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Rare homozygosity in amyotrophic lateral sclerosis suggests the contribution of recessive variants to disease genetics

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ABSTRACT

Objective: to determine the occurrence of homozygous rare, *in-silico* damaging variants in a genetically relatively homogenous group of amyotrophic lateral sclerosis (ALS) patients.
Methods: Whole-exome-sequencing of 43 ALS patients of North-Africa Jewish origin was performed. Data were filtered to identify very rare homozygous recessive *in-silico* damaging variants, in genes annotated to ALS-associated cellular pathways.
Results: We identified a rare missense homozygous variant, p.Arg663Cys in *MFN2*, predicted to be damaging, in a patient with an early age at disease onset (36 years) and fast progression. An additional ALS patient carried the mutation and together established its association to ALS ($p = .01$). Additional homozygous variants were identified, including the risk allele p.Arg261His in *NEK1,* as well as variants in genes known to be associated with other neurodegenerative diseases, such as *HTT* (Huntington's disease), *ATM* (Ataxia-Telangiectasia), and *ZFYVE26* (SPG15), and variants in genes previously reported as upregulated (*LZTS3*) or downregulated (*ARMC4*, *CFAP54*, and *MTHFSD*) in ALS patients. Altogether, 13 patients (30%) carried at least one homozygous rare *in-silico* damaging variant, of them 10 carried either another rare homozygous variant and/or a variant in a known ALS gene, which is categorized as pathogenic, likely-pathogenic or variant of uncertain significance.
Conclusions: Our results suggest the contribution of recessive alleles to ALS and the possibility of burden of mutations, emphasizing the complexity of ALS genetics.

1. Introduction

Amyotrophic lateral sclerosis (ALS) affects different populations and ethnic groups worldwide with a similar incidence, with few known clusters in secluded populations [1,2]. It may appear as familial or sporadic, with a wide range of age at disease onset (AAO), from early 20s to late 80s. All three modes of inheritance were reported [3], though recessive inheritance is the least common and reported only in a few genes and rare cases such as *NEK1* [4], *OPTN* [5,6], *FUS* [7], *ALS2* [8,9], *SPG11* [10], and *SOD1* [11]. These reports described mainly cases of familial ALS, however recessive inheritance is rare and difficult to establish as it may appear as sporadic disease. The homozygous alleles that contribute to ALS might be numerous but rare, making their discovery by population-based methods difficult without large sample sizes. Homozygosity analysis is a relevant and powerful tool to identify rare recessive alleles in ALS. Recently, two population-based studies identified new risk alleles in ALS [12,13]. These studies show that there are more regions of homozygosity segments per case compared to controls, a greater proportion of cases harbor homozygosity, and a higher rate of these segments overlap with RefSeq gene regions, all statistically significant. Applying this analysis on a UK case-control cohort [13] identified positive association to ALS in three regions (chromosome 21 *SOD1* region, chromosome 1 and chromosome 5) encompassing more than 20 potential genes; and on an Irish case-control cohort [12] identified positive association to ALS in more than 30 loci. These studies point to further possible rare recessive genetic causes of ALS.

North Africa Jews (NAJ) constitute the second largest Jewish diaspora group, and genome-wide analysis shows that they create a distinct cluster, with proximity to other Jewish populations, and high

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Table 1
Clinical and epidemiological details of 43 Jewish ALS patients for whom whole-exomes were sequenced.

Phenotype class/study groups	MJ-ALS	NAJ-nm-ALS	Total
Number of patients (% of total)	31 (72.1)	12[a] (27.9)	43
Age at onset, yrs. (± SD)	52.7 (± 10.4)	45.8 (± 12.1)	50.8 (± 11.2)
Males (%)	19 (61.3)	10 (83.3)	29 (67.4)
Familial ALS[b] (%)	5[c] (16.1)	3[d] (25)	8 (18.6)
Disease duration, months[e] (± SD), n	42.7 (± 29.5), $n = 27$	36.6 (± 22.8), $n = 11$	41.0 (± 27.6), $n = 38$
Site of onset (%)			
Bulbar	6 (19.3)	4 (33.3)	10 (23.3)
Limb	25 (80.7)	8 (66.7)	33 (76.7)

MJ-Moroccan Jews, NAJ-nm- North African non-Moroccan Jews.

