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C9orf72 expansion differentially affects males with spinal onset

Amyotrophic Lateral Sclerosis

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Ethics Statement:

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.

Abstract

Introduction:

The *C9orf72* repeat expansion has been reported as a negative prognostic factor in amyotrophic lateral sclerosis (ALS). Here 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 ALS patients drawn from three prospective ALS registers (Ireland, Italy and The Netherlands), and clinical datasets 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 hazard ratios 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 hazard ratio (HR) of 1.36 (1.18 – 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 – 1.96)).

Conclusions:

This study represents the largest combined analysis of the prognostic characteristics of the *C9orf72* expansion. We have shown for this first time that the negative prognostic implication of this variant is driven by males with spinal onset disease, indicating a hitherto unrecognized 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 pathogenesis¹. 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 fronto-temporal dementia in some instances⁴⁻¹⁰. Although the *C9orf72* repeat expansion has been shown to be an important negative prognostic factor in survival analyses⁴⁻¹⁰, to date 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 datasets 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, The Netherlands and the United Kingdom. All patients fulfilled the diagnostic criteria for ALS, and core data elements as defined by the ENCALS consortium were harmonised across data-sets for consistency based on existing consortia agreements¹¹. Due to the lack of an agreed international definition of 'familial ALS', and given a previous population based familial aggregation analysis from Ireland demonstrated a much higher familial ALS occurrence (16%) than usually recognised¹², 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 registers¹³⁻¹⁹. 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.

.A base model using Royston-Parmar flexible parametric regression²⁰ was built on the preferred timescale, with a proportional hazards scale and a number of degrees of freedom selected by comparison of the AIC (Akaike Information Criterion) and BIC (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 14.0 were used to perform the survival analysis and produce the meta-analysis graphs, whilst the *ggplot2*²³ package in R 3.1.1 was used to generate selected final graphs.

Ethics

Ethical approval for this study and data sharing was obtained at each participating centre, and data **was** shared through a legal agreement under the auspices of the EU JPND STRENGTH consortium, administered through King's College London **and approved for combined analyses locally by each participating institution. 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).**

Results

Descriptive statistics and basic survival model

In total, 5,106 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%). Supplementary information 1 summarizes the basic survival model. An individual patient data (IPD) meta-analysis of *C9orf72* status in the base model estimated a hazard ratio of 1.36 (1.18 – 1.57) for those carrying the expansion vs 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 (Fig 1) which suggested three-way interaction. Therefore gender, site of onset and *C9orf72* status were categorised into one variable with eight levels as demonstrated in Table 1. Through comparison of survival curves, redundant sub-groups were combined (Supplementary Information 2) leaving 3 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 (Fig 2). Meta-analysis calculated a survival hazard ratio of 1.56 (95% CI: 1.25 – 1.96) for this group (figure 3). Adjustment for El Escorial category did not substantially alter the hazard ratio (1.57 CI:1.26 – 1.97). 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 sub-groups 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.

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 to date 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 unrecognized 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 yrs 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.

Gender has previously been reported as an independent predictor of functional decline in ALS²⁴, however our observation of an interaction between site of onset and gender has not been previously noted possibly due to 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 haplo-insufficiency, toxic RNA interfering with the function of RNA-binding proteins or other cellular factors, and the presence of toxic dipeptide repeat proteins through RAN translation^{25,26}. Recent work has pointed also towards *C9orf72* induced pathology of nucleocytoplasmic transport processes²⁶⁻²⁹. 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 numbers³⁰. The mechanism for this gender effect in animals, although well recognized has not been characterized, but can be attenuated when mice are bred on a different genetic background³⁰.

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 our knowledge to date, no gender mediated effect has been demonstrated in *C9orf72* related cognitive profiling. Moreover, as *C9orf72* is part of the causal pathway for some 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 tissue³²⁻³⁴, diagnostic testing within a clinical setting uses blood samples from which all previous prognostic and clinical correlative studies have been performed.

In conclusion, we have performed an analysis of the effect of the *C9orf72* expansion on survival in almost 5000 European ALS patients. 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 unrecognized interaction between the C9orf repeat expansion, site of onset and gender. This has important implications in understanding both the pathobiology of C9orf-mediated disease, and in the development of future disease-related prognostic models.

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Author contributions:

James Rooney 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.

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Drafting of the manuscript: James Rooney, Orla Hardiman

Critical revision of the manuscript for important intellectual content: All authors.

