ISSN 0120-4157

Biomédica

Revista del Instituto Nacional de Salud

PUBLICACIÓN ANTICIPADA EN LINEA

El Comité Editorial de *Biomédica* ya aprobó para publicación este manuscrito, teniendo en cuenta los conceptos de los pares académicos que lo evaluaron. Se publica anticipadamente en versión pdf en forma provisional con base en la última versión electrónica del manuscrito pero sin que aún haya sido diagramado ni se le haya hecho la corrección de estilo.

Siéntase libre de descargar, usar, distribuir y citar esta versión preliminar tal y como lo indicamos pero, por favor, recuerde que la versión impresa final y en formato pdf pueden ser diferentes.

Citación provisional:

Jaramillo J, Solano JM, Aristizabal A, Martínez J. Mutations study of SOD1 and C9orf72 genes in patients with amyotrophic lateral sclerosis in Antioquia, Colombia. Biomédica. 2022;42 (4).

Recibido: 31-03-22

Aceptado: 20-09-22

Publicación en línea: 22-10-22

Mutations study of SOD1 and C9orf72 genes in patients with amyotrophic lateral sclerosis in Antioquia, Colombia

Estudio de mutaciones en los genes SOD1 y C9orf72 en pacientes con esclerosis lateral amiotrófica en Antioquia, Colombia

Genetic amyotrophic lateral sclerosis in Colombia

Jimena Jaramillo [1], Juan M. Solano [2], Alejandra Aristizabal [2], Juliana Martínez [1]

[1] Facultad de Ciencias y Biotecnología, Universidad CES, Medellín, Colombia

[2] Grupo de Investigación en Enfermedades Neuromusculares, Universidad de Antioquia, Medellín, Colombia

Correspondencia:

Juliana Martínez, Calle 10A # 22-04, Medellín, Colombia

Teléfono: (4) 4440555 ext. 1546

jmartinezg@ces.edu.co

Contribución de los autores:

Jimena Jaramillo: conceptualizacion, análisis de resultados, consecusión de recuros, toma de muestras, análisis de laboratorio, financiación y escritura del artículo.

Juan M. Solano: conceptualizacion, diagnóstico, contacto con pacientes, toma de muestra y análisis de resultados.

Alejandra Aristizabal: diagnóstico, contacto con pacientes y toma de muestra.

Juliana Martínez: conceptualizacion, análisis de resultados, consecusión de recuros, análisis de laboratorio, financiación, administración, validación, supervisión y escritura.

Todos los autores participaron en la revisión del articulo.

Introductión: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with a possible multifactorial origin characterized by the progressive degeneration of motor neurons. There is a relatively high prevalence of this disease in Antioquia; however, there is no published genetics study of ALS to date in Colombia. Despite its unknown etiopathogenesis, ever more genetic risk factors for the development of the disease are constantly found. In this context.

Objetives: To evaluate the G93A and D90A mutations of the SOD1 gene and a Short Tandem Repeat (STR) in C9orf72 in a cohort of ALS patients from Antioquia, Colombia.

Materials y methods: 34 patients previously diagnosed with ALS were included in the study and, peripheral blood samples were drawn from them for use in DNA extraction and genotyping.

Results: No mutations were found in SOD1 (G93A and D90A) in any of the patients, while C9orf72 exhibited an allele with a statistically significant high prevalence in the study sample ((CAGCAG)8).

Conclusions: These results suggest an association between this STR in C9orf72 and the presence of ALS in the study population. However, this association should be established in larger studies and with controls from the same population; in addition, there also seems to be a genetic anticipation effect for the disease with regard to this locus, since patients with this genotype present an earlier onset of the disease.

Keywords: amyotrophic lateral sclerosis/genetics; neurodegenerative disease; genes; mutations.

Introducción. La esclerosis lateral amiotrófica (ELA) es una enfermedad neurodegenerativa con un posible origen multifactorial, caracterizado por una degeneración progresiva de las neuronas motoras. Hay una alta prevalencia relativa de esta enfermedad en Antioquia; sin embargo, no hay publicaciones de estudios genéticos sobre ELA en Colombia. A pesar de su etiopatogenesis desconocida, hay varios factores de riesgo genético que son encontrados constantemente en el desarrollo de esta enfermedad.

Objetivo. Evaluar las mutaciones G93A y D90A del gen SOD1 y una repetición en tándem (STR) en el locus C9ORF72 en una cohorte de pacientes con ELA en Antioquia, Colombia.

Materiales y métodos. 34 pacientes previamente diagnosticados fueron incluidos en el estudio, una muestra de sangre periférica se uso para extraer ADN y genotipificar.