[a] Libya = 3, Tunisia = 5, Libya/Tunisia = 1, Morocco/Algeria = 2, Morocco/Egypt = 1.

[b] Familial ALS was defined as having at least one first- or second-degree relative with a diagnosis of ALS.

[c] Additional three and

[d] one patients had a questionable positive family history.

[e] Disease duration, defined as time from first symptoms to death or tracheostomy, was calculated only for patients deceased at the time of the data analysis (n, number of patients).

degree of endogamy due to religious constraints [14]. ALS is observed in this population apparently with a similar incidence as worldwide, but with earlier AAO [15]. We recently showed that *OPTN* 691_692insAG is a founder mutation causing recessive ALS in Jews of Morocco origin (MJ), as well as of Ashkenazi descent [5], with high frequency of homozygous (5.8%) among MJ-ALS patients. These findings suggest this unique patients' cohort as a powerful group to identify recessive mutations and risk factors for ALS. We used whole-exome-sequencing (WES) to reveal very rare recessive genetic variants in ALS in this patient group.

2. Material and methods

2.1. Population

Whole-exome-sequencing was carried out on 43 patients with ALS of North Africa Jewish origin, 40 were unrelated. Thirty-one patients were of Morocco descent, and 12 were from other North Africa countries (Table 1). Four patients carried the *C9orf72* hexanucleotide repeat expansion [16], three carried the *OPTN* 691_692insAG mutation in a heterozygous state [5], and one carried both. We previously demonstrated that our cohorts of NAJ patients and controls do not show a hidden- relatedness [5].

Two variants were genotyped on the complete cohort of unrelated 135 ALS patients of North Africa origin (that included an additional 95 unrelated ALS patients) and 400 controls of full Jewish Moroccan origin. The control samples were anonymous DNA samples from young healthy individuals, aged 20–45, mostly women who underwent routine genetic screening tests and were randomly selected.

All ALS patients were followed at the ALS Clinic at Tel-Aviv Medical Center, Tel-Aviv, Israel. All patients had a diagnosis of clinically definite or probable ALS according to the revised El Escorial criteria [17]. The recruitment interval spanned from 2004 to 2018.

For all patients, the following demographic and clinical data were collected: ancestry, family history of ALS, dementia or other neurodegenerative diseases, AAO, and affected site at disease onset. Disease duration (defined from first recalled symptom to death or tracheostomy) was recorded for all patients.

2.2. Standard protocol approvals, registrations, and patients consents

All participants provided informed consent before DNA collection. DNA samples were coded and tested in an anonymous manner. The Institutional and National Supreme Helsinki Committees for Genetics Studies approved the study protocol and the informed consent form.

2.3. Whole-exome-sequencing and data analysis

Exome libraries were prepared using Illumina Nextera Rapid Capture Exome kit or Illumina TruSeq Rapid Exome kit following manufacture protocol, and sequenced on the Illumina NextSeq500 sequencing platform, either with 300 cycles or with 150 cycles (Illumina Inc. San Diego, CA, USA).

The NextSeq500 system generated .bcl files, followed by demultiplexing of indexed reads and generation of FASTQ files. Alignment to the hg19 reference genome was done using BWA version 2.1 (BaseSpace OnSite, version 2.13, Illumina Inc. San Diego, CA, USA). Variants analysis and filtering was done using SNP & Variation Suite v8.7 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). We filtered-in variants in which at least one individual was homozygous. We then filtered based on quality scores, read depth, annotation, frequency, functional prediction scores by 11 algorithms (SIFT, Polyphen2 HDIV, Polyphen2 HVAR, LTR, MutationTaster, Mutation Assessor, FATHMM, PROVEAN, FATHMM MKL, MetaSVM, MetaLR), and gene ontology (Supplement Fig. 1). Filtering by gene ontology was done to include only variants in genes that are involved in the 5 main pathways implicated in ALS: autophagy, RNA-binding, mitochondria cell component, axonal transport, and cytoskeleton, a list that was generated using AmiGO2 (Supplement Table 1, http://amigo.geneontology.org/amigo). Finally, we applied allele frequency cut off from Chia *et al*, 2018 [18] and adjusted it to homozygosity inheritance to exclude variants with allele frequency higher than 0.007 in samples from individuals who were not ascertained for a neurological condition in a neurological case/control study in Genome Aggregation Database (non-neuro cases, gnomAD v2.1, [19]).

