
20. Pesiridis, G.S., Lee, V.M. and Trojanowski, J.Q. (2009) Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **18**, R156–R162.
21. Kuhnlein, P., Sperfeld, A.D., Vanmassenhove, B., Van Deerlin, V., Lee, V.M., Trojanowski, J.Q., Kretschmar, H.A., Ludolph, A.C. and Neumann, M. (2008) Two German kindreds with familial amyotrophic lateral sclerosis due to TARDBP mutations. *Arch. Neurol.*, **65**, 1185–1189.
22. Kamada, M., Maruyama, H., Tanaka, E., Morino, H., Wate, R., Ito, H., Kusaka, H., Kawano, Y., Miki, T., Nodera, H. *et al.* (2009) Screening for TARDBP mutations in Japanese familial amyotrophic lateral sclerosis. *J. Neurol. Sci.*, **284**, 69–71.
23. Iida, A., Kamei, T., Sano, M., Oshima, S., Tokuda, T., Nakamura, Y. and Ikegawa, S. (2012) Large-scale screening of TARDBP mutation in amyotrophic lateral sclerosis in Japanese. *Neurobiol. Aging*, **33**, 786–790.
24. Zaldivar, T., Gutierrez, J., Lara, G., Carbonara, M., Logroscino, G. and Hardiman, O. (2009) Reduced frequency of ALS in an ethnically mixed population: a population-based mortality study. *Neurology*, **72**, 1640–1645.
25. Cronin, S., Hardiman, O. and Traynor, B.J. (2007) Ethnic variation in the incidence of ALS: a systematic review. *Neurology*, **68**, 1002–1007.
26. Fernandez-Santiago, R., Hoening, S., Lichtner, P., Sperfeld, A.D., Sharma, M., Berg, D., Weichenrieder, O., Illig, T., Eger, K., Meyer, T. *et al.* (2009) Identification of novel angiogenin (ANG) gene missense variants in German patients with amyotrophic lateral sclerosis. *J. Neurol.*, **256**, 1337–1342.
27. Gellera, C., Colombrita, C., Ticozzi, N., Castellotti, B., Bragato, C., Ratti, A., Taroni, F. and Silani, V. (2008) Identification of new ANG gene mutations in a large cohort of Italian patients with amyotrophic lateral sclerosis. *Neurogenetics*, **9**, 33–40.
28. Greenway, M.J., Andersen, P.M., Russ, C., Ennis, S., Cashman, S., Donaghy, C., Patterson, V., Swinger, R., Kieran, D., Prehn, J. *et al.* (2006) ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat. Genet.*, **38**, 411–413.
29. Conforti, F.L., Sprovieri, T., Mazzei, R., Ungaro, C., La Bella, V., Tessitore, A., Patitucci, A., Magariello, A., Gabriele, A.L., Tedeschi, G. *et al.* (2008) A novel angiogenin gene mutation in a sporadic patient with amyotrophic lateral sclerosis from southern Italy. *Neuromuscul. Disord.*, **18**, 68–70.
30. van Es, M.A., Schelhaas, H.J., van Vught, P.W., Ticozzi, N., Andersen, P.M., Groen, E.J., Schulte, C., Blauw, H.M., Koppers, M., Diekstra, F.P. *et al.* (2011) Angiogenin variants in Parkinson disease and amyotrophic lateral sclerosis. *Ann. Neurol.*, **70**, 964–973.
31. Sebastia, J., Kieran, D., Breen, B., King, M.A., Nettelband, D.F., Joyce, D., Fitzpatrick, S.F., Taylor, C.T. and Prehn, J.H. (2009) Angiogenin protects motoneurons against hypoxic injury. *Cell Death. Differ.*, **16**, 1238–1247.
32. Wu, D., Yu, W., Kishikawa, H., Folkert, R.D., Iafrate, A.J., Shen, Y., Xin, W., Sims, K. and Hu, G.F. (2007) Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. *Ann. Neurol.*, **62**, 609–617.
33. Subramanian, V. and Feng, Y. (2007) A new role for angiogenin in neurite growth and pathfinding: implications for amyotrophic lateral sclerosis. *Hum. Mol. Genet.*, **16**, 1445–1453.
34. Subramanian, V., Crabtree, B. and Acharya, K.R. (2008) Human angiogenin is a neuroprotective factor and amyotrophic lateral sclerosis associated angiogenin variants affect neurite extension/pathfinding and survival of motor neurons. *Hum. Mol. Genet.*, **17**, 130–149.
35. Giannini, F., Battistini, S., Mancuso, M., Greco, G., Ricci, C., Volpi, N., Del Corrona, A., Piazza, S. and Siciliano, G. (2010) D90A-SOD1 mutation in ALS: The first report of heterozygous Italian patients and unusual findings. *Amyotroph. Lateral Scler.*, **11**, 216–219.
36. Rabe, M., Felbecker, A., Waibel, S., Steinbach, P., Winter, P., Muller, U. and Ludolph, A.C. (2010) The epidemiology of CuZn-SOD mutations in Germany: a study of 217 families. *J. Neurol.*, **257**, 1298–1302.
37. Andersen, P.M., Nilsson, P., Ala-Hurula, V., Keranen, M.L., Tarvainen, I., Haltia, T., Nilsson, L., Binzer, M., Forsgren, L. and Marklund, S.L. (1995) Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase. *Nat. Genet.*, **10**, 61–66.
38. Kwiatkowski, T.J. Jr., Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E.J., Munsat, T. *et al.* (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*, **323**, 1205–1208.
39. Vance, C., Rogelj, B., Hortobagyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., Hu, X., Smith, B., Ruddy, D., Wright, P. *et al.* (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*, **323**, 1208–1211.