Resultados. No se encontraron mutaciones en el gen SOD1 (G93A y D90A), mientras que C9orf72 exhibe un alelo con una significativa prevalencia en los pacientes del estudio (8 repeticiones del hexanucleotido G_4C_2).

Conclusiones. Se sugiere asociación entre el STR en C9orf72 y la presencia de ELA en la población. Sin embargo, se sugiere estudios adicionales y la inclusión de un grupo control de la misma población. Además, También se detecta un fenómeno de anticipación genética de la enfermedad, dado que los pacientes con el alelo de 8 repeticiones en C9orf72 presentan una edad temprana de aparición de síntomas.

Palabras clave: Esclerosis lateral amiotrófica/genética; enfermedades neurodegenerativas; genes; mutaciones.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive death of motor neurons, producing muscular paralysis(1,2). Its clinical presentation is variable and depends on the anatomical site of onset and the type of motor neuron affected (3-5). There is no cure for ALS and most of the patients die of respiratory failure about 3 to 5 years after onset of the symptoms (1,6). ALS etiology is not completely understood but, like other neurodegenerative diseases, it is thought to be the result of a complex interaction between multiple cellular mechanisms, and the interaction of environmental and genetic factors (7-9).

The genes with the strongest association to the development of both familiar and sporadic ALS are *SOD1* and *C9orf72* (1,4,10). *SOD1* encodes the enzyme superoxide dismutase 1, responsible for the degradation of superoxide ions via reactions with Zn and Cu ions (1,7,11), while *C9orf72* encodes a protein involved in both nuclear membrane transport and as a guanine nucleotide exchange factor that activates GTPases (5,12,13).

Mutations *G93A* and *D90A* in *SOD1*, cause oxidative stress and subsequent neuronal death (1,11,14). However, the most common genetic anomaly found in ALS patients is a repeat of a hexanucleotide sequence or STR in the *C9orf72* gene which , explains 23.5% of familial cases worldwide and up to 60% of familial cases in Spain (1,3,15). This *C9orf72* expansion causes a loss of the functional protein, progressively generating cellular death (5,12,13). Healthy individuals, generally have a maximum of two repetitions (16) while a pathological form of the gene in ALS patients typically has 20 or more repetitions of the hexanucleotide; however, the number of pathogenic repetitions varies depending on the population (17).

The ALS incidence in Europe has been stimated at 2.8 cases per 100,000 people, per year. While there is few data for Latin America, reports from some countries in the region suggest a range between 0.2 and 3.17 cases per 100,000 people, per year. In Colombia national reports propose the prevalence of ALS to be of 0.5 cases per 100,000 people (18), but in Antioquia (a state within Colombia) this prevalence is much higher, being reported as 4.9 cases per 100,000 people (19,20). Despite this, no study has been published, in the country, on the genetics of ALS.

The population of Antioquia (Colombia), due to historical processes of its foundation, may have a component of ancestry of European origin, with a proportion of 70%, more precisely from the Iberian peninsula (3,21,22). Additionally, Antioquia is considered a genetic isolate due to population bottlenecks and endogamy (23). Hence, this population and others with similar characteristics are recommended for the genetic analysis of complex diseases such as ALS, since they tend to show lower etiologic heterogeneity (6,21). All of this could explain the relative high prevalence of this and other neurodegenerative diseases in Antioquia (19).

Knowing ALS genetic traits provides advantages for the development of diagnostic and prognostic biomarkers with utility both in the clinical practice and in the development of therapeutic trials for this disease, so far incurable. To date there is no published genetics study of ALS in Colombia. The purpose of this research was to evaluate the presence of mutations *D90A, G93A* [*SOD1*] and the hexanucleotide repetition [*C9orf72*] in ALS patients in Antioquia (Colombia).

Materials and methods

Population sample

Patients found in the databases of the Neuromuscular Disorders Service at Neuroclínica IPS Medellín, Antioquia whofulfilled the Escorial and Awaji-Shima diagnostic criteria (24) were included in the study. Only patients from Antioquia, diagnosed in 2017 and whose ancestors had been born in Antioquia, were included. Informed consent was obtained from all participants. This study was approved by the CES University Medical Ethics Committee

DNA extraction and genotyping

Blood samples were used to obtain DNA through extraction with the Wizard Genomic DNA Purification Kit (Promega, USA) and following manufacturer's instructions. For the genotyping of *SOD1* variants, *D90A* and *G93A* [*rs80265967* SNP, *rs121912438* SNP], besides the samples to evaluate, positive and negative controls were used for the experiments. For these, 10% of the data was repeated for a blind evaluation, with the purpose of confirming the reproducibility of the genotypes obtained through the KASP-PCR methodology (performed by LGC Genomics Inc., USA). Conventional PCR was performed to genotype the *C9orf72* STR; the primers and methodology are described by DeJesus-Hernández *et al*., (2011) (25), PCR was amplified in all patient using the genotyping primers F: FAM-CAAGGAGGGAAACAACCGCAGCC and R: GCAGGCACCGCAACCGCAG. DNA amplification was confirmed through electrophoresis in 1% agarose gel stained with ethidium bromide; alleles were resolved through capillary electrophoresis using an ABI PRISM 3130 (IdentiGEN Laboratory, Colombia).

