RESEARCH PAPER

C9orf72 expansion differentially affects males with spinal onset amyotrophic lateral sclerosis

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ABSTRACT

Introduction The *C9orf72* repeat expansion has been reported as a negative prognostic factor in amyotrophic lateral sclerosis (ALS). We have examined the prognostic impact of the *C9orf72* repeat expansion in European subgroups based on gender and site of onset.

Methods *C9orf72* status and demographic/clinical data from 4925 patients with ALS drawn from 3 prospective ALS registers (Ireland, Italy and the Netherlands), and clinical data sets in the UK and Belgium. Flexible parametric survival models were built including known prognostic factors (age, diagnostic delay and site of onset), gender and the presence of an expanded repeat in *C9orf72*. These were used to explore the effects of *C9orf72* on survival by gender and site of onset. Individual patient data (IPD) meta-analysis was used to estimate HRs for results of particular importance.

Results 457 (8.95%) of 4925 ALS cases carried the *C9orf72* repeat expansion. A meta-analysis of *C9orf72* estimated a survival HR of 1.36 (1.18 to 1.57) for those carrying the expansion. Models evaluating interaction between gender and *C9orf72* repeat expansions demonstrated that the reduced survival due to *C9orf72* expansion was being driven by spinal onset males (HR 1.56 (95% CI 1.25 to 1.96).

Conclusions This study represents the largest combined analysis of the prognostic characteristics of the *C9orf72* expansion. We have shown for the first time that the negative prognostic implication of this variant is driven by males with spinal onset disease, indicating a hitherto unrecognised gender-mediated effect of the variant that requires further exploration.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a debilitating disease with a poor prognosis. Progress towards developing new treatments has been limited both by disease heterogeneity, and by the likely interaction between genetic and environmental factors in disease pathogenesis.[1] A pathological expansion of a hexanucleotide repeat in the *C9orf72* gene[2 3] accounts for up to 10% of those with ALS in populations of European extraction, and is associated with a distinctive clinical phenotype that includes frontotemporal dementia (FTD) in some instances.[4–10] Although the *C9orf72* repeat expansion has been shown to be an important negative prognostic factor in survival analyses,[4–10] until now no study has been sufficiently large to permit robust analysis of interactions between the variant and demographic features including age, gender and site of onset. Here, we used our combined clinical data sets to determine whether the presence of the expanded variant differentially modulates survival based on gender and site of onset.

METHODS

Data sources/case capture

Clinical data from ALS cases incident from January 2000 to April 2015 were collected from Belgium, Ireland, Italy, Netherlands and the UK. All patients fulfilled the diagnostic criteria for ALS, and core data elements as defined by the European Network for the Cure of ALS (ENCALS consortium) were harmonised across data sets for consistency based on existing consortia agreements.[11] Owing to the lack of an agreed international definition of 'familial ALS', and given that a previous population-based familial aggregation analysis from Ireland demonstrated a much higher familial ALS occurrence (16%) than usually recognised,[12] we did not exclude cases based on family history. The Belgian and UK cases were collected from clinical research centre cohorts, while the Dutch, Irish and Italian cases were sourced from the prospective population-based national registers.[13–19]

In accordance with existing Consortia agreements, data were collated using the following variables: age of onset, date of onset, date of diagnosis, date of death/last known follow-up date, site of onset, revised El-Escorial diagnostic category (except Belgium) and *C9orf72* status (normal or expanded). For all study participants, *C9orf72* status was determined by repeat primed PCR as described previously (with individual laboratory-based validation and quality control by Southern blot analyses).[3]

Survival analysis strategy

Initially exploratory models were constructed using Cox proportional hazards regression to explore the effect of different time of entry to the studies. Cox models were generated including known important survival covariates including age of onset, site of onset, diagnostic delay and *C9orf72*. Cox models were compared using a likelihood ratio test, and by testing the validity of the proportional hazards assumption of each covariate at each timescale.

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A base model using Royston-Parmar flexible parametric regression[20] was built on the preferred timescale, with a proportional hazards scale and a number of degrees of freedom selected by comparison of the Akaike information criterion and Bayesian information criterion from models with increasing degrees of freedom, and the variance–covariance matrix clustered by country. Survival follow-up was limited to 5 years from entry. Models were then built to explore the effect of *C9orf72* status in sex and site of onset subgroups. The *stpm2*[21] and *ipdmetan*[22] commands from Stata MP V.14.0 were used to perform the survival analysis and produce the meta-analysis graphs, while the *ggplot2*[23] package in R V.3.1.1 was used to generate selected final graphs.

