# Appendix A : Supplementary Material

# Introduction

More than 180 mutations in the *SOD1* gene have been identified worldwide (ALS Online Genetic Database, ALSOD: <http://alsod.iop.kcl.ac.uk>) (**1**) in ALS cases. A genotype-phenotype correlation has been defined for only a few mutations. Some *SOD1* mutations occur as recurrent or founder mutations (**2**), however few data have been reported in the literature regarding haplotype studies (summarized in Table 1).

Here, we describe the phenotypic and genotypic features of two unrelated Italian ALS patients, both originating from South-East Tuscany, a familial and an apparently sporadic case, with pure lower motor neuron phenotype, carrying the *SOD1* D124G mutation (formally designated p.D125G), an extremely rare mutation, reported in only one patient (without clinical information) to date (**1**). Furthermore, we provide evidence for a common founder for the D124G mutation in the Italian population and we discuss the effect of the mutation on the protein structure and stability.

# Methods

##  Molecular analysis

After obtaining written informed consent, genomic DNA was extracted from peripheral blood by standard procedures. After DNA amplification of the five *SOD1* exons and flanking intronic sequences by PCR and sequenced bidirectionally by an automated sequencing system (ABI 3730 DNA Analyzer, Applied Biosystems). Other ALS related genes (*C9orf72, TARDBP, FUS, VCP, and ANG)* were analysed according to the methods previously reported (**13**).

## Haplotype analysis and estimate of mutation age

Genotyping was carried out using eight polymorphic markers (*D21S263*, *Hamlet*, *Macbeth*, *Shylock*, *Yorick*, *Goneril*, *D21S223*, *D21S63*) flanking the *SOD1* gene (**4**). Microsatellites were amplified by fluorescent PCR and analyzed with an automated sequencer (3730 DNA analyzer, Applied Biosystems) using Gene Mapper software Version 4.0. Control allele frequencies were defined by genotyping 100 control subjects from the same ethnic background. Haplotype frequencies and association statistics for the markers were constructed using PHASE version 2 software (**14**) (http://stephenslab.uchicago.edu/software.html#phase). Estimate of mutation age was performed using DMLE+ version 2.3 software (**15**) (www.dmle.org). The DMLE+ program allows multipoint LD mapping using an arbitrary number of SNPs or microsatellite markers. It implements Markov chain Monte Carlo (MCMC) methods to allow Bayesian estimation of the posterior probability density of the position of a disease mutation relative to a set of markers. In general, the coalescent model implemented in DMLE is probably the best representation of the gene genealogies and their variability, as demonstrated in a study where the date of origin of a recent mutation was known, and the DMLE estimate was very accurate (**16**). DMLE+ generates the marginal posterior probability density of mutation age, based on the following parameters: observed haplotypes (or genotypes) in normal and affected chromosomes, map distances between markers and mutation site, fraction of mutated chromosomes sampled, and estimated population growth rate. Chromosome map distances were derived from those originally reported by Parton and colleagues (**8**), estimated population growth rate in Tuscany and fraction of mutated chromosomes sampled were calculated as previously described (**17**).

# Results

## Case report

**Case 1**. The patient, a 68-year-old male, came to our observation due to a progressive weakness in his lower left limb which arose about 2 months before, without any pain or other sensory disturbance; in the previous 3 months he had lost about 3 kg of body weight. The patient (III-13) had a positive family history of motor neuron disease (Figure 1A): his father (II-12) died at the age of 70 years from spinal onset ALS with spinal onset, after a 5-year disease course; one of the patient’s brothers (III-22) developed spinal onset ALS at the age of 66 and died 3 years later; a first cousin (III-2) died from spinal onset ALS after a 2-year disease course, at the age of 70 years. Another brother (III-18) died from respiratory failure caused by acute neurological disease, likely due to Guillain Barre’ Syndrome.

Patient's neurological examination showed widespread weakness and atrophy in his lower left limb, which was more marked proximally, and fasciculations in both lower limbs and upper left limb; no sensory loss was clinically detectable; deep-tendon reflexes were absent in the lower limbs and no upper motor neuron signs were present; the examination was normal in all other districts.