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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	United Kingdom n= 929	P (Chi 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%)	<0.001	2053 (41.7%)
Male	297 (62.3)	374 (58.4%)	456 (52.6%)	1193 (58.6%)	552 (61.1%)		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%)	0.559	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%)		1636 (33.2%)
Site of Onset							
Spinal	326 (68.3%)	437 (68.3%)	583 (67.2%)	1333 (65.4%)	610 (67.5%)	0.540	3289 (66.8%)
Bulbar	151 (31.7%)	203 (31.7%)	284 (32.8%)	704 (34.6%)	294 (32.5%)		1636 (33.2%)
C9orf72							
Normal	392 (82.2%)	578 (90.3%)	805 (92.8%)	1861 (91.4%)	841 (93.0%)	<0.001	4477 (90.9%)
Expanded	85 (17.8%)	62 (9.7%)	62 (7.2%)	176 (8.6%)	63 (7%)		448 (9.1%)

Table 1 Legend:

Diagnostic delay is defined by three tertiles per country labeled “short”, “medium” and “long” diagnostic delay to allow for variation in diagnostic delay between countries.

[¶] Kruskal-Wallis test

IQR = inter-quartile range

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

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

[¶] Kwallis test

[†] Chi² test

Table 3 – Hazard ratios for El Escorial criteria after inclusion in final model

El Escorial Category	HR	95% CI	Wald test
Suspected	0.86	0.68 – 1.08	0.199
Possible	1	-	-
Probable – Lab supported	1.38	1.31 – 1.44	<0.001
Probable	1.49	1.39 – 1.60	<0.001
Definite	2.09	1.99 – 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 – 1.45	<0.001
Male C9orf72 expanded cases only	1.57	1.26 – 1.96	<0.001

Table 3 Legend:

Hazard ratios 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 hybridized variable with variance-covariance matrix allowed to pool by country. Note that Belgium was omitted as the El-Escorial criteria was not available, the UK data included only Probable and Definite category ALS, whilst Ireland does not use the “Probable lab supported” category as shown in table 1. Nevertheless, hazard ratios are in line with expectations showing a gradually increasing hazard with increasing category severity.

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

Study	Population	<i>C9orf72</i>		Median survival delta [¶]	Median age at onset delta [¶]	HR (CI)
		Normal	Expanded			
Byrne et al ⁴	Ireland	170	21	-6 months	-3.2 yrs	1.9 (1.1 – 3.7)
Van Rheenen et al ⁵	The Netherlands	1422	78	-2.5 months	-2.6 yrs	1.46 (1.17 – 1.83)
Sabatelli et al ⁶	Italy & Sardinia	1688	69	-1 yr	-3.8 yrs	1.79 (1.26 – 2.98)
Borghero et al ⁷	Sardinia	375	51	-1.5 yrs ^{¶¶}	-0.9 yrs	na
Debray et al ⁸	Belgium	513	77	fALS -38.3 months sALS -5.8 months	fALS -5.9 yrs sALS -0.3 yrs	fALS 2.5 (1.5 – 4.3) sALS 1.1 (0.8 – 1.5)
García-Redondo et al ⁹	Spanish	936	67	-12 months	-2.6 yrs ^{¶¶¶}	na
Irwin et al ¹⁰ ¶¶¶¶	United States (Pennsylvania)	69	64	-6 months ^{¶¶¶}	-3.0 yrs ^{¶¶¶}	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 & FTD cases

fALS = familial ALS ; sALS = sporadic ALS

Figure 1 – Predicted survival by sex and *C9orf72* status after multivariate Royston-Parmar regression including interaction terms

Figure 1 Legend: C9 Normal = group not carrying the *C9orf72* expansion; C9 Exp. = group carrying the *C9orf72* expansion present. Shaded areas represent 95% confidence intervals.

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 (3 d.f.), 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 shows a wider spread by *C9orf72* status in males compared to females, while the lower shows a wider spread in spinal onset cases vs bulbar onset cases – however hazard ratios for these interactions were not significant.

Figure 2 - Predicted survival function for male spinal onset *C9orf72* expanded patients

Figure 2 legend: C9 exp. = group carrying the *C9orf72* expansion. Shaded areas represent 95% confidence intervals.

Survival curves show three subgroups of patients categorized 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 (95%CI 2.67 – 2.87); bulbar onset 2.38 (95% CI 2.33 – 2.42); spinal onset males with *C9orf72* repeat expansion 2.29 (95%CI 2.15 – 2.49).

Figure 3 - Individual Patient Data meta-analysis of the hazard ratio of male spinal onset ALS patients carrying the *C9orf72* repeat expansion

Figure 3 legend: C9orf72exp. = group carrying the C9orf72 expansion.

IPD meta-analysis of *C9orf72* repeat expansion in male spinal onset ALS patients versus spinal onset *C9orf72* normal patients pooled by country and analysed using a Royston-Parmar flexible parametric model with 3 degrees of freedom on the hazard scale correcting for age at onset (time varying), site of onset and diagnostic delay, based on the 3 level categorical breakdown of sex, site, and *C9orf72* status described in Suppl. Info. 2.