RESULTS

Descriptive statistics and basic survival model

In total, 5106 ALS cases met the inclusion criteria, of which 457 (8.95%) carried the *C9orf72* repeat expansion. Breakdown of the demographics of the overall cohort by country is shown in table 1. Missing values were minimal affecting 181 cases (3.5%). Online supplementary information 1 summarises the basic survival model. An individual patient data (IPD) meta-analysis of *C9orf72* status in the base model estimated an HR of 1.36 (1.18 to 1.57) for those carrying the expansion versus those not.

C9orf72, gender and site of onset subgroup analysis

Survival curves were generated to evaluate the effect of *C9orf72* status on gender and site of onset (figure 1) which suggested a three-way interaction. Therefore, gender, site of onset and *C9orf72* status were categorised into one variable with eight levels as demonstrated in Online supplementary information 2 table 1. Through comparison of survival curves, redundant subgroups were combined (see online supplementary information 2), leaving three groups: spinal onset males with the *C9orf72* expansion, other spinal onset patients and all bulbar onset patients. Survival curves for these groups showed that male spinal onset patients with the *C9orf72* repeat expansion had a prognosis distinct from other spinal onset patients and similar to bulbar onset patients (figure 2). Meta-analysis calculated a survival HR of 1.56 (95% CI 1.25 to 1.96) for male spinal onset patients with the *C9orf72* repeat expansion (figure 3). The finding was in the same direction in each country, although only the pooled estimate was statistically significant (figure 3).

The median ages and distribution of diagnostic delay across the final subgroups is shown in table 2. While age of onset was oldest in the bulbar onset group and youngest in the male spinal onset *C9orf72* expanded group, the male spinal onset *C9orf72* expanded group also had the highest proportion in the 'short' diagnostic delay category, consistent with the finding that the *C9orf72* expansion differentially affects disease course in a gender-specific manner. Adjustment for the El-Escorial category (table 3) did not substantially alter the HR (1.57 CI 1.26 to 1.97).

DISCUSSION

Previously, studies have shown that people with ALS carrying a *C9orf72* repeat expansion in blood present at a younger age and have reduced survival when compared to patients without the expanded variant (table 4). However, studies until now have not been sufficiently powered to determine whether the expanded variant differentially affects outcome in subgroups based on gender and site of onset. Our findings demonstrate an intriguing and previously unrecognised interaction between the expanded variant and male patients with spinal onset disease, which appears to drive the overall survival effect. Within this cohort, the median age of onset was 59.3 and the median survival was 2.29 years. This compared to a median age of onset of 62.3 and median survival of 2.77 years in all other spinal onset disease, and a median age of onset of 65 and median survival of 2.38 years in all bulbar onset disease. Moreover, and contrary to the usual pattern in young onset disease, male spinal onset C9orf72 expanded cases were also more likely to have experienced a shorter diagnostic delay, suggesting rapidly progressing disease.

Female gender has previously been reported as an independent predictor of faster functional decline in ALS;[24] however, our observation of an interaction between site of onset, gender and *C9orf72* has not been previously noted, possibly due to