Statistical analysis

Correlation between the ALS status and allele variants was performed descriptively and mathematically using Fisher's Exact Test (26). Population genetics parameters such as allele and genotype frequencies as well as Hardy-Weinberg Equilibrium, were also calculated using Genepop version 4.2 [GENEPOP, RRID:SCR_009194] (27,28). The endogamy model was evaluated through a X^2 test and the selection model through calculations for different selection coefficients. All of this was done considering that by having chosen individuals already diagnosed with ALS an artificial selection was exerted over the study sample.

Results

Out of a total of 63 cases diagnosed and confirmed that year for the disease, 34 ALS patients, evaluated and followed during 2017, were included in the study. The size of this study's sample represents 54% of ALS cases diagnosed in 2017 in Antioquia, Colombia.

Clinical characteristics

Approximately 32% of the patients present more than 5 years of disease duration. No patient had a family history of ALS although 26% reported a family history of other neurodegenerative diseases. In 19% of the cases the disease began before 35 years of age.

Table 1 shows the characteristics of the patients classified by age ranges of onset of symptoms. An earlier ages of symptoms onset were correlated with longer duration of disease (CDC= -0,37 and P=0,03). a separation by age group was made to show the difference in the age of onset of ALS in the population of

Antioquia, with regard to the usual age of onset described in other populations (figure 1).

Allele frequencies

With regard to the mutations of the *SOD1* gene: *G93A* and *D90A*, all patients evaluated presented the ancestral genotypes (GG and AA, respectively), thus being these monomorphic locus.

For the *C9orf72* gene, 6 different alleles were found in 33 samples analyzed. (table 2).

The allele of 8 repeats of the hexanucleotide expansion GGGGCC, was found in 84,85% (28 of 33 people) of the samples from patients with ALS (figure 2).

The *C9orf72* STR no was found to be in Hardy-Weinberg Disequilibrium ($P < 0,01$). *SOD1* variants could not be evaluated with this parameters due to the finding of ancestral (no mutated) genotype on all the patients. Upon performing the calculations to evaluate the selection model against the genotypes not carrying Allele of 8 repeats, with different values of selection coefficient, it was found that when selection coefficient = 0,9 the genotype frequencies are similar to the frequencies observed in the data from the patients (table 3).

Correlations between variables and the predominant genotype

Upon performing Fisher's Exact Test it was found that there is a greater frequency of genotypes with Allele of 8 repeats in patients below 65 years of age ($X^2 = 6,72$, $P < 0,01$), that this genotype is more frequent in patients with spinal onset (with a $X^2 = 8,90$ and $P < 0,01$), and that there is no difference in its distribution with respect to the duration of the disease (with a $X^2 = 0,22$ and $P = 0,64$).

Discussion

This study evaluated the association between variants in *SOD1* and *C9orf72* and the occurrence of ALS, and found the existence of an 8 repeat allele in the *C9orf72* locus with a high frequency amongst ALS cases in Antioquia, Colombia.

All cases included in this study were sporadic ALS diagnosed at youngers ages (<- 65 years old) than the average 65 years of age reported in other populations. None of the patients had a history of ALS in their family. This is of remarkable importance since it is very likely that the genetic bases differ between familial and sporadic ALS (29).

In this investigation we had 78,13% of patients whose age of onset for the disease was between 20 - 65 years (figure 1) and an average age of 54 +/- 15,23 years, which differs from what has been reported in the literature; an age of onset of 65 years, in other populations (30). The duration of the disease in the study group was higher than that reported in global literature, which can be explained by a larger proportion of young patients. As it has been described, an earlier age of onset of the symptoms, a greater life expectancy of the patients (31,32); a phenomenon observed in this research.

