Genetic epidemiology of motor neuron disease-associated variants in the Scottish population

Holly A. Black a,1, Danielle J. Leighton b,c,1, Elaine M. Cleary b,c,d, Elaine Rose a, Laura Stephenson b,c, Shuna Colville b,c, David Ross a, Jon Warner d, Mary Porteous d, George H. Gorrie b, Robert Swingler b, David Goldstein e, Matthew B. Harms e, Peter Connick c, Suvankar Pal b,c, Timothy J. Aitman a,* Siddharthan Chandran b,c,**

1. Introduction

Motor neuron disease (MND) is a rapidly progressive and fatal neurodegenerative disorder, characterized by loss of motor neuron function. Presentations can include limb onset, bulbar onset, or cognitive/behavioural disease, reflecting variable involvement of the upper motor neurons, lower motor neurons and frontotemporal cortex (Turner and Swash, 2015). Age and site of onset, rate of disease progression and clinical syndrome vary considerably between cases, presenting difficulties for diagnosis and disease management.

Phenotype data from Scottish MND cases are available through the Scottish Motor Neuron Disease Register (SMNDR), a prospective population-based record of all cases diagnosed with MND, which has been operational since 1989 (The Scottish Motor Neurone Disease Research Group, 1992). In Scotland, there is a predicted annual crude incidence of MND of 2.38 per 100,000 of the population (Forbes et al., 2007). Mean survival from symptom onset to death is 2.8 years (Forbes et al., 2007), albeit with a variable trajectory depending on the clinical syndrome.

Substantial progress in understanding the genetic landscape of MND has occurred over the last 10 years, including association with variants in NEK1 and TBK1 (Abel, 2016; Abel et al., 2013). In recent series, pathogenic variants in known genes contribute to the burden of MND in the Scottish population. SOD1, TARDBP, OPTN, TBK1, and NEK1 were sequenced in 441 cases and 400 controls. In addition to 44 cases known to carry a C9orf72 hexanucleotide repeat expansion, we identified 31 cases and 2 controls that carried a loss-of-function or pathogenic variant. Loss-of-function variants were found in TBK1 in 3 cases and no controls and, separately, in NEK1 in 3 cases and no controls. This study provides an accurate description of the genetic epidemiology of MND in Scotland and provides support for the contribution of both TBK1 and NEK1 to MND susceptibility in the Scottish population.

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variants specifically in the Scottish population is unknown, screening of an unselected Scottish cohort for variants in SOD1 identified a high frequency of the 1114T variant, which was found in 9% cases (Jones et al., 1995), and more recently C9orf72 expansions were found in 11% of Scottish cases (Cleary et al., 2016). Two genes, TBK1 and NEK1, have recently been associated with MND, with each thought to account for a small proportion of cases, although they have not been screened in the Scottish MND population (Brenner et al., 2016; Cirilli et al., 2015; Freischmidt et al., 2015; Kenna et al., 2016).

In families where affected individuals carry the same MND-associated variant, for example SOD1 1114T, the phenotype can be extremely variable (Lopate et al., 2010). This phenotypic heterogeneity, along with previous reports of pathogenic variants in more than one MND gene in the same affected individual (oligogenic cases; Bury et al., 2016; Cady et al., 2015; Chio et al., 2012b; Kenna et al., 2013; Lattante et al., 2012; van Blitterswijk et al., 2012), has led to a hypothesis that disease risk and subsequent phenotype is determined by a combination of genetic factors and modifiers, rather than a single genetic variant, in combination with environmental triggers, as described in the multistep hypothesis of MND (Al-Chalabi et al., 2014).

In this study, we report a multi-gene screen of MND cases and controls from the Scottish population. Our aim was to determine the contribution of variants in different genes to cases of MND in Scotland, to investigate whether variants in TBK1 and NEK1 contribute to the burden of cases and to assess the association of variants in MND genes with disease phenotype.

2. Materials and methods

2.1. MND case recruitment and control samples

MND cases were recruited through the SMNDR. The registration process achieved high ascertainment coverage (98% in 1989–98; Forbes et al., 2007), providing a cohort representative of the national MND population. Details of methodology of recruitment have been reported previously (The Scottish Motor Neuron Disease Research Group, 1992); latterly the El Escorial classification system was adopted (Brooks, 1994; Brooks et al., 2000). Recruited cases included individuals aged ≥16 with probable or definite amyotrophic lateral sclerosis and individuals with MND subtypes (progressive bulbar palsy, progressive muscular atrophy and primary lateral sclerosis). Individuals provided written consent for DNA extraction and genetic studies.