The neurophysiological examination showed signs of acute and chronic denervation with multi-metameric distribution in the lower left limb, fasciculation potentials in some muscles of the lower right limb; normal findings in the upper limbs. A lumbar and sacral MRI revealed no alterations that could justify the clinical and the electrophysiological findings. Three months later, the patient showed further worsening of the weakness in the lower left limb, mild weakness in the lower right limb and motor impairment in the left hand. The EMG follow up revealed a clear spreading of lower motor neuron involvement also in the lower right limb and in the upper left limb. MEP study showed a central conduction time within the normal range in all four limbs. There were no signs or symptoms in the cranial district and respiratory functions were normal. The clinical follow-up, 3 months later, showed a further reduction in strength in the four limbs, with the left side more affected. Twenty-two months from the onset, he could not walk or stand (even using a support) and he showed initial reduction of respiratory volumes. At 29 months from the onset he developed bulbar symptoms whereas cognitive functions remained intact. He died from acute respiratory failure 35 months after the onset of symptoms.

**Case 2**. The patient, a 52-year-old male, was admitted to the Neurology ward of another hospital due to the onset of weakness in the lower right limb 3 months before. The neurological examination showed fasciculations in the shoulder girdle, which were more evident on the right side, widespread weakness in the lower right limb with deep-tendon reflexes reduced. Sensory examination was normal and no signs of upper motor neuron impairment were present. The EMG examination showed acute denervation in the muscles of the lower right limb and reduced amplitude of the CMAPs and the SAPs in both lower limbs. Brain and cervical MRI were normal. In the hypothesis of a sensory motor polyneuropathy, a lumbar puncture was carried out. CSF analysis showed a slight increase in protein level and in the blood-brain barrier index; anti-ganglioside antibodies were absent. The disease progressed rapidly to the lower left limb. Seven months from the onset of symptoms, the patient began to develop respiratory symptoms. At this time he came to our observation for the first time and the neurological examination showed flaccid tetraparesis, dysarthria, tongue hypotrophy and fasciculations on the left side. The ALSFRS-R was 27; deep tendon reflexes were absent in all limbs. Sensory examination remained normal. The EMG showed severe signs of acute and chronic involvement of lower motor neuron, widespread and symmetrical in four limbs, in the paravertebral dorsal muscles and in the muscles of the left-side cranial district. The Magnetic Transcranial Stimulation showed MEPs with normal central conduction times in the upper limbs and non-recordable MEPs in the lower limbs, due to the serious peripheral damage. Respiratory function tests confirmed the involvement of the respiratory muscles with an approximate 50% reduction in volumes. Cognitive functions were not compromised and there were no behavioural disturbances. The patient began non-invasive mechanical ventilation. Nine months from the onset of the symptoms, the patient underwent a tracheotomy and invasive mechanical ventilation. The patient died from cardio-respiratory complications 28 months after the onset.

The patient’s mother (II-4) and the patient’s sister (III-2), aged 75 and 58 years, respectively, were both clinically unaffected at the time of our examination (Fig.1B).

## Molecular analysis

Automatic sequencing revealed the presence of the c.374A> G mutation in heterozygous state in both patients. The modification of the GGA triplet in GGT in the codon 124 of the exon 5 leads to the replacement of aspartic acid with glycine (D124G). The mother and the sister of case 2, both clinically unaffected, had consented to genetic testing for research purposes after informed consent. Genetic analysis showed the D124G *SOD1* mutation in heterozygous state in both of them (Figure 1B).

No alterations were detected in *C9orf72*, *TARDBP*, *FUS*, *VCP* and *ANG* genes.

## Haplotype analysis

To assess whether patients carrying the D124G mutation may descend from a common ancestor, genotyping was carried out using 8 polymorphic markers flanking the *SOD1* gene. This analysis revealed the presence of the same haplotype in both patients. For each marker, Table 2 shows the alleles present in the two patients, the total number of alleles found in the control group and the frequency of the alleles common to both patients in the general population. In particular, for *D21S263*, *D21S63*, *Yorick* and *Goneril* markers, the alleles shared by the patients were particularly rare in the control population (with frequencies ranging from 1% to 2%). Allele phasing for haplotype construction in Case 2 was obtained comparing genotyping results in proband's mother and sister, and confirmed the association of the shared haplotype with the D124G *SOD1* mutation (data not shown).For control subjects, haplotype frequencies were constructed using PHASE software. The evaluation of the mutation age was carried out using the DMLE method, that estimates the time of origin of the mutation. The analysis revealed that the D124G mutation originated approximately 16 generations ago (95% confidence intervals: 7-49), that, if we assume 25 years per generation, corresponds to about 400 years ago, therefore at the start of the 17th century.