Table 1 Baseline demographics of *C9orf72*-tested ALS cases by country

Variable	Belgium n=482	Ireland n=645	Italy n=897	The Netherlands n=2153	UK n=929	p Value(χ^{2})	Combined N=5106
Included after missing values removed	477 (99.0%)	640 (99.2%)	867 (96.7%)	2037 (94.6%)	904 (97.3%)		4925 (96.5%)
Sex							
Female	180 (37.3)	266 (41.6%)	411 (47.4%)	844 (41.4%)	352 (38.9%)		2053 (41.7%)
Male	297 (62.3)	374 (58.4%)	456 (52.6%)	1193 (58.6%)	552 (61.1%)	<0.001	2872 (58.3%)
Age of onset							
Median	61.4	63.0	66.8	63.0	61.3		63.1
Diagnostic delay							
Short	161 (33.8%)	193 (30.2%)	285 (32.9%)	702 (43.5%)	300 (33.2%)		1641 (33.3%)
Medium	163 (34.2%)	211 (33.0%)	293 (33.8%)	683 (33.5%)	298 (33.0%)		1648 (33.5%)
Long	153 (32.1%)	236 (36.9%)	289 (33.3%)	652 (32%)	306 (33.8%)	0.559	1636 (33.2%)
Site of onset							
Spinal	326 (68.3%)	437 (68.3%)	583 (67.2%)	1333 (65.4%)	610 (67.5%)		3289 (66.8%)
Bulbar	151 (31.7%)	203 (31.7%)	284 (32.8%)	704 (34.6%)	294 (32.5%)	0.540	1636 (33.2%)
C9orf72							
Normal	392 (82.2%)	578 (90.3%)	805 (92.8%)	1861 (91.4%)	841 (93.0%)		4477 (90.9%)
Expanded	85 (17.8%)	62 (9.7%)	62 (7.2%)	176 (8.6%)	63 (7%)	<0.001	448 (9.1%)

Diagnostic delay is defined by three tertiles per country labelled 'short', 'medium' and 'long' diagnostic delay to allow for variation in diagnostic delay between countries.
ALS, amyotrophic lateral sclerosis.

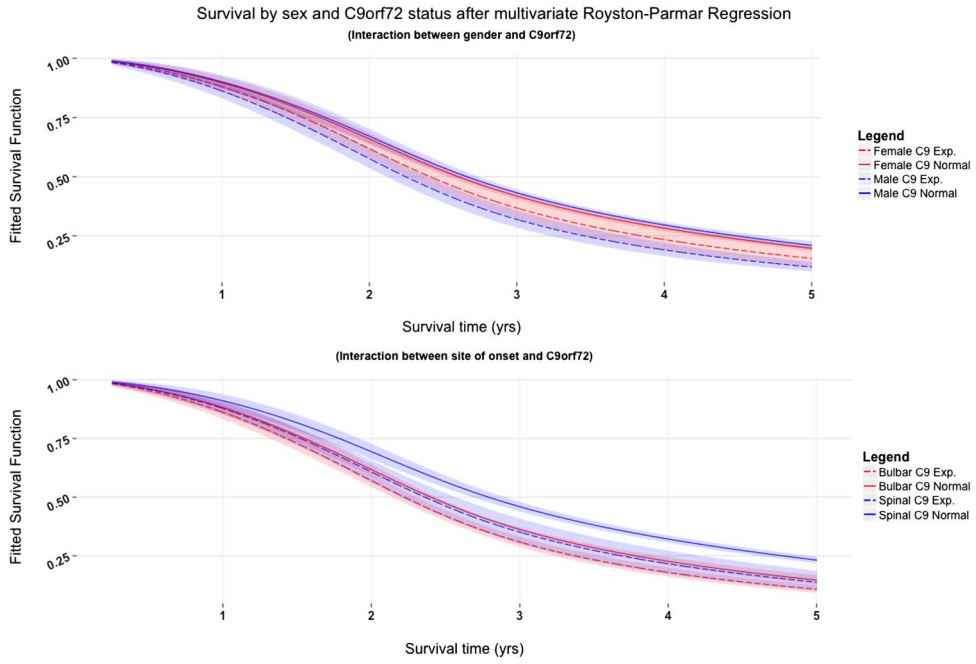

Figure 1 Predicted survival by sex and *C9orf72* status after multivariate Royston-Parmar regression including interaction terms. C9 normal=group not carrying the *C9orf72* expansion; C9 Exp=group carrying the *C9orf72* expansion present. Shaded areas represent 95% CIs. Predicted survival curves for interaction models between gender and *C9orf72* status (upper), and site of onset and *C9orf72* status (lower) after multivariate regression using a Royston-Parmar model on the hazard scale (three degrees of freedom), correcting for age of onset (time varying), diagnostic delay group (time varying), site of onset, country and using a variance–covariance matrix clustered by country. The upper graph showing a wider spread by *C9orf72* status in males compared to females, while the lower graph showing a wider spread in spinal onset cases versus bulbar onset cases; however, HRs for these interactions were not significant.