Four hundred forty-one samples, obtained from cases diagnosed with MND in Scotland in the years 1989–2014, were included in this study, which included 3 pairs of related individuals (2 brothers, 2 first cousins, and 2 first cousins once removed). Case records were examined for 7 phenotypic characteristics: sex, age at onset, age at diagnosis, diagnosis to diagnosis, duration of disease (until death or final data review [20th April 2016]), site of onset (bulbar or spinal), and family history of MND. Individuals were classified as having a family history of MND if a first, second, or third degree relative had been known to have MND, as for cases. The study did not include the presence of frontotemporal dementia (FTD) alone as a criterion for positive family history. Five individuals were lost to follow-up due to relocation from Scotland, and survival dates were censored to date of last contact. The cohort was screened for expansions of the C9orf72 intronic hexanucleotide repeat, as described by Cleary et al. (2016). A subset of the cohort had been screened for variants in SOD1 in several previous studies (Hayward et al., 1998; Jones et al., 1995; Swingler et al., 1995).

Five MND cases, each with a variant in 1 of the 5 genes sequenced, were included as positive controls; these were taken from the cohort described by Cirilli et al. (2015). Four hundred ethnicity and sex-matched healthy controls were selected from the Generation Scotland Donor DNA databank (Kerr et al., 2010). The selected controls were aged ≥56 at the time of collection (20% aged 50–56, 66% aged 60–65, 14% aged 66+); an older cohort was chosen to minimize inclusion of young subjects who could go onto develop MND later in life.

2.2. Targeted amplification and sequencing

Five genes were sequenced: SOD1, TARDBP, OPTN, TBK1, and NEK1; these will be referred to hereafter as the MND gene panel. The panel includes the recently associated TBK1 and NEK1, alongside genes that are among the largest contributors to cases in UK and European populations (SOD1, TARDBP, and OPTN), after C9orf72 (Abel, 2016; Abel et al., 2013; Renton et al., 2014).

Primers for 120 amplicons (Supplementary Table 1) were designed according to the Fluidigm Access Array protocol (Fluidigm). The primers amplified the coding regions of these 5 genes (Supplementary Table 2), excluding a total of 312bp of coding sequence across the 5 genes, due to primer design constraints. The Fluidigm Access Array was used for amplification, following the manufacturer’s multiplex amplicon tagging protocol, with 2 modifications (Martyna Adamowicz-Brice, personal communication); an additional 1:1 AMPure XP (Agencourt) cleanup step was introduced prior to harvest and the subsequent dilution step was removed. The amplicon library was sequenced on an Illumina MiSeq with 2 × 150-bp reads. Each batch of 48 samples included at least one water blank as a negative control. The positive control samples were each run in 2 independent batches.

2.3. Read mapping, sample filtering, variant calling, and annotation

Quality control of the amplicon sequencing data was performed using FastQC (version 0.11.2) (Andrews, 2010). Primer sequences were removed from the 5’ ends of reads using the cutadapt tool (version 1.7.1) with the ‘anchor’ option (Martin, 2011). The maximum error rate for primer sequences was set to 10%. Reads were mapped to the human genome reference sequence hs37d5 using BWA MEM (version 0.7.10; Li, 2013). Picard (version 1.85; Broad Institute), and DepthOfCoverage GATK tool (version 3.3–0; McKenna et al., 2010) were used to collect alignment and amplicon coverage statistics. The median amplicon coverage of the negative control sample was used to determine the threshold for including samples from the same batch in further analysis. Only samples with a median coverage that was >10 × that of the negative control were included. This removed 7 cases and 11 controls, leaving 434 cases (432 independent) and 389 controls for further analysis. The UniﬁedGenotyper tool, as implemented in GATK version 2.6 (McKenna et al., 2010), was used for variant calling. ANNOVAR (version 2014 Nov 12) was used to provide functional annotation of the variants (Wang et al., 2010). Variants were annotated with reference to the transcripts listed in Supplementary Table 2.

2.4. Variant filtering and validation

Intronic and synonymous variants, variants with a population frequency greater than 1% in either the 1000 Genomes (October 2014 release; 1000 Genomes Project Consortium et al., 2015) or ExAC v0.2 (Lek et al., 2016) data sets and variants with a frequency >5% in our cohort were excluded from further analysis. Per-sample variant calls were filtered to exclude calls with a read depth <50 or an allele balance <0.3. Filtered variants were validated by Sanger-sequencing. One sample failed to amplify using PCR at variant validation stage and was excluded from further analysis, leaving...
433 (431 independent) cases. Nine false positives were identified in SOD1 exon 1, and this exon was excluded from the analysis. Following exclusion of this exon, 5 false positives remained, which were excluded from downstream analysis. Validated variants were submitted to Clinvar. The known variants in the positive controls were identified in both assays in which they were tested.