# Discussion

We identified the D124G mutation in two unrelated Italian patients, a FALS case and an apparently sporadic case. Among the more than 180 mutations of the *SOD1* gene reported in literature, the D124G mutation is extremely rare, having been identified in only one patient to date, as reported in the ALS Online Genetic Database (ALSOD: <http://alsod.iop.kcl.ac.uk/>, **1**). Unfortunately, no clinical or demographic data are described for this patient, therefore no information is available about the disease phenotype and the geographical origin.

The clinical picture of both patients was quite uniform, characterized by a spinal onset in the lower limbs and a progressive muscular atrophy (PMA) phenotype, which has a frequency of about 3% in the entire ALS population (**18**). The progression of the disease however was partially different. In the patient 1 (FALS case), the duration of the disease was approximately three years and a clinical homogeneity was found within the family, where all the other affected members displayed a spinal onset and a duration of the disease between 2 and 5 years. Patient 2 (SALS case) had a very rapid progression of the disease, less than one year, similar to that described for the A4V (**2**) and the G41S (**10**) mutations of *SOD1* gene. This progression is unusual for the PMA, that is characterized by a longer survival (median survival time 7.3 years) than the majority of ALS phenotypes (**18**). It can therefore be hypothesized that the more aggressive progression in our cases, compared to that reported for the PMA phenotype, is associated with this mutation. On the other hand, it is not possible to exclude the role of other unknown modulator genes or environmental factors, especially for patient 2.

As already seen in other *SOD1* mutations, the D124G shows incomplete penetrance. In patient 1’s family, there are few affected members compared to the size of the pedigree and all of them are male, while in the case of patient 2, patient’s mother and sister, both clinically unaffected at the age of 75 and 58 years, respectively, carried the D124G mutation in heterozygous state. As in the present study, it has been observed that in ALS-SOD1 families with reduced penetrance, the unaffected individual is often a woman of advanced age who has affected sons (**19**).

From a structural viewpoint, several experimental studies have shown that the residual D124, located in the protein electrostatic loop, is essential for the structural integrity of the binding site for metals, in particular for stabilisation of the zinc site (**20**). The importance of the lateral chain of the D124 residue for the correct positioning of the zinc in its binding site has been seen in experimental systems, where mutations in position 124 produced proteins that could not bind the zinc ion in physiological conditions (**21**). More recently, it has been proven that the mutation D124V, associated with familial ALS (**22**), causes a considerable destabilisation of the zinc loop, but also of the electrostatic loop (**23**). A similar alteration may be caused by the mutation D124G. It is interesting to note that destabilisation of the electrostatic loop is a common characteristic for many of FALS linked to *SOD1* mutations, that probably leads to the gain of a toxic function of the protein (**24**) and to the formation of complex aggregates, subject to oligomerization inside motor neurons (**23**).

Both patients originated from South-East Tuscany and haplotype analysis showed that they shared the same alleles, some of which had a low frequency in the reference population. So, we propose that the D124G mutation in Tuscany may originate from a common founder. Estimation of the age of the mutation indicated that the D124G mutation arose approximately 400 years ago, at the beginning of the 17th century. The DMLE method attempts to identify the time in history at which the mutation in question arose, rather than the most recent common ancestor, thus this is an estimate of the first appearance of D124G mutation in the Tuscany population. However, it is not possible to establish whether the mutation was a de novo event that occurred in an autochthonous individual, or it was older and was introduced in Tuscany by an individual from other geographical areas. The lack of information about the ALS patient carrying the D124G mutation, reported in ALS Online Genetic Database, does not allow us to formulate hypotheses about the possible geographic origin of the mutation.

In conclusion, in this study we have defined for the first time the clinical profile associated with the D124G mutation in *SOD1* gene and provided evidence that this mutation in Italy originates from a common founder. Further studies on ALS patients carrying the D124G mutation may confirm the genotype-phenotype correlation.

**References**

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