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The multistep hypothesis of ALS revisited: the role of genetic mutations

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Abstract

Objective. ALS incidence rates are consistent with the hypothesis that ALS is a multistep process. We tested the hypothesis that carrying a large effect mutation might account for one or more steps through the effect of the mutation, thus leaving fewer remaining steps before ALS begins.

Methods. We generated incidence data from an ALS population register in Italy (2007-2015) for which genetic analysis for C9orf72, SOD1, TARDBP and FUS genes was performed in 82% of incident cases. As confirmation, we used data from ALS cases diagnosed in the Republic of Ireland (2006-2014). We regressed the log of age-specific incidence against the log of age with least squares regression for the subpopulation carrying disease-associated variation in each separate gene.

Results. Of the 1077 genetically-tested cases, 74 (6.9%) carried C9orf72 mutations, 20 (1.9%) SOD1 mutations, 15 (1.4%) TARDBP mutations and 3 (0.3%) FUS mutations. In the whole population there was a linear relationship between log incidence and log age ($r^2=0.98$) with a slope estimate of 4.65 (4.37-4.95), consistent with a 6-step process. The analysis for C9orf72 mutated patients confirmed a linear relationship ($r^2 = 0.94$) with a slope estimate of 2.22 (1.74-2.29), suggesting a 3-step process. This estimate was confirmed by data from the Irish ALS register. The slope estimate was consistent with a 2-step process for SOD1 and with a 4-step process for TARDBP.

Conclusions. The identification of a reduced number of steps in ALS patients with genetic mutations compared to those without mutations supports the idea of ALS as a multistep process and is an important advance for dissecting the pathogenic process in ALS.
**Introduction**

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by a progressive loss of cortical, bulbar and spinal motor neurons, often associated with an involvement of prefrontal cortex. There are indications that the degenerative process in ALS is the consequence of a combination of genetic and environmental factors. More than 20 genes have been detected as causes of ALS.\(^1\) Several environmental factors have been proposed, but none of them, with the possible exception of cigarette smoking and military service, are consistently associated with ALS.\(^2\)-\(^5\) About 20% of ALS heritability is attributable to common genetic variation compared with an overall heritability of 60% in studies based on concordance of monozygotic twin pairs.\(^6\)-\(^8\)

In a previous study, we utilized the Armitage-Doll model derived from cancer research to assess whether ALS incidence is consistent with a multistep process, and if so, to estimate the number of steps, \(n\), required for ALS to develop.\(^9\),\(^10\) The model can be briefly conceptualized as follows: if we assume that ALS is caused in a single step molecular process, then the incidence in a particular year will be proportional to the risk of undergoing the step, which in turn depends on exposure to the relevant disease-causing factor. The probability a second molecular step has occurred by that year is dependent on the risk of exposure to the relevant factor per year and the number of years of exposure, or age, and this is true for any subsequent step. Thus incidence is proportional to the product of the risks of undergoing the first step and the subsequent steps. This concept implies a logarithmic increase in incidence with age, obeying a power law in which one less than the number of steps, \(n-1\), relates to the rate of increase. As a result, taking logs of the age of onset and incidence rates has the form of a straight line equation with slope \(n-1\) if a multistep model applies. Our previous study found a linear relationship, with a slope estimate of 5, indicating that the process leading to ALS needs on average 6 steps.\(^9\) Considering the large heterogeneity of ALS in terms of clinical
presentation, progression and outcome, it is likely that the number of steps varies in specific subgroups of patients. For example, those carrying a large effect mutation might have one or more steps accounted for by the effect of the mutation, and thus have fewer remaining steps before ALS is established. We therefore tested this hypothesis using the Armitage-Doll model in genetically defined patient subgroups from a population-based cohort.
Methods

All people with ALS diagnosed in Piemonte and Valle d’Aosta, Italy, in the period 2007-2015 were eligible to be enrolled in the study. Cases were identified through the Piemonte and Valle d’Aosta register for ALS (PARALS). PARALS is a prospective epidemiological register based on the collaboration of the neurological departments of the two Italian regions. ALS cases are ascertained through several concurrent sources (hospital admission, etc.). ALS diagnosis is based on El Escorial revised criteria. Cases with definite, probable and probable laboratory supported El Escorial diagnosis during the course of the disease are included in the register. A detailed description of register methodology is reported elsewhere. The cohort included in this study is different from that included in the previous one, which was based on patients incident in the 1995-2004 period.