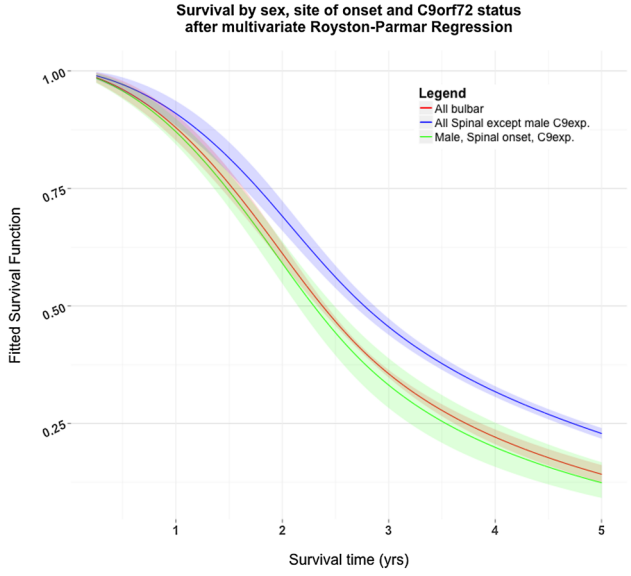

Figure 2 Predicted survival function for male spinal onset *C9orf72* expanded patients. C9 exp=group carrying the *C9orf72* expansion. Shaded areas represent 95% CIs. Survival curves show three subgroups of patients categorised by sex, site of onset and *C9orf72* status. Survival curves for male spinal onset patients with the *C9orf72* repeat expansion are markedly worse than other spinal onset patients and are in fact inseparable from bulbar onset patients. Median predicted survival in the three groups were: spinal excluding *C9orf72* expanded males=2.77 years (95% CI 2.67 to 2.87); bulbar onset=2.38 years (95% CI 2.33 to 2.42); spinal onset males with *C9orf72* repeat expansion=2.29 years (95% CI 2.15 to 2.49).

limitations in the power of previous studies due to lower numbers (table 4). Taken together, our findings and those of previous studies imply that distinct processes may operate in differing subgroups of ALS even when a known genetic factor is present as the underlying cause, and demonstrate that male gender is likely to be an important interacting factor in the biology of C9orf72-related disease.

A number of pathogenic mechanisms have been proposed to explain the role of the *C9orf72* repeat expansion in ALS. These include haploinsufficiency, toxic RNA interfering with the function of RNA-binding proteins or other cellular factors, and the presence of toxic dipeptide repeat proteins through Repeat-associated non-ATG (RAN) translation.[25 26] Recent work has also pointed towards *C9orf72*-induced pathology of nucleocytoplasmic transport processes.[26–29] However, the pathobiology of the observed interaction between the *C9orf72* variant and gender remains unclear, but it is congruent with observations in the SOD1 mouse model, in which transgenic mutant males have shorter survival compared to their transgenic female littermates with similar copy numbers.[30] The mechanism for this gender effect in animals, although well recognised, has not been characterised, but can be attenuated when mice are bred on a different genetic background.[30]

A potential weakness of our study is that it did not include clinical scores for the presence of cognitive change, which is a known prognostic indicator in ALS. We and others have shown that those with C9orf72 repeat expansions are more likely to experience cognitive and behavioural change; however, to the best of our knowledge, until now no gender-mediated effect has been demonstrated in *C9orf72*-related cognitive profiling. Moreover, since *C9orf72* is part of the causal pathway for some