2.5. Assessing variant pathogenicity

Stop-gain, frameshift, and splice site variants were categorized according to their predicted effect on protein function. Missense variants reported as disease-causing in association with MND in the Human Gene Mutation Database (Stenson et al., 2008) were categorized as pathogenic. The remaining missense variants were categorized according to in silico scores of pathogenicity and conservation. The scores used are listed below, with the thresholds for supporting pathogenicity/conservation in brackets: SIFT (D), PolyPhen HDIV (P/D), LRT (D), Mutation Taster (D), Mutation Assessor (M/H), PATHMM (D), CADD phred (25), GERP (2), phyloP (20), and SiPhy (10). Variants for which 7–10 in silico measures supported pathogenicity/conservation were categorized as likely pathogenic, variants with 4–6 measures supporting pathogenicity/conservation were categorized as uncertain significance, and variants with 0–3 measures supporting pathogenicity/conservation were categorized as likely benign (Supplementary Table 3).

2.6. ExAC reference exomes

The ExAC v0.3.1 reference exome data (Exome Aggregation Consortium (ExAC); Lek et al., 2016) were used to provide an additional, larger control population. It contains exome-wide variants identified in 60,706 individuals. For both TBK1 and NEK1, the total number of loss-of-function or loss-of-function + missense alleles in ExAC were recorded (including filtered [non-pass] variants), excluding variants found in >1% individuals. The number of ExAC individuals was reduced to reflect the average percentage of individuals covered at 30× across each gene. For TBK1, an average of ~69% of individuals was covered at 30×, giving 41,848 individuals assumed to have sufficient depth for variant calling. For NEK1, it was ~50%, giving 30,298 individuals.

2.7. Statistical analysis

Fisher’s exact tests were used in comparisons between the number of variants in cases and controls (Sections 3.2, 3.4 and 3.5). One-tailed tests were used because of the prior assumption that cases contain more pathogenic variants than controls. Genotype-phenotype association testing was used to compare clinical cases with different MND genotypes (Section 3.7). Only univariate association testing was used to compare clinical phenotypes with different MND genotypes (Section 3.7). Only significant univariate association at $p \leq 0.1$ were inputted into binomial logistic regression models dependent on gene. Logistic regression analysis was used to test the hypothesis that significant variables were independently associated with having a pathogenic or loss-of-function variant in one of the genes tested. For the purposes of the model, age of onset was grouped by decade. Results from logistic regression modeling were considered significant if $p < 0.05$ (Section 3.7). SPSS Statistics version 21 was used for all statistical analysis.

3. Results

3.1. MND case phenotypes

After sample filtering, 433 MND cases (431 independent) remained, of which complete phenotypic data were obtained for 428 (99%; Table 1). Site of onset was unrecorded for 1 case. Family history was unrecorded for 3 cases and was unknown for 1 case, who was adopted. The cohort had a male to female ratio of 1.4:1 (Table 1). The mean age of onset was 59.5 years. Of the 443 cases in this cohort, 367 (85%) had died before censorship date; median duration of disease from onset for all the cases was 42 months. Of the 430 independent cases for which site of onset was recorded, 304 (71%) had spinal onset MND. Of the 425 cases with recorded family history, 44 (10%) had a family history of MND.

3.2. Identification of variants in MND genes

Targeted sequencing with the MND gene panel achieved a mean coverage of 6280× per amplicon per sample. Following variant filtering and validation (Section 2.4), at least one rare stop-gain, splice site, frameshift, or missense variant was identified in 57/433 cases and 19/389 controls (Supplementary Tables 4 and 5). Following sample filtering (Section 2.3), the case population contained 2 pairs of related individuals. The first pair (brothers) both carried the SOD1 G94R variant, whereas the second pair (first cousins) did not carry any variants in the MND gene panel that passed variant filtering. Therefore, the results for the related individuals were concordant. Thirty-seven unique variants were identified in either cases or controls across the MND gene panel; of these, 3 were stop-gain, 3 were splice site, 28 were missense variants (Supplementary Table 4). All stop-gain, splice site, and frameshift variants were categorized as loss-of-function, except for 3 variants. A stop-gain variant in TARDBP was categorized as uncertain.