As a confirmation cohort, we used the data from ALS cases diagnosed in the Republic of Ireland in the period 2006-2014. ALS cases were identified though the Irish ALS register. Although similar cohorts exist for the other registers studied in our original report, the genetic data are either not complete enough or do not overlap enough with the population data to allow similar analysis.

Genetic analysis. All cases were tested for mutations in SOD1 (all exons), TARDBP (exon 6), FUS (exons 14 and 15), and C9orf72 using standard methodology described elsewhere. C9orf72 repeat length was determined using repeat primed PCR. Normal was defined as 28 or fewer repeats.

Statistical analysis. The Armitage-Doll methodology was used, under the same assumptions as our previous paper. In brief, a plot of the log of ALS incidence against log age will be linear if a multistep model applies, and will have slope $n-1$, i.e. one less than the number of steps needed for disease onset. According to the pattern identified in cancer, the model predicts that the slope will be approximately linear, but will decrease (and therefore will be less than linear)
at older age groups due to a substantial proportion of the population having undergone one or more of the earlier steps.

Following this model, we calculated the incidence rates per 100,000 person-years in 5-year age-groups for people aged 35-74 years. We excluded the youngest age-groups (those younger than 35) because of the small number of patients, and the older age-groups (those over 74) because of the risk of under-ascertainment or cohort-effect; this reflects also the finding in some cancers where the log incidence and log age association is non-linear in the older age groups.10

We then performed a preliminary analysis of the log incidence against log age on all cases (i.e., both mutated and non-mutated), in order to verify if our population followed a multistep model and to replicate our previous findings. Second, we assessed separately familial and non-familial ALS patients. Third, we assessed the incidence of ALS for cases involving each single gene. To correctly calculate incidence, the population used for the denominator should correspond to the population used for the numerator. For example, for ALS incidence in those carrying a C9orf72 mutation, the correct denominator to use would be the count of all people in the population carrying a C9orf72 mutation. This information was only available for the cases but not for the general population. However, since the relevant mutations do not in general markedly increase mortality apart from their effects on ALS, we assumed that the proportions of the population carrying a specific mutation would not differ substantially by age-group (e.g. the proportions with the C9orf72 mutation would be similar in the 40-44 and 60-64 years age-groups). Under this assumption, it is then reasonable to use the total population as the denominators in the analyses for specific genes (e.g. cases involving the C9orf72 mutation), since this would involve multiplying the relevant age-specific population denominator by an unknown but fixed constant (e.g. if 5% of the population carry a particular mutation, then the total population denominator would be 20 times that of the unknown population subgroup carrying this
Thus, all of the age-specific incidence rates would be overestimated by an unknown but fixed multiplying factor; this in turn would affect the age-specific incidence rates, but would have no effect on the slope of the graph of log incidence against log age.

**Standard Protocol Approvals, Registrations, and Patient Consents**

The Piedmont regional government has recognized the Piemonte ALS Registry as a ‘Registry of High Sanitary Interest’ (Regional Law, April 11, 2012, n. 4). Accordingly, PARALS has the right to access to all the existing databases owned by the regional administration and to obtain clinical information about ALS patients from public and private hospitals, and general practitioners. The study was approved by the Ethical Committee of the Città della Salute e della Scienza of Turin. The register database is anonymized and treated according to Italian Data Protection Code. Patients sign a written informed consent. The Irish ALS Register complies with Irish Data protection legislation (1988 and 2003), and has been approved by the Beaumont Hospital Ethics Committee (02/28 and 05/49).

