RESEARCH ARTICLE

REVISED Analysis of *C9orf72* repeat expansions in Georgian patients with Amyotrophic lateral sclerosis (ALS) [version 2; peer review: 2 approved]

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Abstract

Background

Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disorder that affects the upper and lower motor neurons. Several genetic risk factors have been identified in the past decade with a hexanucleotide repeat expansion in the *C9orf72* gene being the most significant. However, the presence of *C9orf72* repeat expansion has not been examined in the Transcaucasian region, therefore we aimed to analyse its frequency in Georgian patients with ALS.

Methods

We included 64 self-reported Georgian patients with ALS from different parts of the country, fulfilling the Gold Coast criteria. To investigate the presence of an expanded GGGGCC hexanucleotide repeat in the non-coding region of the *C9orf72* gene, we performed Repeat-Primed PCR (RP-PCR).

Results

Open Peer Review

Approval Status ✓ ✓

	1	2
version 2 (revision) 06 Mar 2024		✓ view
version 1 07 Sep 2023	✓ view	? view

1. **Karri Kaivola**, University of Helsinki, Helsinki, Finland

2. **Naoki Suzuki**, Tohoku University Graduate School of Medicine, Sendai, Japan

Any reports and responses or comments on the article can be found at the end of the article.

In total, 62 sporadic and two familial ALS cases were identified. Patients were aged 26 to 84 years with a mean age of 58.3 years at disease onset. Bulbar onset was observed in 21.88%, upper limb onset in 34.38%, and lower limb onset in 43.75% of the patients. Frontotemporal dementia (FTD) fulfilling the Strong criteria was diagnosed in seven patients (10.94%). *C9orf72* repeat expansion was detected in only one case using RP-PCR; the patient had a family history of dementia.

Conclusions

Our results indicate that *C9orf72* hexanucleotide expansion does not belong to the major genetic risk factor of ALS in Georgian patients. Further genetic studies in a bigger study population are needed to reveal the genetic causes of ALS in the Transcaucasian population.

Keywords
ALS, MND, Gene, C9orf72 , Georgia, DNA, Genetics, Genomics

This article is included in the Genomics and Genetics gateway.

This article is included in the University College London collection.

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REVISED **Amendments from Version 1**

Changes were made in version 2. Abstract: specifically in result section number of sporadic cases was 62, and in previous abstract there was a typo.

As reviewer 2 suggested, we have added demographic information about Georgia with the reference N4, and the reason why 50 participants would be scientifically significant number in the methods section. Changes were made in accordance of reviewer comments.

Any further responses from the reviewers can be found at the end of the article

List of Abbreviations

ALS: Amyotrophic Lateral Sclerosis
FTD: Frontotemporal dementia
LL-ALS: Lower Limb onset Amyotrophic Lateral Sclerosis
LMN: Lower Motor Neuron
PBP: Progressive Bulbar Palsy
PLS: Primary Lateral Sclerosis
PMA: Progressive Muscular Atrophy
RP-PCR: Repeat-Primed Polymerase Chain Reaction
TSMU: Tbilisi State Medical University
UCL: University College London
UL-ALS: Upper Limb onset Amyotrophic Lateral Sclerosis
UMN: Upper Motor Neuron

Introduction

"Does it take place through simple propagation, extending gradually across the neuroglia?".[1] This is what French neurologist J. M Charcot questioned regarding the disease development of amyotrophic lateral sclerosis (ALS) almost 150 years ago. Although the pathogenesis of ALS is still unknown, extensive studies have revealed important genetic risk factors in the past decade. *C9orf72* (#MIM 105550), *SOD1* (#MIM105400), *TARDBP* (#MIM612069, #MIM612069), and *FUS* (#MIM608030) are the most frequently mutated genes that have been shown in ALS,[2,3] with the hexanucleotide repeat expansion in *C9orf72* being the most significant and accounting for 30–50% of familial and 7% of sporadic ALS cases in the European population.[2,3] However, the genetic basis of ALS has not been investigated in the Transcaucasian region. Therefore, we aimed to determine the frequency of *C9orf72* repeat expansion in Georgian patients with ALS.