<table>
<thead>
<tr>
<th>Phenotypic characteristic</th>
<th>Summary statistic</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female (%)</td>
<td>177/433 (41)</td>
</tr>
<tr>
<td>Age of onset (y)</td>
<td>Mean (SD)</td>
<td>59.5 (12.9)</td>
</tr>
<tr>
<td>Age of diagnosis (y)</td>
<td>Mean (SD)</td>
<td>61.1 (12.6)</td>
</tr>
<tr>
<td>Time to diagnosis (mo)</td>
<td>Median (IQR)</td>
<td>12 (6, 22)</td>
</tr>
<tr>
<td>Duration of disease from onset (mo)</td>
<td>Median (IQR)</td>
<td>42 (25, 73.5)</td>
</tr>
<tr>
<td>Site of onset</td>
<td>Bulbar (%)</td>
<td>268/432 (62)</td>
</tr>
<tr>
<td>Family history of MND</td>
<td>Yes (%)</td>
<td>44/429 (10)</td>
</tr>
</tbody>
</table>

Duration of disease: survival until (i) death (n = 367), (ii) data censurship at 20th April 2016 (n = 61), or (iii) date of last contact (n = 5). Includes 2 pairs of related individuals. Key: SD, standard deviation.
significance, as it is in the final exon of the gene and therefore not expected to result in nonsense-mediated decay. There were also 2 splice site variants in NEK1 predicted to result in the in-frame loss of a single exon (Brunak et al., 1991), which were categorized as uncertain significance. Therefore, of the 37 unique variants identified, 6 were categorized as loss-of-function, 7 pathogenic, 11 likely pathogenic, 8 uncertain significance, and 5 likely benign (Supplementary Table 4).

In total, 31 MND cases and 2 controls carried at least one loss-of-function or pathogenic variant across the MND gene panel (Table 2 and Supplementary Tables 4 and 5; Fisher’s $p = 1.761 \times 10^{-7}$). Taken together with prior findings that 44 of the 431 independent cases contain a pathogenic intronic hexanucleotide repeat expansion in C9orf72 ($>100$ repeats; Cleary et al., 2016), 74 independent cases (17%) carried at least one pathogenic or loss-of-function variant (Table 2 and Supplementary Table 5). This represented 26/42 (62%) of cases with a family history and 47/385 (12%) of cases with no family history (Fig. 1).

### 3.3. Variants in SOD1, OPTN, and TARDBP

After C9orf72, the largest genetic contributor to cases in our study was SOD1, with pathogenic or likely pathogenic variants observed in 22/431 independent MND cases (5%) and 1/389 controls (0.3%; Table 2 and Supplementary Tables 4 and 5). The I114T Scottish founder mutation was observed in 18 cases (4%) and 1 control (0.3%). Of all cases, 29% of familial and 2% of sporadic carry a pathogenic variant in SOD1.

Pathogenic variants in TARDBP were observed in 4/431 (1%) cases and no controls. This is in addition to a stop-gain variant of uncertain significance, observed in one case. For OPTN, at least one pathogenic or likely pathogenic variant was observed in 3/431 (1%) cases and 1/389 controls (0.3%). The single pathogenic variant in OPTN, Q314L, was observed in 1 case and 1 control. There is no functional evidence to support the association of this variant with MND and, although in previous studies in silico evidence has suggested a pathogenic role for the variant in MND (Del Bo et al., 2011), the occurrence of the variant in 1 case and 1 control in our data suggests that the variant either has variable penetrance or is not pathogenic for MND. OPTN therefore appears to contribute to very few cases of MND in the Scottish population.

### 3.4. Assessing the association of variants in TBK1 with MND

Several previous studies have reported dominant variants in TBK1 in cases of MND, FTD, and MND-FTD, accounting for

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**Table 2**

Variants identified across the MND gene panel, with their pathogenicity classification and frequency in cases and controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Loss-of-function</th>
<th>Pathogenic</th>
<th>Likely pathogenic</th>
<th>Uncertain significance</th>
<th>Likely benign</th>
<th>Totala</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
</tr>
<tr>
<td>SOD1</td>
<td>0</td>
<td>0</td>
<td>22$^a$</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>TARDBP</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OPTN</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TBK1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>NEK1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Includes 1 pair of brothers.

The total includes 1 case with a pathogenic variant in OPTN and a loss-of-function variant in TBK1; a case with a pathogenic variant in TARDBP and a variant of uncertain significance in NEK1; and a control with a pathogenic and likely pathogenic variant in OPTN.