**Data availability**

Anonymized data will be shared by request from any qualified investigator.
Results

Of the 1309 cases incident during the 2007-2015 period, 1077 (82.2%) underwent genetic analysis of all four genes, 93.5% (1030) of those followed by the two ALS multidisciplinary centers and 21.7% (47) of those followed by general neurology departments. Patients who did not undergo genetic analysis were older and more frequently had a bulbar onset than those who were tested (Table 1). C9orf72 mutations were detected in 74 cases (6.9%), SOD1 in 20 (1.9%), TARDBP in 15 (1.4%) and FUS in 3 (0.3%). One patient carried both a C9orf72 expansion and the p.Asns90Ser heterozygous missense mutation of the TARDBP gene. A list of SOD1, TARDBP and FUS mutations is reported in Table 2.

In the 1077 patients with genetic test data, there was a linear relationship between log incidence and log age ($r^2=0.98$) with a slope estimate of 4.65 (95% CI 4.37-4.95), consistent with a 6-step process (Figure 1a), replicating our previous findings. A similar result ($r^2=0.99$) was obtained when including all 1309 incident cases (Figure 1b). There was no effect of sex (data not shown).

When considering the 109 patients with definite or probable familial ALS (10.1% of the total), there was a linear relationship between log incidence and log age, with a slope estimate of 2.95 (2.43-3.57), consistent with a 4-step process (Figure 2).

The analysis for C9orf72 mutated patients confirmed a linear relationship ($r^2 = 0.94$) with a slope estimate of 2.22 (1.74-2.79) suggesting a 3-step process (Figure 3a). Similarly, a linear relationship was found for SOD1 mutated patients ($r^2 = 0.53; n-1 = 0.76, 95\% \text{ CI} 0.46-1.17$) consistent with a 2-step process (Figure 3b) and for TARDBP ($r^2 = 0.93; n-1 = 3.24, 95\% \text{ CI} 2.21-4.13$) consistent with a 4-step process (Figure 3c). Due to the very small number of cases carrying FUS mutations we did not estimate the slope. When considering the 45 patients with familial ALS but negative for the 4 tested genes, the linear relationship was confirmed ($r^2 =$
0.95; n-1 = 3.71, 95% CI 2.67-4.53) consistent with a 5-step process (Figure not shown). These data are summarized in Table 3.

We next analyzed patients from the Irish ALS register. The register includes 597 genetically-tested patients (56.3% of incident patients in the 2006-2014 period), of whom 67 carried a C9orf72 expansion. In the 597 patients with genetic test data, there was a linear relationship between log incidence and log age \((r^2=0.93)\) with a slope estimate of 5.09 (4.69-5.52), consistent with a 6-step process. In the C9orf72 expanded cases results were similar to those of the Piemonte register \((r^2 = 0.66; \text{ slope estimate } 2.47, 95\% \text{ CI } 1.91-3.13, \text{ consistent with a 3 step process})\). Finally, the 530 Irish patients without a C9orf72 expansion had a slope estimate of 5.35 (4.92-5.82) \((r^2=0.95)\). No patients with SOD1 mutations and only two with TARDBP missense mutations were identified in the Irish ALS register, making it impossible to assess the effects of these genes.

For comparison, we assessed the slope for type 1 and type 2 diabetes mellitus, using the data of the Piemonte register for diabetes for the age-groups 30 to 49 years (Table 4).\(^{15}\) In keeping with our findings on ALS, the slope estimate for diabetes type 1, a highly genetically determined disease, was 0.96 (0.62-1.13) \((r^2=1.0)\) consistent with a 2 step process, while that of diabetes type 2, a multifactorial disease with a polygenic architecture, was 5.27 (4.50-6.18) \((r^2=0.98)\), consistent with a 6 step process.
Discussion

We have found that in patients carrying a genetic mutation, the slope of the graph of log incidence and log age is lower than that of cases who do not carry these mutations. This in turn implies that the number of steps necessary to start the neurodegenerative process in genetically mediated ALS is reduced compared to non-mutated cases. The number of steps varies according to the mutated gene, and is lower for SOD1 (2), intermediate for C9orf72 (3) and higher for TARDBP (4). The number of steps identified in non-mutated patients is 6, consistent with our previous paper. In particular, the slope in the patients from the Piemonte register reported in that paper, which was based on incident cases in the 1995-2004 period, is almost identical to that found in the present paper, which was based on the incident cases in the 2007-2015 period. Furthermore, the slope for C9orf72, as well the overall slope of genetically-tested patients, was confirmed in the Irish ALS population. These findings suggest that a genetic lesion alone might account for up to four molecular steps, leaving only two further, likely environmental, steps for those with SOD1 mutation for example. This argues for the concentration of efforts in dissecting environmental risk factors in individuals with identified mutations rather than those with apparently sporadic ALS, since such environmental factors will be fewer in number per person, and likely of larger effect size as a result.