40. Belzil, V.V., Valdmanis, P.N., Dion, P.A., Daoud, H., Kabashi, E., Noreau, A., Gauthier, J., Hince, P., Desjarlais, A., Bouchard, J.P. *et al.* (2009) Mutations in FUS cause FALS and SALS in French and French Canadian populations. *Neurology*, **73**, 1176–1179.
41. Drepper, C., Herrmann, T., Wessig, C., Beck, M. and Sendtner, M. (2011) C-terminal FUS/TLS mutations in familial and sporadic ALS in Germany. *Neurobiol. Aging*, **32**, 548.e1–548.e4.
42. Tateishi, T., Hokenohara, T., Yamasaki, R., Miura, S., Kikuchi, H., Iwaki, A., Tashiro, H., Furuya, H., Nagara, Y., Ohyagi, Y. *et al.* (2010) Multiple system degeneration with basophilic inclusions in Japanese ALS patients with FUS mutation. *Acta Neuropathol.*, **119**, 355–364.
43. Ticozzi, N., Silani, V., Leclerc, A.L., Keagle, P., Gellera, C., Ratti, A., Taroni, F., Kwiatkowski, T.J. Jr., McKenna-Yasek, D.M., Sapp, P.C. *et al.* (2009) Analysis of FUS gene mutation in familial amyotrophic lateral sclerosis within an Italian cohort. *Neurology*, **73**, 1180–1185.
44. Syriani, E., Morales, M. and Gamez, J. (2011) FUS/TLS gene mutations are the second most frequent cause of familial ALS in the Spanish population. *Amyotroph. Lateral Scler.*, **12**, 118–123.
45. Yamamoto-Watanabe, Y., Watanabe, M., Okamoto, K., Fujita, Y., Jackson, M., Ikeda, M., Nakazato, Y., Ikeda, Y., Matsubara, E., Kawarabayashi, T. and Shoji, M. (2010) A Japanese ALS6 family with mutation R521C in the FUS/TLS gene: a clinical, pathological and genetic report. *J. Neurol. Sci.*, **296**, 59–63.
46. Lai, S.L., Abramzon, Y., Schymick, J.C., Stephan, D.A., Dunckley, T., Dillman, A., Cookson, M., Calvo, A., Battistini, S., Giannini, F. *et al.* (2011) FUS mutations in sporadic amyotrophic lateral sclerosis. *Neurobiol. Aging*, **32**, 550.e1–550.e4.
47. Rademakers, R., Stewart, H., DeJesus-Hernandez, M., Krieger, C., Graff-Radford, N., Fabros, M., Briemberg, H., Cashman, N., Eisen, A. and Mackenzie, I.R. (2010) Fus gene mutations in familial and sporadic amyotrophic lateral sclerosis. *Muscle Nerve*, **42**, 170–176.



48. Blair, I.P., Williams, K.L., Warraich, S.T., Durnall, J.C., Thoeng, A.D., Manavis, J., Blumbergs, P.C., Vucic, S., Kiernan, M.C. and Nicholson, G.A. (2010) FUS mutations in amyotrophic lateral sclerosis: clinical, pathological, neurophysiological and genetic analysis. *J. Neurol. Neurosurg. Psychiatry*, **81**, 639–645.
49. Corrado, L., Del Bo, R., Castellotti, B., Ratti, A., Cereda, C., Penco, S., Soraru, G., Carlomagno, Y., Ghezzi, S., Pensato, V. *et al.* (2010) Mutations of FUS gene in sporadic amyotrophic lateral sclerosis. *J. Med. Genet.*, **47**, 190–194.
50. van Rheenen, W., van Blitterswijk, M., Huisman, M.H.B., Vlam, L., van Doormaal, P.T.C., Seelen, M., Medic, J., Dooijes, D., de Visser, M., van der Kooi, A.J. *et al.* (2012) Hexanucleotide repeat expansions in C9orf72 in the spectrum of motor neuron diseases. *Neurology*, in press.
51. Brooks, B.R., Miller, R.G., Swash, M. and Munsat, T.L. (2000) El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.*, **1**, 293–299.
52. Brooks, B.R. (1994) El Escorial World Federation of Neurology criteria for the diagnosis of amyotrophic lateral sclerosis. Subcommittee on Motor Neuron Diseases/Amyotrophic Lateral Sclerosis of the World Federation of Neurology Research Group on Neuromuscular Diseases and the El Escorial 'Clinical limits of amyotrophic lateral sclerosis' workshop contributors. *J. Neurol. Sci.*, **124** (Suppl) 96–107.
53. Huisman, M.H., de Jong, S.W., van Doormaal, P.T., Weinreich, S.S., Schelhaas, H.J., van der Kooi, A.J., de Visser, M., Veldink, J.H. and van den Berg, L.H. (2011) Population based epidemiology of amyotrophic lateral sclerosis using capture-recapture methodology. *J. Neurol. Neurosurg. Psychiatry*, **82**, 1165–1170.
54. van Es, M.A., Van Vught, P.W., Blauw, H.M., Franke, L., Saris, C.G., Andersen, P.M., Van Den Bosch, L., de Jong, S.W., van 't Slot, R., Birve, A. *et al.* (2007) ITPR2 as a susceptibility gene in sporadic amyotrophic lateral sclerosis: a genome-wide association study. *Lancet Neurol.*, **6**, 869–877.
55. Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J.C., Williams, K.L., Buratti, E. *et al.* (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*, **319**, 1668–1672.
56. Nickerson, D.A., Tobe, V.O. and Taylor, S.L. (1997) PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res.*, **25**, 2745–2751.