Although environmental factors that explain the particularities of the group of patients must always be taken into account, it is possible genetics has a crucial role. First, Antioquia is a genetic isolate and has experienced population bottlenecks and endogamy, which produce an effect of reducing etiologic heterogeneity and leads to an increase in hereditary or rare diseases prevalence (21,33). Adding to this is the action of a phenomenon called genetic anticipation, which produces an effect of greater severity of a phenotype and/or makes its time

of onset come earlier (34). In general, anticipation occurs when there is repetition and expansion on triplets of DNA sequences, as is the case of the *C9orf72* marker (17), hexanucleotide repeat elongation with variants in other ALS causative genes are associated with a younger age of onset, suggesting that both mutations affect the onset of the disease (35). Allele (CAGCAG)8, present in either homozygous or heterozygous condition, was found in 88,46% of the patients, suggesting a dominant allele effect on the ALS phenotype. This same gene, has been previously associated with ALS It has been proposed that the *C9orf72* hexanucleotide repeat be considered the cause of ALS at 20-25 repeats (25,36); however, in recent research it has been reported that the number of repeats considered as pathologic varies according to the population (17,19,37). Given the reported unusual genetic characteristics of the population of Antioquia, we do not rule out a phenotypic association of ALS to an allele with a number of repeats lower than that reported for the *C9orf72* gene, nor do we rule out this allele as a risk factor for the disease. For this reason, it is necessary to complement the current study with a control population. This will allow determining the distribution of alleles in the general population and confirm that the observations registered are not due to chance. Despite it was expected to find mutations *G93A* and *D90A* of the *SOD1* gene, they were not present in any of the patients of the sample studied. Hypotheses that could explain this phenomenon are: the migration of these mutations to the study population is scarce or nil; or that the mutations are expressing a different phenotype due to additional genetic and environmental factors (3,4,22).

Given that the *C9orf72* locus was, of the three studied in the diagnosed patients, the polymorphism related to ALS in Antioquia, corresponding analyses were

performed. It was found that the data do not fit the Hardy-Weinberg Equilibrium model (table 3), which could be indicative of a direct association with ALS (38,39). It is known that when an allele/genotype is associated to a phenotype this one can be augmented (risk) or diminished (protection) causing a deviation from the model, just as is shown in our research data (40). However, there are multiple causes for which a population may be out Hardy-Weinberg Equilibrium, such as selection, endogamy, genetic flux or genotyping errors (39,40). However, several of them can be ruled out by the use of controls in the execution of the experiments and by the differences between observed and expected heterozygotes, which are not significant in our data. With these alleles, 21 possible combinations could have been generated, but of them only 5 were found in the study population, which suggests an effect of the selection made on the samples (previously diagnosed patients) and this reinforces the possibility of an association between this loci and the occurrence of ALS.

Furthermore, allele C9orf7 (CAGCAG)8 was most commonly observed in patients below 65 years of age than in their older counterparts (> 65 yo) supporting the effect of genetic anticipation in this population. In our sample, allele C9orf7 (CAGCAG)8 carriers were more likely to have an spinal onset; This predominance of spinal over the bulbar ALS in carriers of the C9orf72 variant in our cohort can be explained by the small sample of patients, since in international reports this variant has been associated with both spinal and bulbar ALS (16). The number of hexanucleotide repeats in *C9orf72* that has been considered pathologic or as the cause of ALS is of 20-25 (25,36). In our study, the allele candidate for being related to the disease has only 8 repeats. While this variant has not been associated

previously with ALS cases, the relationship of the disease with the number of repeats is highly variable between different populations, lacking a definitive correlation between specific number and occurrence (37); analysis of common causal variants in different populations are important in investigating the pathophysiology of ALS in each population would have valuable implications (41,42).

This, along with the complete absence of *G93A* and *D90A* mutations of *SOD1* in the patients, is an unexpected and relevant finding about ALS genetics, its physiopathology and other neurodegenerative conditions. It is therefore necessary to confirm whether this genotype of 8 repeats in *C9orf72* is pathologic in our population and determine its importance as a diagnostic biomarker or as a risk factor for ALS, as is currently being done in other populations (43,44). It is also important to define the influence of homozygosity or heterozygosity of these repetitions on the risk of having the disease. Considering the great relevance of these findings for ALS genetics, and their clinical implications, it is necessary to carry out further studies to confirm the hypotheses we have presented.

Acknowledgements

We thank Universidad CES for its support in the realization of this work; Andrés Pareja López, Nicolás González Castro and Pablo Andrés Guzmán for their comments on the manuscript; Adriana Monsalve and Eliana Giraldo for their help in the collection of samples. We also thank Neuroclínica IPS in Medellín for their willingness to review clinical records, and cooperation for clinical evaluations and patient recruitment.

We thank the non-for-profit organization Huitaca (www.projecthuitaca.org) for providing English editing services to the final draft of this manuscript. Finally, we thank all the patients, who voluntarily participated and allowed this study to be carried out.