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**Fig. 1.** Proportion of cases with a pathogenic or loss-of-function variant in genes on the MND gene panel or in C9orf72. (A): cases with a family history; (B): sporadic cases with no known family history. Each pie chart includes one digenic case that is counted for both of the genes in which a pathogenic or loss-of-function variant is found.
approximately 1% of all cases (Borghero et al., 2016; Cirulli et al., 2015; Exome Aggregation Consortium (ExAC); Freischmidt et al., 2015; Gijselink et al., 2015; Le Ber et al., 2015; Pottier et al., 2015; Shu et al., 2016; Tsai et al., 2016; van Rheenen et al., 2016; Williams et al., 2015). In our study, we observed 6 cases and 1 control with a rare stop-gain, splice site frameshift, or missense variant in TBK1 (Section 2.4; Table 2 and Supplementary Table 5; Fisher’s $p = 0.080$). When compared with the ExAC reference data set (Exome Aggregation Consortium (ExAC); Lek et al., 2016), this result is not significant (Fisher’s $p = 0.238$). When looking only at loss-of-function variants, 3 cases and no controls contained a loss-of-function variant in TBK1 (Fisher’s $p = 0.145$). When compared with the ExAC reference data set, where the frequency of loss-of-function variants was 0.03%, this represents a statistically significant excess (Fisher’s $p = 4.370 \times 10^{-4}$; Exome Aggregation Consortium (ExAC); Lek et al., 2016).

3.5. Assessing the association of variants in NEK1 with MND

Loss-of-function variants in NEK1 have been associated with MND in two recent studies of both familial and sporadic MND cases (Brenner et al., 2016; Kenna et al., 2016), following an earlier study of mostly sporadic cases that highlighted NEK1 as a candidate gene (Cirulli et al., 2015; Exome Aggregation Consortium (ExAC)). These 3 studies estimate that loss-of-function variants in NEK1 contribute to approximately 1% of cases. In this study, we observe 3 cases and no controls with a variant predicted to result in loss-of-function of NEK1 (Table 2 and Supplementary Table 5; Fisher’s $p = 0.145$). When compared with the ExAC reference data set, where the frequency of loss-of-function variants was 0.3%, our result remains nonsignificant (Fisher’s $p = 0.143$; Exome Aggregation Consortium (ExAC); Lek et al., 2016). Regarding missense variants in NEK1 (Section 2.4; Table 2 and Supplementary Table 5), we observe 16 cases and 16 controls with an NEK1 missense variant, which is not statistically significant (Fisher’s $p = 0.453$).

3.6. Cases carrying 2 variants in MND-associated genes

There are several reports of MND cases that carry MND-associated variants in more than one gene (Bury et al., 2016; Cady et al., 2015; Chio et al., 2012b; Kenna et al., 2013; Lattante et al., 2012; van Blitterswijk et al., 2012). We observed 5 cases and 1 control carrying 2 different rare stop-gain, frameshift, splice site, or missense variants (Section 2.4) either across the MND gene panel or in C9orf72 (Table 3). Two cases, MND-0040 and MND-0434, and no controls carry 2 variants that are predicted to be either pathogenic or loss-of-function; these will be referred to as digenic cases (Table 3). Both digenic cases have an age of onset within 2 standard deviations of the mean and a typical disease duration (Table 3). However, as discussed in Section 3.3, the pathogenicity of the variant Q314L in OPTN in MND-0040 is unclear.

Of the remaining cases with two rare variants in MND-associated genes, case MND-0119 carries 1 pathogenic variant and 1 variant of uncertain significance and has a typical age of onset and disease duration. Case MND-0158, who carries 1 pathogenic and 1 likely pathogenic variant, had a young age of onset (26 years, >2.5 standard deviations from the mean) and long disease duration (142 months, above 90th percentile). MND-0211 also carries 1 pathogenic and 1 likely pathogenic variant and had a typical age of onset, but disease duration at the lower end of the spectrum (16 months, equal to the 10th percentile). Interestingly, 3 of the 5 cases (60%) carrying 2 rare stop-gain, splice site, frameshift, or missense variants in MND genes (including C9orf72 expansions) had a bulbar site of onset, which is higher than the 29% observed across all MND cases in the cohort.