Methods

In total, 64 self-reported ethnically Georgian patients with ALS have been included in the study. Georgians are the predominant ethnic group in Georgia (almost 90% of the population).[4] There was no prior dataset of ALS patients in Georgia, nor epidemiological data about the disease, despite considering small size of Georgian population and rarity of the disease worldwide, we estimated that at least 50 participants would be scientifically significant number for this research considering the low incidence of the disease worldwide and the population number of Georgians (3-3.5 million).[4] Ethical approval was obtained from Tbilisi State Medical University (TSMU) ethics committee (Date: 8th June 2020, approval no. N3-2020/80) and University College London (UCL) institutional board (Short Title:IGC, CI:Prof H Houlden, Sponsor EDGE ID:146653,IRAS Approval Number:310045, Protocol V1.12 22.06.2019). Before the study, written informed consent was obtained from all subjects or their legal representatives in cases where participants were unable to write and sign the form.

Patients were recruited via phone during the years of 2019-2023 and the database of the First University Clinic of TSMU as well as of collaborative clinics were utilized for the study. Participants were contacted first of all to get information if they were still alive or not, and ask if they would agree on genetic testing in the future. No information was collected at that point, nor blood samples, before ethical approval was granted and consent taken from participants.

Diagnosis of ALS was based on the new Gold Coast criteria, incorporating progressive motor impairment documented by history or repeated clinical assessment, preceded by normal motor function, and the presence of upper and lower motor neuron dysfunction in at least one body region, or lower motor neuron dysfunction in at least two body regions, most importantly excluding other diseases.[5] Patients were reevaluated according to the Gold Coast criteria, however prior diagnosis and assessments were done by involved clinics. Patients diagnosed with conditions such as spinal muscular atrophy, Kennedy disease, monomelic amyotrophy, Hirayama syndrome, or multifocal motor neuropathy were excluded

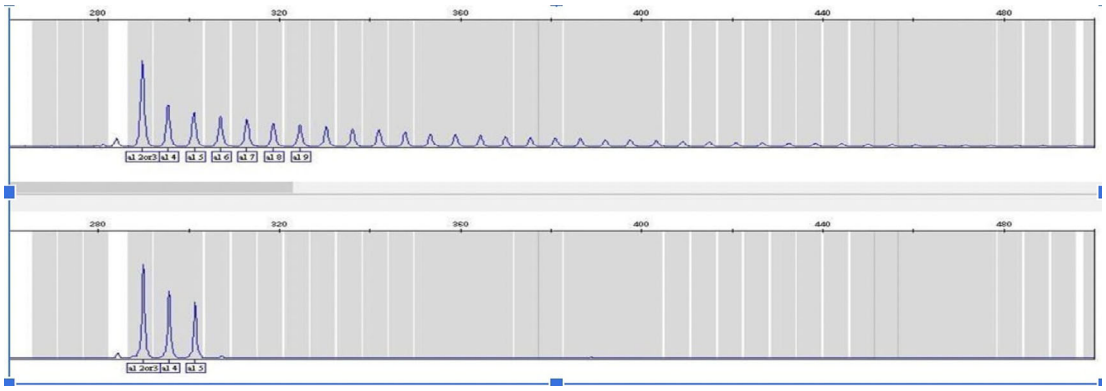

Figure 1. RP-PCR targeting the GGGGCC repeated hexanucleotide the plot in the top panel shows results from a positive control with the expanded repeats, and the bottom panel shows results from one of the non-expanded Georgian cases. This figure is an original figure produced by the authors for this article. RP-PCR, Repeat-Primed PCR.

from the study, there were no other exclusion criteria. Also, clinical symptom studies were conducted using the Mayo Clinic Laboratory Neurological Questionnaire. Patients were distributed into clinical subtypes of ALS: typical, progressive muscular atrophy, primary lateral sclerosis, and progressive bulbar palsy. Furthermore, based on site of onset they were divided into three groups: bulbar onset, lower limb onset, upper limb onset. Cognitive changes were assessed using Addenbrooke's Cognitive Examination Scale (ACE III) and the Frontal Behavioral Questionnaire, Frontotemporal dementia (FTD) was diagnosed according to the Strong critiera,[6] and the patient's quality of life was assessed by ALSFRS-R.