It is generally recognized that ALS is a multifactorial disease, characterized by interplay between genetic and environmental factors. Although several ALS-related genes are known, it is increasingly clear that genetic mutations alone cannot fully explain the pathological process in ALS, but that genes can rather be considered triggers of the degenerative process. A similar role can be attributed to environmental toxins. However, we have very little information about the possible exogenous factors involved in ALS. Cigarette smoking may be a risk factor in ALS, other suggested factors are physical activity, participating in professional sports, and physical trauma. Protective factors have also been hypothesized, such as diabetes mellitus
and an unfavorable lipid profile. All these factors could act on the genome through epigenomic interactions. For example, smoking induces DNA hypermethylation in specific CpG sites, which persists for years after cessation of smoking, or may induce somatic nucleic acid changes. It is likely that the remaining steps in different genetic subgroups may originate from one or more of these risk factors.

Besides our current results, there are more observations that fit the multistep hypothesis in ALS. First, there are indications that ALS can be an oligogenic disease. In fact, there are several reports of patients carrying two or more mutations of different ALS-related genes. In a study of 391 ALS patients which assessed variants in 17 genes, 3.8% had variants in more than one gene. In that series, the burden of rare variants in known ALS genes significantly reduced the age of onset of symptoms. In the present series one patient had both a \textit{C9orf72} expansion and a heterozygous mutation of the \textit{TARDBP} gene, even though we assessed only four genes. Second, besides ‘causative’ genes, several other genes have been reported to modify ALS phenotype, such as \textit{UNC13A}, \textit{ATXN2} and \textit{CAMTA1}; suggesting that variants in these genes modify the sequential process, either accelerating or slowing it.

Non-genetic elements such as environmental factors and aging also likely trigger molecular steps. However, consistent with other reports, the slope is the same between sexes in all analyses suggesting there is no effect of sex on the assumed cascade.

There appears to be some relationship between the number of remaining steps identified for each mutated gene and the penetrance of mutation in the gene. Such a relationship is consistent with a multi-step model, since a greater number of remaining steps will correspond to a lower probability of exposure to all the steps and therefore reduce the probability of disease given a specific genotype. For example, \textit{C9orf72} expansion mutation penetrance has been estimated to be 60% at the age of 60 and 91% at the age of 80 years and corresponds to 3 remaining steps. At least three mechanisms might regulate \textit{C9orf72} penetrance: first, the size of the
GGGGCC expansion; second, DNA methylation and transcriptional downregulation of the promoter,\textsuperscript{32} and third the presence of additional mutations.\textsuperscript{33}

The penetrance of \textit{TARDBP} mutation is much lower than that of \textit{C9orf72} (60\% at 80 years for the p.Ala382Thr mutation)\textsuperscript{34} and it leaves 4 remaining steps, more than for the other two genes. The lowest number of remaining steps, 2, has been estimated for \textit{SOD1} mutation. However, \textit{SOD1} penetrance varies across the different mutations. For example, in a study on pedigrees dating back to the 18\textsuperscript{th} century, carriers of p.Glu101Gly, p.Ile114Thr and p.Val149Gly \textit{SOD1} mutations were reported to have a penetrance of more than 95\% at the age of 78.\textsuperscript{35} Similarly, the penetrance of the p.Ala5Val mutation, the commonest in the USA, has been estimated to be 91\% at the age of 80.\textsuperscript{36} Other mutations have a much reduced penetrance; an example is the p.Asp91Ala mutation, which is transmitted with a recessive inheritance in people of Scandinavian ancestry and with a dominant inheritance, albeit with a low penetrance, in the other populations.\textsuperscript{37, 38} Most of the \textit{SOD1} mutations we identified are regarded as having very high penetrance, and would therefore be expected to account for more steps than low penetrance mutations.