### Table 3: Samples containing 2 variants that potentially contribute to MND pathogenicity

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Phenotype information</th>
<th>Variant 1</th>
<th>Variant 2</th>
<th>Gene</th>
<th>Pathogenicity</th>
<th>Sample type</th>
<th>Family history</th>
<th>Site of onset (mo)</th>
<th>Duration of disease from onset (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MND-0040</td>
<td>Case 46</td>
<td>c.A941T;</td>
<td>c.G1021A;</td>
<td>TARDBP</td>
<td>Pathogenic</td>
<td>Bulbar</td>
<td>Yes</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>MND-0434</td>
<td>Case 44</td>
<td>c.A941T;</td>
<td>c.G1021A;</td>
<td>C9orf72</td>
<td>Pathogenic</td>
<td>Limb</td>
<td>No</td>
<td>34</td>
<td>44</td>
</tr>
<tr>
<td>MND-0158</td>
<td>Case 26</td>
<td>c.G859A;</td>
<td>c.G1021A;</td>
<td>TARDBP</td>
<td>Pathogenic</td>
<td>Limb</td>
<td>No</td>
<td>142</td>
<td>26</td>
</tr>
<tr>
<td>MND-0211</td>
<td>Case 65</td>
<td>c.A941T;</td>
<td>c.G1021A;</td>
<td>OPTN</td>
<td>Pathogenic</td>
<td>Bulbar</td>
<td>No</td>
<td>16</td>
<td>65</td>
</tr>
<tr>
<td>MND-0119</td>
<td>Case 64</td>
<td>c.A941T;</td>
<td>c.G1021A;</td>
<td>OPTN</td>
<td>Pathogenic</td>
<td>Bulbar</td>
<td>No</td>
<td>44</td>
<td>64</td>
</tr>
<tr>
<td>CONTROL-0325</td>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The table includes samples that carry 2 rare stop-gain, splice site, frameshift, or missense variants in genes on the MND gene panel or samples that carry an expansion in C9orf72 alongside a variant in a gene on the MND gene panel.
CONTROL-0325, who was aged 56–60 at the time of collection, carries 2 missense variants in OPTN; one considered pathogenic; and 1 likely pathogenic. The pathogenicity of variant Q314L, as discussed in Section 3.3, is unclear. The data available do not allow confirmation of whether the 2 OPTN variants are found in cis or in trans and, therefore, if both allelic copies of OPTN carry a variant. It is also unclear whether both variants are expected to contribute to MND pathogenesis; if so, they would be expected to be associated with variable penetrance. As no follow-up information is available for controls, there is a small chance the control developed MND after sample donation, although this is unlikely given the lifetime risk of MND.

3.7. Genotype-phenotype associations

Genotype-phenotype association testing was used to determine the relationship between carrying a variant in a specific gene and 5 phenotypic markers (Table 4 and Supplementary Table 6). MND cases carrying the SOD1 I114T founder variant were also analyzed independently of other SOD1 variants in view of the high incidence of this specific variant in the Scottish population. Only unrelated cases were included in the analysis (n = 431).

Univariate analyses comparing cases with a pathogenic or loss-of-function variant (including C9orf72 expansions) to cases without such a variant found associations between carrying a pathogenic or loss-of-function variant and a family history of MND (Fisher’s p < 5 × 10⁻⁷), a younger age of onset (t-test p = 0.018), and female sex (Fisher’s p = 0.028; Table 4). A logistic regression model evaluating the possible independent associations of these variables with variant status explained 21% of the variance between cases with and without a pathogenic or loss-of-function variant (Nagelkerke R²). Independent associations were found for positive family history (p = 5.5 × 10⁻⁷, 95% CI 5.38–22.01), decreasing age of onset by decade (p = 0.018, OR 0.77, 95% CI 0.62–0.96), and female sex (p = 0.028, OR 1.88, 95% CI 1.07–3.30; Table 5).

A high proportion of cases carrying a pathogenic SOD1 variant had spinal onset disease (95%) compared with the frequency of spinal onset in all other case (Fisher’s p = 0.012). Carrying a pathogenic SOD1 variant was also associated with having a family history of MND, compared to all other cases (Fisher’s p < 5 × 10⁻⁷; Table 4). When examining SOD1 I114T carriers in isolation, these 2 factors remained significant (site of onset Fisher’s p = 0.031, family history Fisher’s p = 9 × 10⁻⁶; Table 4). Twenty-seven percent of the variance between SOD1 pathogenic variant carriers and all other cases was explained by the model (Nagelkerke R²). Spinal onset disease (p = 0.044, OR 8.25, 95% CI 1.06–64.49) and family history of MND (p = 5 × 10⁻⁷, OR 16.21, 95% CI 6.21–42.33) were independently associated with carrying a SOD1 pathogenic variant. After initial modeling for the SOD1 I114T variant (using sex, site of onset, and family history), sex and site of onset did not achieve significance at the p = 0.1 threshold and were removed. On revised modeling, family history remained significant (p = 1 × 10⁻⁵, OR 11.39, 95% CI 4.23–30.67; Table 5), explaining 16% of the variance (Nagelkerke R²).