Venous blood samples were collected in Georgia by MK, 5 ml venous blood samples were taken from the median vein of the forearm, EDTA k2 Vacutainers were used for storage and transferred to UCL Queen Square Institute of Neurology, Neurogenetics Laboratory for further research. The genomic DNA of the included subjects was extracted from whole blood using the Promega ReliaPrep™ Blood gDNA Miniprep System using manufacturers instructions.

To investigate the presence of an expanded GGGGCC hexanucleotide repeat in the non-coding region of the *C9orf72* gene, we performed Repeat-Primed PCR (RP-PCR) in all patients and in three positive controls. The primers and thermocycling conditions used for the assay have been previously described.[7] RP-PCR is able to determine whether an expanded allele is present in an individual, in which case a characteristic stutter pattern is seen[8] (Figure 1).

All primers were used with the same molar concentrations. A PCR Mastermix was prepared by mixing 12.5 µl of Amplitaq Gold 360 Master Mix (ThermoFisher), 9.5 µl of 5M Betaine (ThermoFisher), 1 µl of 10 pmol/µl FAM labelled Forward primer (5′-TGTAAAACGACGGCCAGTCAAGGAGGGAAACAACCGCAGCC-3′), 1 µl of 10 pmol/µl Reverse primer (5′-CAGGAAACAGCTATGACC-3′), 1 µl of 10 pmol/µl repeat specific reverse primer (5′-CAGGA AACAGCTATGACCGGGCCCGCCCCGACCACGCCCCGGCCCCGGCCCCGG-3′) and 1 µl of 100 ng/µl DNA. Samples were amplified with an initial heat denaturation of 95°C for 10 minutes, followed by 10 cycles of 95°C for 30 seconds, 58°C for 2 minutes, 72°C for 2 minutes, and then 25 cycles of 95°C for 10 minutes, followed by 10 cycles of 95°C for 30 seconds, 58°C for 2 minutes, 72°C for 2 minutes with a 20 seconds increase per cycle. The final extension step was 72°C for 7 minutes. The PCR was run on a 9700 Block, at ramp speed 9600. After PCR, 1 µl of the reaction product was added to a mix with 9.2 µl of Formamide (Roche) and 0.1 µl of GeneScan 500 LIZ Size Standard (ThermoFisher). After a denaturation step at 95°C for 5 minutes, samples were analyzed using the ABI 3739 Genetic Analyser. Data were analyzed with the GeneMapper (RRID:SCR_014290) software (v. 4.0, Applied Biosystems).

Results

Patients with ALS were aged 26 to 84 years with a mean age of 58.3 years at disease onset. In total, 63.8% of the patients were 50–69 years old. A total of 51% of the patients were male, 49% were female with a male-to-female ratio of 1:1.

After initial examination of 70 patients for eligibility, two patients were not confirmed to be eligible in the first stage, due to misdiagnosis of ALS, one of them had myasthenia gravis (MG) with anti-MUSK antibodies and the second patient had SMA (Spinal muscular atrophy) type 4. Four patients were not included in the study after being confirmed eligible, due to geographical conditions researchers were not able to get samples, and patients were not mobile, thus it was impossible for them to be transferred to the hospital (Figure 2).