Conflict of interest

The authors declare no conflict of interest.

Financing

The authors certify the reception of funding for the research Project. Monetary funding was received from the Direction of Knowledge Management (*Dirección de Gestión del Conocimiento*) of Universidad CES, for the research with the project code INV032016008. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. **Yamashita S, Ando Y.** Genotype-phenotype relationship in hereditary amyotrophic lateral sclerosis. Transl Neurodegener. 2015;4:13. https://doi.org/10.1186/s40035-015-0036-y

2. **Turner MR, Verstraete E.** What does imaging reveal about the pathology of amyotrophic lateral sclerosis? Curr Neurol Neurosci Rep. 2015;15:45. https://doi.org/10.1007/s11910-015-0569-6

3. **Hamosh A.** Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. Nucleic Acids Res. 2004;33:D514–7. https://doi.org/10.1093/nar/gki033

4. **Yu W, Gwinn M, Clyne M, Yesupriya A, Khoury MJ.** A navigator for human genome epidemiology. Nat Genet. 2008;40:124–5. https://doi.org/10.1038/ng0208-124

5. **Rohrer JD, Isaacs AM, Mizielinska S, Mead S, Lashley T, Wray S,** et al. C9orf72 expansions in frontotemporal dementia and amyotrophic lateral sclerosis. Lancet Neurol. 2015;14:291–301. https://doi.org/10.1016/S1474-4422(14)70233-9

6. **Wijesekera LC, Leigh PN.** Amyotrophic lateral sclerosis. Orphanet J. Rare Dis. 2009;4:3. https://doi.org/10.1186/1750-1172-4-3

7. **Ajroud-Driss S, Siddique T.** Sporadic and hereditary amyotrophic lateral sclerosis (ALS). BBA -Mol Basis of Dis. 2015;1852:679–84. https://doi.org/10.1016/j.bbadis.2014.08.010

8. **De Jong SW, Huisman MHB, Sutedja NA, van der Kooi AJ, de Visser M, Schelhaas HJ**, et al. Smoking, alcohol consumption, and the risk of amyotrophic lateral sclerosis: a population-based study. Am J Epidemiol. 2012;176:233–9. https://doi.org/ 10.1093/aje/kws015

9. **Saez-Atienzar S, Bandres-Ciga S, Langston RG, Kim JJ, Choi SW, Reynolds RH, et al**. Genetic analysis of amyotrophic lateral sclerosis identifies contributing pathways and cell types. Sci Adv. 2021;7:eabd9036. https://doi.org/10.1126/sciadv.abd9036

10. **Özoğuz A, Uyan Ö, Birdal G, Iskender C, Kartal E, Lahut S, et al.** The distinct genetic pattern of ALS in Turkey and novel mutations. Neurobiol Aging. 2015;36:1764.e9-1764.e18. https://doi.org/10.1016/j.neurobiolaging.2014.12.032

11. **Bertolin C, D'Ascenzo C, Querin G, Gaiani A, Boaretto F, Salvoro C, et al.** Improving the knowledge of amyotrophic lateral sclerosis genetics: novel SOD1 and FUS variants. Neurob Aging. 2014;35:1212

12. **Byrne S, Elamin M, Bede P, Shatunov A, Walsh C, Corr B, et al.** Cognitive and clinical characteristics of patients with amyotrophic lateral sclerosis carrying a C9orf72 repeat expansion: a population-based cohort study. Lancet Neurol. 2012;11:232–40. https://doi.org/10.1016/S1474-4422(12)70014-5

13. **Ugolino J, Ji YJ, Conchina K, Chu J, Nirujogi RS, Pandey A, et al.** Loss of C9orf72 Enhances autophagic activity via deregulated mTOR and TFEB signaling. PLOS Genet. 2016;12:e1006443. https://doi.org/10.1371/journal.pgen.1006443

14. **Isobe T, Tooi N, Nakatsuji N, Aiba K.** Amyotrophic lateral sclerosis models derived from human embryonic stem cells with different superoxide dismutase 1 mutations exhibit differential drug responses. Stem Cell Res. 2015;15:459–68. https://doi.org/10.1016/j.scr.2015.09.006

15. **Mancuso R, Navarro X.** Amyotrophic lateral sclerosis: Current perspectives from basic research to the clinic. Prog Neurobiol. 2015;133:1–26. https://doi.org/10.1016/j.pneurobio.2015.07.004

16. **Renton AE, Majounie E, Waite A, Simón-Sánchez J, Rollinson S, Gibbs JR, et al.** A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-Linked ALS-FTD. Neuron. 2011;72:257–68. https://doi.org/10.1016/j.neuron.2011.09.010