Univariate analysis showed carrying a C9orf72 expansion was associated with having a family history of MND (Fisher’s p = 1 × 10⁻⁵; Table 4). Site of onset did not reach statistical significance (p = 0.05), but met the criteria required for inclusion in the logistic regression model (Fisher’s p = 0.082; Table 4). The logistic regression model explained 12% of the variance between carriers of C9orf72 expansions and those without (Nagelkerke R²). Bulbar onset disease (p = 0.021, OR 2.23, 95% CI 1.13–4.42) and family history (p = 1 × 10⁻⁶, OR 6.86, 95% CI 3.19–14.77) were significantly and independently associated with carrying a C9orf72 expansion (Table 5).
The influence of carrying a pathogenic or loss-of-function variant in one of the MND genes on survival was visualized using a Kaplan-Meier plot (Fig. 2). Cases were grouped by genotype. Survival was plotted for all cases, including the 15% of cases for whom duration of disease was calculated from onset to date of last contact/study truncation. No significant difference was found between groups (log rank \( p = 0.276 \)).

4. Discussion

4.1. Genetic epidemiology of MND in Scotland

This study provides a detailed description of the genetic pathology of MND in Scotland. We have identified 31 independent cases carrying a pathogenic or loss-of-function variant across our MND gene panel. The identification of pathogenic variants in 2 controls (0.5%) is comparable to previous studies and is explained by the variable penetrance observed with variants in genes associated with MND (Lopate et al., 2010; van Blitterswijk et al., 2012).

The Scottish MND population were comparable with other European MND populations on demographic and disease characteristics (Li et al., 1990; Logroscino et al., 2008; Traynor et al., 2000). A positive family history in 10% of Scottish MND cases was comparable recent population estimates (16% in the Irish population and 9% in a US population of European ancestry; Byrne et al., 2013; Gibson et al., 2014). Our data show that, after C9orf72, variants in SOD1 contribute to the largest proportion of MND cases (5%) in the Scottish population, which is in the upper range compared with other populations of European ancestry (Andersen, 2006; Renton et al., 2014). This reflects the high frequency of the I114T variant, which had previously been identified as a founder mutation with variable penetrance in the Scottish population (Hayward et al., 1996; Jones et al., 1993, 1995; Lopate et al., 2010). Although this variant is less frequent than was observed previously in a smaller cohort (Hayward et al., 1996), it is still found in 4% of cases when assessed across our larger cohort and is found in 24% of those cases in which a pathogenic variant was identified. In comparison, pathogenic variants in TARDBP contribute to \(<1\%\) of cases, comparable to previous estimates in populations of European ancestry (Renton et al., 2014), and pathogenic variants in OPTN are extremely rare, which is similar to a previous study of the UK population (Johnson et al., 2012).

Our data support a growing literature associating loss-of-function variants in TBK1 with MND. Several previous studies have associated dominant coding variants in TBK1 with MND. The first study reported a significant excess of coding variants in TBK1, excluding those predicted to be benign, in mostly sporadic cases compared to controls (Cirulli et al., 2015). However, the biggest excess was observed for loss-of-function variants. This was supported by a second study, which found a significant excess of loss-of-function variants in TBK1, but only in familial cases (Freischmidt et al., 2015). Several other studies, looking at MND, frontotemporal dementia (FTD) and MND-FTD cases from both European and Asian populations, have also reported variants in TBK1, mostly loss-of-function (Borghero et al., 2016; Gijselinck et al., 2015; Le Ber et al., 2015; Pottier et al., 2015; Shu et al., 2016; Tsai et al., 2016; van Rheenen et al., 2016; Williams et al., 2015). In our study, although the number of loss-of-function variants identified in TBK1 was not statistically significant when compared with our 389 controls, a significant difference was observed when compared with the larger ExAC reference data set and the percentage of cases carrying a loss-of-function variant in TBK1 was comparable to previous studies (0.7% vs. \(<1\%\) in previous studies [Cirulli et al., 2015; Freischmidt et al., 2015]). Therefore, our data support the association of loss-of-function variants in TBK1 with MND.

Similarly, the frequency of NEK1 loss-of-function variants in our cases (0.7%) is comparable to previous studies (1%; Brenner et al., 2016; Cirulli et al., 2015; Kenna et al., 2016); however, the observation of 3 loss-of-function variants in NEK1 in our cases was not significantly different to that of our 389 controls or the ExAC reference data set. Although the result does not provide formal statistical significance for the association of loss-of-function variants in NEK1 with MND, as the frequency is comparable to other studies, our result is supportive of these previous findings. We observed the same number of missense variants in NEK1 in both cases and controls, with little difference in the assigned pathogenicity classifications. This is comparable to a recent study which showed that, although one specific missense variant, R261H (classified as likely pathogenic in our study), was associated with MND, collectively, missense variants showed very little difference in frequency between sporadic cases and controls (Kenna et al., 2016). In addition, the other variants in NEK1 causally related to the
development of MND in previous studies are loss-of-function variants. There may therefore be some specific missense variants that result in impaired NEK1 function, but collectively, this appears not to be the case.