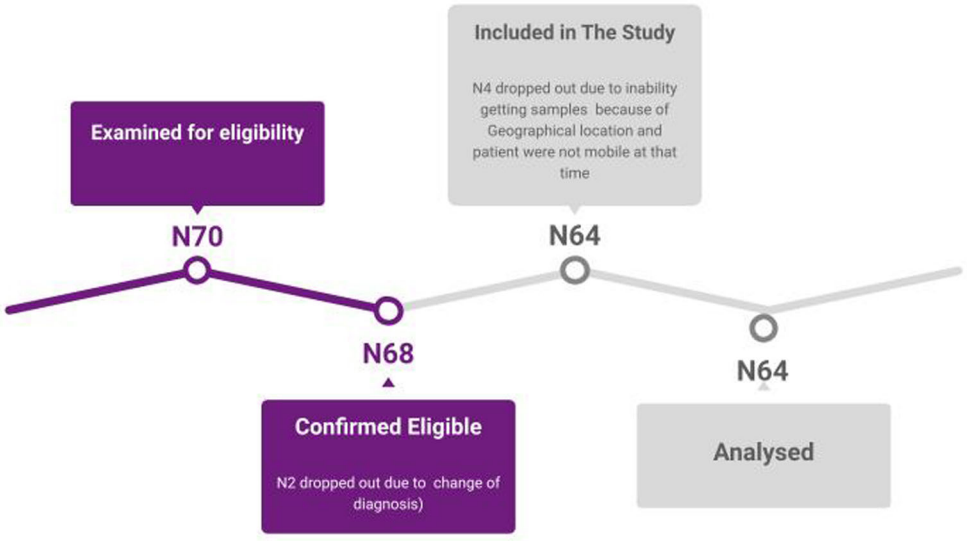

Figure 2. Representation of the number of participants at each stage. This figure is an original figure produced by the authors for this article.

Table 1. Numbers and percentages of different ALS phenotypes in Georgian patients according to neuronal level, site of onset, and presence of ALS/FTD.

Phenotypic variant	UMN	LMN	n	%
Neuronal level				
Typical ALS	+	+	60	93.7
PLS	++	-	1	1.56
PMA	-	++	3	4.69
PBP	+	+	0	0
Site of onset				
Bulbar ALS			14	21.88
UL-ALS			22	34.38
LL-ALS			28	43.75
Mill's (hemiplegic) variant			0	0
Flail Arm			0	0
Flail Leg			0	0
Presence of FTD				
ALS/FTD			7	10.94

ALS, amyotrophic lateral sclerosis; UMN, upper motor neuron; LMN, lower motor neuron; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; PBP, progressive bulbar palsy; UL-ALS, upper limb onset ALS; LL-ALS, lower limb onset ALS; FTD, frontotemporal dementia. + typical to a variable degree; ++ primary feature, - not a feature.

Bulbar onset ALS was observed in 21.3%, upper limb onset (UL-ALS) in 38.3%, and lower limb onset (LL-ALS) in- 40.4% of the patients (Table 1). FTD fulfilling the Strong criteria was diagnosed in seven patients (10.94%). Two patients (3.13%) have been identified to have familial ALS (FALS) based on family history.[9]

We screened all patients for GGGGCC hexanucleotide expansions between two 5′ non-coding exons of the *C9orf72* locus using RP-PCR. We used a reliable assay that confidently differentiates between positive and negative cases by detecting up to 40 repeats, thus categorizing them as pathogenic expansions. An accurate number of repeats in each allele can be detected in the negative cases. The repeat size in the general population has been observed to vary between two to 30 for healthy individuals, while affected people present at least one expanded allele with repeats ranging between 30 to

several hundred hexanucleotides,[7,8] please see Figure N1 for comparison After performing RP-PCR, GGGGCC expansion was observed in only one patient. Most of our cases presented a homozygous two-repeats expansion. The mean expansion in our cohort was 2+3.11 repeats (Allele1 2-2, Allele2 2-12).[10]

Discussion

A pathogenic repeat expansion in the non-coding region of the *C9orf72* gene has been described to be the most common risk to develop familial ALS. However, most studies have been performed in European study populations[11–14] and little is known from non-European countries. Based on these, a significant variation in c9orf-ALS frequency and distribution throughout Eurasia can be observed, with east Asian populations experiencing lesser cases, and India and Taiwan being exceptions (Figure 3).

In our study, we aimed to investigate the frequency of c9orf-ALS in Georgian patients. However, from our 64 patients only one tested positive for pathogenic GGGGCC repeat expansion, and the patient demonstrated bulbar onset ALS, with a family history of severe dementia, in particular, her sibling suffered from FTD, and her mother was diagnosed with dementia as well, however, the type was not specified in reports. FTD was suspected by clinicians according to reports. Our results possibly indicate a different genetic background and the presence of distinct risk factors for ALS in this ethnic group. The Georgian geography with its isolation and small population size, particularly in the highlands, might have led to the bottleneck effect and enhanced genetic differentiation seen in our data.