17. **Ng ASL, Tan EK**. Intermediate C9orf72 alleles in neurological disorders: does size really matter? J Med Genet. 2017;54:591–7. https://doi.org/10.1136/jmedgenet-2017-104752

18. **Ospina ML, Prieto FE, Pachecho O, Quijano H, LozanoN, Goméz S, et al.** Boletín epidemiológico semana 5. 2019. Fecha de consulta: 4 de febrero de 2021. Disponible en: https://www.ins.gov.co/buscador-eventos/BoletinEpidemiologico/2019%20Bolet%C3%ADn%20epidemiol%C3%B3gico%20semana%205.pdf

19. **Zapata-Zapata C, Franco-Dáger E, Solano-Atehortúa J, Ahunca-Velásquez L.** Esclerosis lateral amiotrófica: actualización. IATREIA. 2016;29:194-205. https://doi.org/10.17533/udea.iatreia.v29n2a08

20. **Zapata-Zapata CH, Dáger EF, Aguirre-Acevedo DC, de Carvalho M, Solano-Atehortua J.** Prevalence, incidence, and clinical-epidemiological characterization of amyotrophic lateral sclerosis in Antioquia: Colombia. Neuroepidemiology. 2020;54:251-8. https://doi.org/ 10.1159/000504549

21. **Nikali K, Vanegas JJ, Burley MW, Martinez J, Lopez LM, Bedoya G, et al**. Extensive founder effect for distal renal tubular acidosis (dRTA) with sensorineural deafness in an isolated South American population. Am J Med Genet. 2008;146A:2709–12. https://doi.org/ 10.1002/ajmg.a.32495

22. **Carvajal-Carmona LG, Soto ID, Pineda N, Ortíz-Barrientos D, Duque C, Ospina-Duque J, et al.** Strong amerind/white sex bias and a possible sephardic contribution among the founders of a population in Northwest Colombia. Am J Hum Genet 2000;67:1287–95. https://doi.org/10.1016/s0002-9297(07)62956-5

23. **Arcos-Burgos M, Muenke M**. Genetics of population isolates. Clin Genet. 2002;61:233–47. https://doi.org/ 10.1034/j.1399-0004.2002.610401.x

24. **De Carvalho M, Dengler R, Eisen A, England JD, Kaji R, Kimura J, et al**. Electrodiagnostic criteria for diagnosis of ALS. Clin Neurophysiol. 2008;119:497-503. https://doi.org/ 10.1016/j.clinph.2007.09.143

25. **DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, et al**. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-Linked FTD and ALS. Neuron. 2011;72:245–56. https://doi.org/10.1016/j.neuron.2011.09.011

26. **Martín Andrés A.** Entry Fisher's exact and Barnard's tests. In: Kotz S, Johnson NL, Read CB, editors. Encyclopedia of Statistical Sciences. Vol. 2. New York, NY, USA: Wiley-Interscience; 1998. p. 250-8. https://doi.org/10.1002/0471667196.ess0642.pub2

27. **Rousset F.** genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Resour. 2008;8:103-6. https://doi.org/10.1111/j.1471-8286.2007.01931.x

28. **Raymond M, Rousset F.** GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. J Hered. 1995;86:248–9. https://doi.org/10.1093/oxfordjournals.jhered.a111573

29. **Couthouis J, Raphael AR, Daneshjou R, Gitler AD.** Targeted exon capture and sequencing in sporadic amyotrophic lateral sclerosis. PLoS Genet. 2014;10:e1004704. https://doi.org/10.1371/journal.pgen.1004704

30. **Marin B, Boumédiene F, Logroscino G, Couratier P, Babron MC, Leutenegger AL, et al.** Variation in worldwide incidence of amyotrophic lateral sclerosis: a meta-analysis. Int J Epidemiol. 2017;46:57-74. https://doi.org/10.1093/ije/dyw061

31. **Chiò A, Logroscino G, Hardiman O, Swingler R, Mitchell D, Beghi E, et al**. Prognostic factors in ALS: A critical review. Amyotroph Lateral Scler. 2009;10:310-23. https://doi.org/10.3109/17482960802566824

32. **Logroscino G, Piccininni M.** Amyotrophic Lateral Sclerosis Descriptive Epidemiology: The Origin of Geographic Difference. Neuroepidemiology. 2019;52(1–2):93–103. https://doi.org/10.1159/000493386.