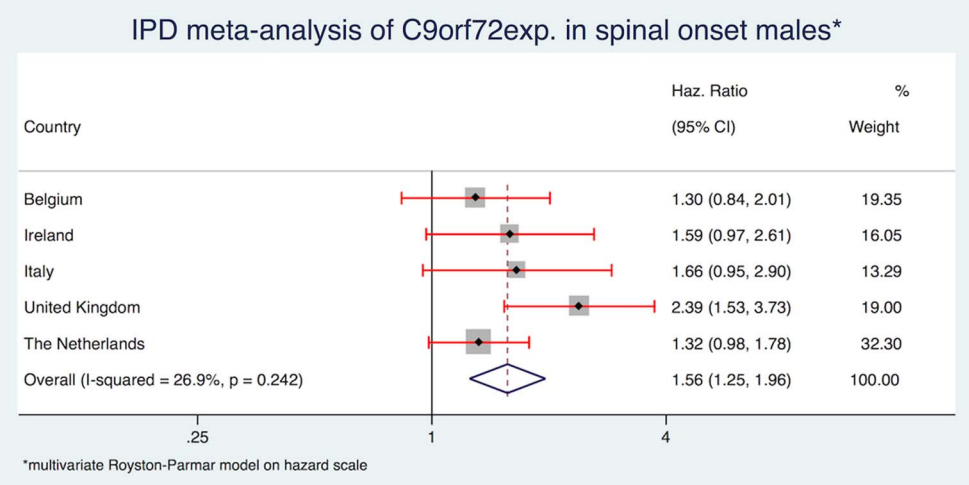

Figure 3 Individual patient data meta-analysis of the HR of male spinal onset patients with amyotrophic lateral sclerosis (ALS) carrying the *C9orf72* repeat expansion. C9orf72exp, group carrying the *C9orf72* expansion. IPD meta-analysis of *C9orf72* repeat expansion in male spinal onset patients with ALS versus spinal onset *C9orf72* normal patients pooled by country and analysed using a Royston-Parmar flexible parametric model with three degrees of freedom on the hazard scale correcting for age at onset (time varying), site of onset and diagnostic delay, based on the three level categorical breakdown of sex, site and *C9orf72* status described in Supplement information 2. IPD, individual patient data.

Table 2 Age of onset and diagnostic delay for hybrid sex/site of onset/C9 variable

Level	Age at onset Median (IQR)	Diagnostic delay Short	Medium	Long
Spinal onset excluding C9orf72 expanded males	62.3 (54.0 to 69.7)	965 (30.8%)	1014 (32.4%)	1151 (36.8%)
All bulbar onset	65.0 (58.4 to 71.9)	611 (37.4%)	590 (36.1%)	435 (26.6%)
Male spinal onset C9orf72 only	59.3 (52.3 to 64.7)	65 (40.8%)	44 (27.7%)	50 (31.5%)
p Value	0.0001*	<0.001†		

*Kruskal-Wallis test.
†χ^2 test.

Table 3 HRs for El-Escorial criteria after inclusion in final model

El-Escorial category	HR	95% CI	Wald test
Suspected	0.86	0.68 to 1.08	0.199
Possible	1	–	–
Probable—laboratory supported	1.38	1.31 to 1.44	<0.001
Probable	1.49	1.39 to 1.60	<0.001
Definite	2.09	1.99 to 2.21	<0.001
Sex/site of onset/C9orf72			
Spinal onset excluding male C9orf72 expanded cases	1	–	–
All bulbar onset cases	1.33	1.21 to 1.45	<0.001
Male C9orf72 expanded cases only	1.57	1.26 to 1.96	<0.001

HRs for each of the El-Escorial criteria from a Royston-Parmar model on the hazard scale with 3 degrees of freedom and including age of onset (time varying), diagnostic delay, site of onset, country and sex-site-C9 as a hybridised variable with a variance–covariance matrix allowed to pool by country. Note that Belgium omitted as the El-Escorial criteria was not available, the UK data included only Probable and Definite category ALS, while Ireland does not use the 'probable laboratory supported'. Nevertheless, HRs are in line with expectations showing a gradually increasing hazard with increasing category severity.

forms of FTD, inclusion of cognitive status as a variable would have introduced a selection bias based on 'conditioning on a common effect'.[31] A further limitation to this study is that our analysis does not include *C9orf72* repeat expansion analysis by Southern blot, although individual definition of pathological expansion performed by each centre using repeat primed PCR was validated by Southern blot. While the length of expansion varies from tissue to tissue,[32–34] diagnostic testing within a clinical setting uses blood samples from which all previous prognostic and clinical correlative studies have been performed. Finally, we did not account for riluzole use in the analysis, as this is routinely prescribed in all patients from the participating countries, and it was not possible to determine the level of compliance using available data.