This study has some weaknesses. First, it was not possible to genotype all incident ALS patients. Non-tested patients were older and more frequently had bulbar onset than those who were tested. However, we could obtain the DNA of >80\% of incident patients, a high proportion in an epidemiological setting. Second, only the four more commonly mutated ALS genes were assessed. However, non-tested genes account for only a fraction of ALS patients in European-derived populations. Third, the estimation of the slope was performed on the relatively small number of genetic cases, in particular for \textit{SOD1} and \textit{TARDBP}, and the slope estimates may therefore be imprecise. Finally, population denominators were not available for specific mutations; however, as noted above, this would have affected our age-specific incidence estimates, but not the slope of the graph of log incidence against log age. It is
therefore important that our findings be replicated in other populations with larger cohorts of patients to confirm our results and determine the extent to which they can be generalized.

The identification of a reduced number of steps in ALS patients with genetic mutations compared to those without mutations strongly supports the idea of ALS as a multistep process and represents a first clue for uncovering the pathogenic process of ALS. Similar patterns have previously been observed in studies of specific cancers in which the relevant mutations and other environmentally-induced steps have been able to be identified, and postulated as being also relevant to neurodegeneration. Our findings support the idea of parallels between the processes leading to carcinogenesis and those leading to ALS. The fact that only 2, 3 or 4 steps are required before disease onset in genetically-mediated ALS is consistent with the concept that up to 4 of the six steps required for disease onset are already accounted for by inherited mutation. This idea is also consistent with the observation that penetrance corresponds to the number of steps accounted for. An alternative explanation is that the underlying etiology must differ in at least one step between genetic and other forms of ALS. The analysis of the influence of non-genetic risk factors should therefore also be performed, to clarify their contribution to the multistep process of ALS. The relatively limited number of steps leading to ALS, as compared, for example, to the complexity of the mechanisms at the base of other multifactorial diseases such as schizophrenia, provides hope for the development of an effective therapy for this devastating disease.
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**Table 1.** Comparison of ALS patients who underwent genetic assessment vs. patients who were not assessed

<table>
<thead>
<tr>
<th></th>
<th>Tested patients (n=1077)</th>
<th>Non-tested patients (n=232)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (female, %)</td>
<td>508 (47.3%)</td>
<td>101 (43.2%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Site of onset (bulbar, %)</td>
<td>348 (32.4%)</td>
<td>106 (45.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean age at onset (years, SD)</td>
<td>65.8 (11.0)</td>
<td>70.9 (10.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Family history of ALS (n, %)</td>
<td>109 (10.1%)*</td>
<td>2 (0.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multidisciplinary ALS center (n and % of genetically tested)</td>
<td>1030 (93.8%)</td>
<td>70 (6.5%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* 39 patients (3.6% of those tested) carried a mutation but did not report a family history for ALS. They are not included in the count of those with a family history.
Table 2. List of missense genetic mutations of SOD1, TARDBP and FUS genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Family history for ALS or FTD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C9orf72 (n=74)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>74 cases with GGGGCC expansion*</td>
<td>Yes = 47; No = 27</td>
</tr>
<tr>
<td><strong>SOD1 (n=20)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leu145Phe (6 cases)</td>
<td>Yes = 5; No = 1</td>
</tr>
<tr>
<td></td>
<td>GLy94Asp (4 cases)</td>
<td>Yes = 4</td>
</tr>
<tr>
<td></td>
<td>Asp91Ala heterozygous (2 cases)</td>
<td>No = 2</td>
</tr>
<tr>
<td></td>
<td>Asp110Tyr (2 cases)</td>
<td>No = 2</td>
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<tr>
<td></td>
<td>Asn20Ser (1 case)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Leu39Val (1 case)</td>
<td>Yes</td>
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<td></td>
<td>Val48Phe (1 case)</td>
<td>Yes</td>
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<tr>
<td></td>
<td>Asn66Ser (1 case)</td>
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<tr>
<td></td>
<td>Gly73Ser (1 case)</td>
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</tr>
<tr>
<td></td>
<td>Asp91Asn (1 case)</td>
<td>No</td>
</tr>
<tr>
<td><strong>TARDBP (n=15)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala382Thr (8 cases)</td>
<td>Yes = 4; No = 4</td>
</tr>
<tr>
<td></td>
<td>Asn276Ser (2 cases)</td>
<td>No = 2</td>
</tr>
<tr>
<td></td>
<td>Asn390Ser (2 cases)*</td>
<td>Yes = 1; No = 1</td>
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<tr>
<td></td>
<td>Ser393Leu (2 cases)</td>
<td>Yes = 1; No = 2</td>
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<td></td>
<td>Gly368Ser (1 case)</td>
<td>No</td>
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<tr>
<td><strong>FUS (n=3)</strong></td>
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<td></td>
<td>Arg495X (1 case)</td>
<td>De novo mutation</td>
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<td>Arg514Ser (1 case)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Gln519Glu (1 case)</td>
<td>De novo mutation</td>
</tr>
</tbody>
</table>