33. **Carvajal-Carmona LG, Ophoff R, Service S, Hartiala J, Molina J, Leon P, et al.** Genetic demography of Antioquia (Colombia) and the Central Valley of Costa Rica. Hum Genet. 2003;112:534-41. https://doi.org/10.1007/s00439-002-0899-8

34. **Chiurazzi P, Oostra BA.** Expanding Mutations/Genetic Anticipation. Encyclopedia of Life Sciences]. Chichester, UK: John Wiley & Sons, Ltd; 2006. https://doi.org/10.1038/npg.els.0001463

35. **Suzuki N, Nishiyama A, Warita H, Aoki M.** Genetics of amyotrophic lateral sclerosis: seeking therapeutic targets in the era of gene therapy. J Hum Genet. 2022. https://doi.org/10.1038/s10038-022-01055-8

36. **Byrne S, Heverin M, Elamin M, Walsh C, Hardiman O**. Intermediate repeat expansion length in C9orf72 may be pathological in amyotrophic lateral sclerosis. Amyotrop Lateral Scler Frontotemporal Degener. 2014;15:148–50. https://doi.org/10.3109/21678421.2013.838586

37. **Yang Q, Jiao B, Shen L.** The Development of C9orf72-Related Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Disorders. Front Genet. 2020. https://doi.org/10.3389/fgene.2020.562758

38. **Mitteroecker P, Cheverud JM, Pavlicev M**. Multivariate analysis of genotype–phenotype association. Genet. 2016;202:1345–63. https://doi.org/10.1534/genetics.115.181339

39. **Namipashaki A, Razaghi-Moghadam Z, Ansari-Pour N.** The essentiality of reporting hardy-weinberg equilibrium calculations in population-based genetic association studies. Cell J. 2015;17:187-92. https://doi.org/10.22074/cellj.2016.3711

40. **Hedrick PW**. Genetics of populations. 4th ed. Sudbury, Mass: Jones and Bartlett Publishers; 2011. p. 65-109.

41. **Nakamura R, Misawa K, Tohnai G, Nakatochi M, Furuhashi S, Atsuta N, et al**. A multi-ethnic meta-analysis identifies novel genes, including ACSL5, associated with amyotrophic lateral sclerosis. Commun Biol. 2020;3:526. https://doi.org/10.1038/s42003-020-01251-2

42. **Mesaros M, Lenz S, Lim W, Brown J, Drury L, Roggenbuck J.** Investigating the genetic profile of the amyotrophic lateral sclerosis/frontotemporal dementia (ALS-FTD) continuum in patients of diverse race, ethnicity and ancestry. Genes (Basel). 2021;13:76. https://doi.org/10.3390/genes13010076

43. **Štětkářová I, Ehler E**. Diagnostics of amyotrophic lateral sclerosis: up to date. Diagnostics (Basel). 2021;11:231. https://doi.org/10.3390/diagnostics11020231

44. **Huang F, Zhu Y, Hsiao-Nakamoto J, Tang X, Dugas JC, Moscovitch-Lopatin M, et al.** Longitudinal biomarkers in amyotrophic lateral sclerosis. Ann Clin Transl Neurol. 2020;7:1103–16. https://doi.org/10.1002/acn3.51078

45. **Alvarez-Fernandez A, Bernal MJ, Fradejas I, Martin Ramírez A, Md Yusuf NA, Lanza M, et al.** KASP: a genotyping method to rapid identification of resistance in Plasmodium falciparum. Malar J. 2021;20:16. https://doi.org/10.1186/s12936-020-03544-7

Table 1. Clinical characteristics of 32 ALS patients group by age of onset.

Age of onset	Number of patients	Spinal onset	*NDD	Tobacco use	Exposure to **substances	Average duration of the disease
20-35	6	4	1	0	3	78 months
36-50	3	3	1	0	1	77 months
51-65	17	13	5	13	6	52 months
66-80	6	2	2	2	2	34 months

*****NDD: Familial history of neurodegenerative diseases

**Substances: Alcohol, cocaine derivatives, solvents and detergents

Table 2. Genotypes for *C9orf72* locus and repeats

Genotypes	Observed	Expected
(CAGCAG)1/(CAGCAG)8	12	8,12
(CAGCAG)8/(CAGCAG)8	16	14,55
(CAGCAG)11/(CAGCAG)12	1	0,02
(CAGCAG)13/(CAGCAG)13	3	0,32
(CAGCAG)13/(CAGCAG)14	1	0,11