Country	fALS (%)	sALS (%)
Belgium	84	0
Sweden	63	14
The Netherlands	37	
Turkey	37	6
Italy	29.9	5.3
UK	26	6
Taiwan	18	2
India	12.5	2.7
Greece	10.95 overall	
Iran	5.9	1.6
Malaysia	3 overall	
Russia	0	2.3
Japan	0	0.4
China	0	0.3
South Korea	0	0
Georgia	0	1.5

Figure 3. Eurasia map depicting c9orf-ALS plus cases across Eurasia colored according to prevalence. The bottom part shows the list of c9orf72 sALS and fALS cases in Eurasian countries reported before.[11,13–26] This figure is an original figure produced by the authors for this article. ALS, Amyotrophic lateral sclerosis; sALS, sporadic ALS; fALS, familial ALS.

However, our results are limited due to the small cohort size. Further, the rare existence of the pathogenic repeat expansion could also be due to a previous single founder mutation. Smith *et al.* (2013) identified a haplotype that proves this point, that all massive GGGGCC hexanucleotide repeat expansion mutations—identified within intron 1 of *C9orf72*—carriers arose from a single common founder.[14] The most sensible explanation would therefore be that the expansion mutation arose on just one occasion in the European population. The results could further be explained by a close link between Georgian and Asian genetic pools, however, researchers reported that Caucasian groups were much closer to European than to West Asian groups with respect to mtDNA, opposite to be true for the Y chromosome, indicating a predominantly West Asian influence.[27]

Conclusions

Further genetic studies in a larger cohort are needed to confirm our results and to reveal genetic risks for ALS in the Transcaucasian population.

Ethics approval and consent to participate

Ethical approval was obtained from Tbilisi State Medical University (TSMU) ethics committee and University College London (UCL) institutional board. All experiments were performed in accordance with WMA declaration of Helsinki – Ethical principles for medical research involving human subjects. Informed consent was obtained from all participants. All participants consented in written form to participate in the study. All authors attest that the participants were aware of the study's purpose, risks, and benefits.

Data availability

Underlying data

Figshare: ALS Patients GEORGIA, https://doi.org/10.6084/m9.figshare.23731674.v2.[9]

This project contains the following underlying data:

- The spreadsheet data for all participants and outcomes underlying the Results section of the paper and Table 1.

Figshare: RP-PCR Traces, https://doi.org/10.5522/04/23661813.v1.[10]

This project contains the following underlying data:

- The raw data of the genetic testing (RP-PCR Traces).

Acknowledgments

Research was conducted as part of the Queen Square Genomics group at the University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. We are also grateful to Queen Square genomics at the Institute of Neurology University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre, for the bioinformatics support. For the purpose of Open Access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 09 March 2024

https://doi.org/10.5256/f1000research.163149.r253260

✔ **Naoki Suzuki**
Tohoku University Graduate School of Medicine, Sendai, Japan

The authors appropriately improved their manuscripts.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 19 January 2024

https://doi.org/10.5256/f1000research.151632.r238668

? **Naoki Suzuki**
Tohoku University Graduate School of Medicine, Sendai, Japan

The report focuses on investigating the frequency of C9ORF72 mutations in ALS cases in Georgia, an area that has not been previously studied. This report holds considerable significance.

Abstract:

In the abstract, it is mentioned that there are 64 self-reported patients; however, in the Results section, it is specified as 64 sporadic and 2 familial cases. To avoid confusion, please correct the total count to 66.

Methods:
In the Methods section, it is stated as "ethnically Georgian." Is it acceptable to assume that there are no individuals with mixed ethnic backgrounds, such as half or quarter Georgian? Additionally, basic demographic information about Georgia's population would be appreciated.

Results:
For cases with C9ORF72 expansion, one instance with a family history of dementia is noted. It would be beneficial to provide details on the type of dementia and its progression.

Participants:
The statement indicating that scientifically significant interpretations can be made with 50 or more participants is made. Could you please provide the basis for this claim?

Table 1:
In Table 1, PBP (Progressive Bulbar Palsy) is listed as zero, while Bulbar onset ALS includes 14 cases. Please clarify the definition of PBP and how Typical ALS is defined.

Figure 3:
Concerns are raised about the data from the Nordic region in Figure 3, as the percentages appear unusually high. Please verify the accuracy of the literature sources.

Typo：a single common founder. 37Themost sensible

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 07 November 2023

https://doi.org/10.5256/f1000research.151632.r218006

Karri Kaivola
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In their manuscript titled ”Analysis of C9orf72 repeat expansions in Georgian patients with Amyotrophic lateral sclerosis (ALS)”, Kekenadze *et al.* assess using repeat-primed PCR the *C9orf72* hexanucleotide repeat expansion frequency in 64 ALS patients from Georgia. The *C9orf72* hexanucleotide repeat expansion is the most common genetic risk factor in populations of European descent, but not in other populations.