We identified 2 cases each carrying 2 variants classified as pathogenic or loss-of-function in different MND genes (digenic cases). As controls were not screened for hexanucleotide repeat expansions in C9orf72, we were biased against identifying digenic controls, so it is not clear whether our 2 digenic cases represent a statistically significant excess. As each digenic case has a different variant combination, it is not possible to obtain a detailed assessment of how digenicity influences MND phenotype. Further investigation of a larger number of digenic cases is required to determine whether each variant contributes to disease pathogenesis, whether variants have an additive effect and to determine how different variant combinations influence disease presentation.

4.2. Genotype-phenotype associations

Our genotype-phenotype analysis confirms the expected association that MND cases with a family history of MND are more likely to carry a pathogenic or loss-of-function variant than cases with no family history. The results also indicate that a young age of onset of MND is a significant independent predictor of carrying a pathogenic variant. Although this finding requires replication, it suggests that, in addition to the presence of a family history, clinicians should use a lower threshold for genetic testing in cases developing symptoms of MND before the mean age of onset. The analysis also suggests that female sex is significantly associated with carrying a pathogenic or loss-of-function variant. This most likely reflects the equal number of males and females carrying variants pathogenic for MND, within an MND case population which overall has a male bias. This result is also consistent with the liability threshold genetic model, which would suggest that, as females have an overall lower risk than males of developing MND, they must accumulate a larger burden of risk factors to pass the threshold for disease onset (Falconer, 1965). The logistic regression model concerned only explains 21% of the difference between cases with a pathogenic or loss-of-function variant and those without, suggesting that additional phenotypic markers are acting as confounders.

Our study confirms that C9orf72 expansion carriers are more likely to present with bulbar disease than other MND cases (Byrne et al., 2012; Chio et al., 2012a; Millecamps et al., 2012). However, our model explained only 12% of the variance, and substantial phenotypic heterogeneity among C9orf72 carriers, particularly in terms of cognitive symptoms, is widely recognized (Rohrer et al., 2015). Cognitive profile may be an important parameter to explore further in characterizing this cohort of cases. SOD1 variant carriers in our cohort were relatively homogeneous in terms of site of onset, with a bias toward spinal onset MND. This mirrors clinical accounts of lower limb onset disease as a common presentation of SOD1 MND (Millecamps et al., 2010). A high proportion of 1114T carriers also had spinal onset disease, despite failing to achieve statistical significance on logistic regression modeling. However, heterogeneity in 1114T carriers for age of onset (range 42–84 years) and duration of disease from onset (11–172 months) was notable and suggests any biological link to these features is absent or extremely weak.

4.3. Limitations

There is increasing need for standardization of methods to assign variant pathogenicity. The recent American College of Medical Genetics guidelines (Richards et al., 2015) sought to address this; however, they are designed principally for monogenic, Mendelian disorders and may not be suitable for complex disorders such as MND, with a preponderance of sporadic cases and considerable variability in the penetrance of pathogenic variants. The lack of segregation data and large number of singleton variants meant that using in silico predictors of variant pathogenicity and conservation were most appropriate for assessing variant pathogenicity in our study, although it is clear that standardization of the methods used to assign pathogenicity for potential MND-associated variants is required.

One limitation of the combined use of the Fluidigm Access Array and Illumina MiSeq is the number of false positives we observed. The large number in SOD1 exon 1 is most likely due to the high GC content of this exon (Timothy J Aitman, unpublished data). Exclusion of this exon could have reduced the overall variant detection rate for SOD1, as many pathogenic missense variants have been reported in this exon (Stenson et al., 2008). After excluding this exon, 6% of the remaining variants also failed to validate. This could be due to the high number of cycles in the 2 rounds of PCR (35 and 15 cycles respectively) required to generate the sequencing library. Therefore, it is essential to use Sanger sequencing to validate variants identified through this protocol, as is typically applied following high-throughput sequencing.

Survival analysis between MND cases carrying pathogenic or loss-of-function variants in different genes did not achieve statistical significance. Possible limitations to this analysis include sample size and lack of data relating to interventions that influence survival (e.g., noninvasive ventilation, gastrostomy insertion, and use of riluzole).

5. Conclusions

In summary, we identified a pathogenic or loss-of-function variant in an MND gene in 17% of our cohort of MND cases from the Scottish population. Our data give supporting evidence for the association of loss-of-function variants in TBK1 and NEK1 with MND. Genotype-phenotype association testing has highlighted that MND cases with a family history or with a young age of onset are significantly more likely to carry a genetic variant pathogenic for MND and suggests that cases presenting with a young age of onset should be referred for genetic testing, in addition to cases with a family history.

Disclosure statement

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Appendix A. Supplementary data

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