Table 3. Observed frequencies v. Calculated model frequencies

Genotype	**Observed frequency**	**Expected frequency with S=0,9 and a dominant model for allele 2**
(CAGCAG)1/(CAGCAG)8	0,3636	0,3195
(CAGCAG)8/(CAGCAG)8	0,4848	0,5858
(CAGCAG)11/(CAGCAG)12	0,0303	0,0001
(CAGCAG)13/(CAGCAG)13	0,0909	0,0015
(CAGCAG)13/(CAGCAG)14	0,0303	0,0004

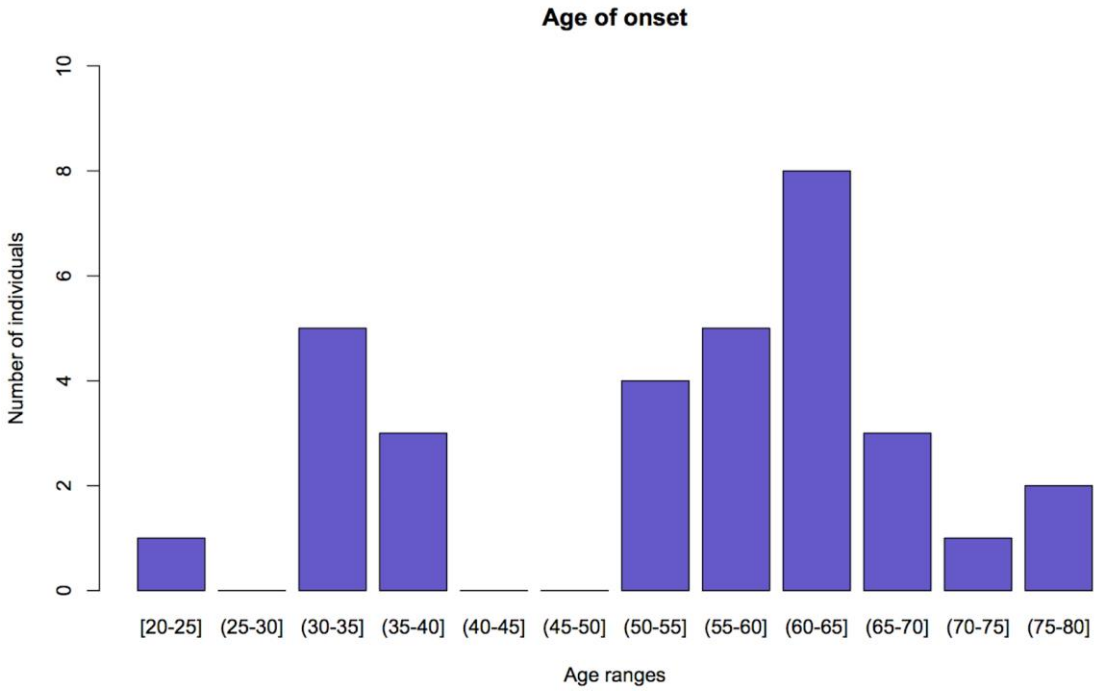

Figure 1. Age of onset and number of patients. The graph shows the distribution of patients according to the age of onset of ALS, presented as age ranges. The Y axis represents the number of patients, as the X axis represents age ranges. Figure made using R version 3.3.2 (R Project for Statistical Computing, RRID:SCR_001905).

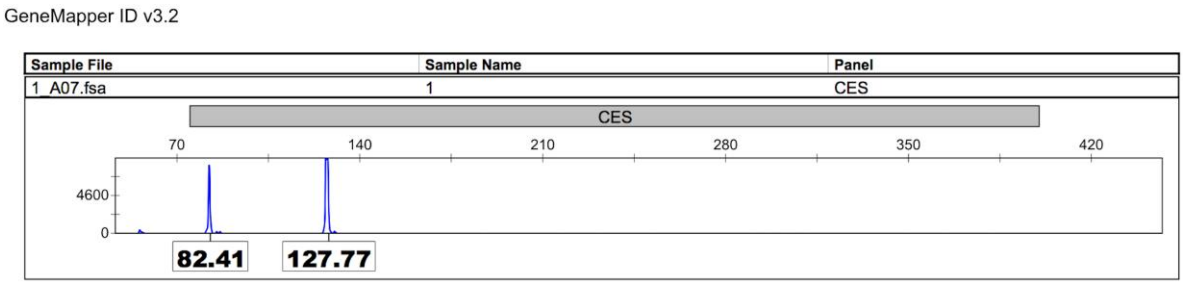

Figure 2. *C9orf72* alleles. Result from the capillary electrophoresis, using an ABI PRISM genetic analyzer. Alleles are represented in the X axis, with relative fluorescence units (RFUs) shown in the Y axis. In the result the alleles are identified with number of base pairs, like this: (82. 41pb): Allele 1; (127.77pb): Allele 2.