Even though the study population is small, it is enough to rule out C9orf72 expansion as a common cause of ALS in Georgia as only one patient carried the mutation. The knowledge gained from this study is pretty much covered in the previous sentence, but the study is methodologically acceptable. There are minor things to address:

1. The introduction is very short and sweet. I was hoping more background on the C9orf72 expansion prevalence in nearby regions and what is known about ALS genetics prior to this study in Georgia. These questions were partly covered at the start of the Methods and Figure 3, I would bring some of that information to Introduction.

2. Also would be useful to know, is the Georgian population homogenous and does the study cohort represent the whole population or just some part of it?

3. In Methods the authors write “we estimated that at least 50 participants would be scientifically significant number for this research.” Estimated how and what does scientifically significant number mean? Is this based on e.g. C9orf72 expansion frequencies in neighboring countries?

4. In Results after listing excluded cases, the authors could repeat the final sample number, even though it is in Figure 2.

5. 1st sentence of 2nd paragraph in Discussion: “c9orf-ALS” is a bit colloquial, I would use the full gene name c9orf72.

6. There is a stray citation number 13 in discussion in sentence “identified within intron 1 of

C9orf72—carriers arose from a single common founder.[13]"

7. The C9orf72 prevalence in Figure 3 seems unbelievably high in Belgium and Sweden (63-84% of fALS cases). I would urge the authors to reassess the evidence for these numbers, as all the numbers I have seen from robust reports have been considerably smaller.

8. In Figshare: ALS Patients GEORGIA table, the full dates of births of the patients are visible. Given the rarity of the disease, this makes the patients very identifiable, and I strongly urge the authors to remove such personally identifiable information and replace them with e.g. age. They could also add the C9orf72 allele lengths to the table.

9. The single founder hypothesis of C9orf72 expansion is very contested[1], I would refrain from making statements about a single founder.

In conclusion, this is a small study that fills a small gap in knowledge, but the study is generally well made and presented.

References

1. Beck J, Poulter M, Hensman D, Rohrer JD, et al.: Large C9orf72 hexanucleotide repeat expansions are seen in multiple neurodegenerative syndromes and are more frequent than expected in the UK population.*Am J Hum Genet*. 2013; **92** (3): 345-53 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurogenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 15 Nov 2023

Mariam Kekenadze

Dear reviewer, Many thanks for your immense effort in reviewing our article, all the comments are much appreciated and looked at in detail. We would like to respond to some of them.

1. Georgia is located in the Caucasus, neighboring countries are Armenia, Azerbaijan, Turkey, and Russia. After a thorough search of the literature using various languages, we were not able to find any relevant information about c9orf72 expansion prevalence in Armenia and Azerbaijan, however, there are some data available from Turkey and Russia, demonstrating low incidence in later countries, we depicted following in Eurasia map, Figure 3. Furthermore, there have not been any studies, including genetics, in the ALS population prior to this one in Georgia, indicating the significance of this research.

2. There is sparse information regarding the genetic architecture of the Georgian population, therefore it is difficult to conclude the homogeneity of the population. The study indeed represents the whole Georgian population, samples were gathered from different parts of Georgia, and results are published in different article: https://pubmed.ncbi.nlm.nih.gov/37166887/

3. We concluded that scientifically significant results would represent identifying a similar number of ALS patients in the Georgian population size according to worldwide incidence/prevalence of the disease. Unfortunately, there is no data available regarding c9orf72 gene expansion prevalence in ALS patients in neighboring countries.

4. We agree with the reviewer.

5. C9orf72 positive cases in familial ALS in Belgium and Sweden, we used the following article: Smith BN, Newhouse S, Shatunov A, et al.: The C9ORF72 expansion mutation is a common cause of ALS+/-FTD in Europe and has a single founder. Eur. J. Hum. Genet. 2013; 21(1): 102–108, who reported these findings.

6. Thank you for pointing out the issue with privacy in Figshare, we will fix it.

Competing Interests: No competing interests were disclosed.