* 1 patient carried both a C9orf72 GGGGCC expansion and the p.N390S missense mutation of the TARDBP gene
Table 3. Comparison of log incidence vs. log age for different ALS subgroups. Only the 1077 genetically tested cases are included.

<table>
<thead>
<tr>
<th></th>
<th>Number included</th>
<th>( r^2 )</th>
<th>( n-1 ) (95% CI)</th>
<th>( n ) steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tested cases</td>
<td>1077</td>
<td>0.98</td>
<td>4.652 (4.368-4.950)</td>
<td>6</td>
</tr>
<tr>
<td>All non-familial non-mutated cases</td>
<td>921</td>
<td>0.98</td>
<td>5.001 (4.681-5.341)</td>
<td>6</td>
</tr>
<tr>
<td>All familial cases</td>
<td>109</td>
<td>0.92</td>
<td>2.945 (2.430-3.566)</td>
<td>4</td>
</tr>
<tr>
<td>All familial cases negative for tested genes</td>
<td>45</td>
<td>0.95</td>
<td>3.710 (2.674-4.532)</td>
<td>5</td>
</tr>
<tr>
<td>SOD1 mutated</td>
<td>20</td>
<td>0.53</td>
<td>0.758 (0.463-1.167)</td>
<td>2</td>
</tr>
<tr>
<td>C9orf72* mutated</td>
<td>74</td>
<td>0.94</td>
<td>2.215 (1.738-2.791)</td>
<td>3</td>
</tr>
<tr>
<td>TARDBP* mutated</td>
<td>14</td>
<td>0.93</td>
<td>3.241 (2.209-4.131)</td>
<td>4</td>
</tr>
</tbody>
</table>

*1 case with both C9orf72 and TARDBP mutation is counted for both genes.
**Figure legends**

**Figure 1. Slope estimation for all ALS patients.**

A. Log incidence vs. log age for all incident ALS patients who have been genetically-tested (n=1077) (y=4.65x - 7.60; r²=0.98).

B. Log incidence vs. log age for all incident ALS patients in the register (n=1309) (y = 4.83x – 7.85; r² = 0.99).

**Figure 2. Slope estimation for all those with familial ALS.** Log incidence vs. log age for all incident familial ALS patients (n=111) (y=2.95x -5.45; r²=0.92).

**Figure 3. Slope estimation for all ALS patients carrying a mutation in one of four tested genes.** Log incidence vs log age for C9orf72 ALS (74 cases) (y=2.22x – 4.33, r²=0.94) (red line) for SOD1 ALS (20 cases) (y=0.758x – 2.43, r²=0.53) (green line) and for TARDBP ALS (15 cases) (y=3.24x – 6.76, r²=0.93) (blue line). The fit to a straight line is good, consistent with a multistep model.

**Figure 4. Slope estimation for patients with type 1 and type 2 diabetes mellitus.** Data from Piemonte diabetes register. Log incidence vs. log age for type 1 diabetes mellitus (y=0.96x – 0.66; r²=0.98) (red line) and type 2 diabetes mellitus (y=5.28x – 6.78; r²=1.0) (blue line).