In conclusion, we have performed an analysis of the effect of the *C9orf72* expansion on survival in almost 5000 European

Table 4 Summary of previous analyses of survival by *C9orf72* status

Study	Population	C9orf72 normal	C9orf72 expanded	Median survival δ* (months)	Median age at onset δ* (years)	HR 95% (CI)
Byrne *et al*[4]	Ireland	170	21	−6	−3.2	1.9 (1.1 to 3.7)
Van Rheenen *et al*[5]	The Netherlands	1422	78	−2.5	−2.6	1.46 (1.17 to 1.83)
Sabatelli *et al*[6]	Italy and Sardinia	1688	69	−12	−3.8	1.79 (1.26 to 2.98)
Borghero *et al*[7]	Sardinia	375	51	−18†	−0.9	NA
Debray *et al*[8]	Belgium	513	77	fALS −38.3 sALS −5.8	fALS −5.9 sALS −0.3	fALS 2.5 (1.5 to 4.3) sALS 1.1 (0.8 to 1.5)
García-Redondo *et al*[9]	Spanish	936	67	−12	−2.6‡	NA
Irwin *et al*[10]§	USA (Pennsylvania)	69	64	−6‡	−3.0‡	NA

*Negative figures imply C9orf72 expanded survive for shorter time, or are younger at onset than C9orf72 normal cases.
†Calculate as median survival in C9orf72 expanded group—median survival in overall cohort median.
‡Calculated from mean data instead of median.
§Mixed ALS and FTD cases.
fALS, familial amyotrophic lateral sclerosis; sALS, sporadic amyotrophic lateral sclerosis.

patients with ALS. We have shown, for the first time, that *C9orf72* repeat expansion is a significant negative prognostic indicator in males with spinal onset disease only. These findings suggest a hitherto unrecognised interaction between the *C9orf* repeat expansion, site of onset and gender. This has important implications in the understanding both the pathobiology of *C9orf72*-mediated disease, and in the development of future disease-related prognostic models.

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Contributors JR had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. JR, IF and OH were involved in study concept and design. H-JW, AV, RML, MH, AJ, RvE, AC, LM, CS, KM, PJS, WR, PVD, AA-C; LvdB; AC; JV and OH were involved in acquisition of data. JR; H-JW and JV contributed to statistical analysis. JR and OH contributed to drafting of the manuscript. All authors were involved in critical revision of the manuscript for important intellectual content.

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Competing interests AA-C is chief investigator of a clinical trial by OrionPharma, served on the scientific advisory board of the Motor Neurone Disease Association, is on the Editorial Board of the journals 'F1000' and 'Amyotrophic Lateral Sclerosis and Frontotemporal Dementia', has consulted for GSK, OrionPharma, Biogen Inc, Cytokinetics, and Treeway, and receives royalties from the books 'The Brain' (Oneworld Publications) and 'The Genetics of Complex Human Diseases" (Cold Spring Harbor Laboratory Press). PJS is Principal Investigator of a clinical trial by OrionPharma, served on the scientific advisory board of the UK Medical Research Council, is on the Editorial Board of the 'Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Journal', has consulted for, OrionPharma, Biogen, Cytokinetics, Treeway and Sanofi Aventis and receives royalties from the books 'Oxford Textbook of Neurology. She has grants from the Motor Neurone Disease Association, National Institute for Health Research, European Commission, Medical Research Council and ALS Worldwide. AC serves on the editorial advisory board of the journal 'Amyotrophic Lateral Sclerosis and Frontotemporal Dementia' and has been a member of advisory panels Biogen Idec, Cytokinetics, Italfarmaco and Neuraltus. OH is funded by the Health Research Board Clinician Scientist Programme. Professor Hardiman has received speaking honoraria from Novarits, Biogen Idec, Sanofi Aventis and Merck-Serono. She has been a member of advisory panels for Biogen Idec, Allergen, Ono pharmaceuticals, Novartis, Cytokinetics and Sanofi Aventis. She serves as Editor-in-Chief of the journal 'Amyotrophic Lateral

Sclerosis and Frontotemporal Dementia'. LvB received travel grants and consultancy fees from Baxalta; serves on scientific advisory boards for Prinses Beatrix Spierfonds, Thierry Latran Foundation, Cytokinetics and Biogen. He serves on the editorial boards of 'Amyotrophic Lateral Sclerosis and Frontotemporal Dementia' and '*Journal of Neurology, Neurosurgery and Psychiatry*'.

Patient consent Obtained.

Ethics approval Ethical approval for this study and data sharing was obtained at each participating centre, and data shared through a legal agreement under the auspices of the EU JPND STRENGTH consortium, administered through King's College London. For the purposes of this analysis, overarching approval was through the Irish Centre (Trinity College Dublin and Beaumont Hospital Research Ethics Committee (02/28; 05/49).

Provenance and peer review Not commissioned; externally peer reviewed.

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