We examined if the 13 ALS patients who carried at least one homozygous rare variant, also carried non-homozygous variants in genes previously established as having an association with ALS. We applied the same pipeline described above to 29 ALS genes [18,20] (Supplement Table 2), while omitting the homozygosity and the functional prediction scores filters, and adjusting the control “non-neuro” gnomAD v2.1 frequency to 1%.

To establish the pathogenicity of all variants, we applied the joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology [21], and categorized them as benign, likely-benign, variant of uncertain significance (VOUS), likely-pathogenic, or pathogenic. We included only variants with VOUS, likely-pathogenic, or pathogenic scores.

2.4. Mutations screening

Validation of the *MFN2* and *NEK1* variants was confirmed by Sanger Sequencing using standard protocols (Supplement Table 3). The ALS patients and controls were genotyped using a TaqMan assay following standard protocols (Applied Biosystems, assay ID C_191333566_10 for *NEK1*, and a custom-made assay for *MFN2*).

2.5. Statistical analysis

Significance of homozygosity frequencies and allelic association were calculated with online calculators using two-tailed Fisher's exact test (http://graphpad.com; http://www.medcalc.org). Goodness of fit test with one degree of freedom was applied to look for any deviation from the Hardy-Weinberg equilibrium (HWE) in the control group.

3. Results

The mean AAO of all 43 patients of North Africa origin was 50.8 years (± 11.2, median = 55, range 22 to 74, Table 1), significantly lower than the AAO of ALS in general, similarly to data previously reported by us [15]. A positive family history (fALS) was noted in 8 patients (18.6%). Whole-exome sequencing depth coverage was between 87.3X and 240.5X in 35 samples sequenced with 300 cycles, and between 53.9X and 84.3X in 8 samples sequenced with 150 cycles.

3.1. An Arg663Cys homozygosity in MFN2 was observed in an early onset ALS patient

One patient carried a rare variant in the *MFN2* gene. The patient was homozygote at position Chr1:12067224 (hg19 assembly), NM_014874.3:c.1987C > T, resulting in a change of a highly conserved amino acid from Arginine to Cysteine (NP_055689.1:p.Arg663Cys). Her parents (from Libya) were not related according to a self-reported statement, and she had 3 healthy siblings. Her neurological disease began at age 36 with one-sided foot drop and recurrent falls, and progressed rapidly. At examination, she presented with severe flaccid weakness in both legs, very brisk reflexes in upper limbs and low to absent in lower limbs, up going toes, atrophy and fasciculations in her thighs and interossei of both hands. She did not have any sensory or bulbar symptoms, but had moderate to severe frontal behavior. EMG showed acute denervation and reinnervation in many muscles, with preserved motor and sensory conduction velocities and SNAP amplitudes. She died 20 months after her first neurological symptoms. She did not carry the repeat expansion in *C9orf72* [16].

This variant was also found in a heterozygous state in one additional fALS patient (AAO = 29) of Morocco/Algeria ancestry (Table 2). The variant was further genotyped on the complete cohort of ALS patients of North Africa origin and 400 MJ controls. No other ALS patient carried this *MFN2* variant and only one heterozygous carrier was identified in the control group (Table 2). Odd ratios for homozygosity and allelic were 8.93, $p = .18$ and 8.98, $p = .058$, respectively. Of note is that no homozygotes were reported in more than 100,000 non-neuro-individuals (genome aggregation database- gnomAD, Table 3). This allele frequency in Ashkenazi-Jews(AJ)-non-neuro cases in gnomAD was not significantly different from the observed frequency in our control cohort of Moroccan Jews (Fisher's exact $p = 1.0$). Therefore, both control groups were combined and compared to the NAJ-ALS patients, resulting in a significant difference for homozygosity (Fisher's exact test, $p = .0359$; Table 2). Allelic analysis shows significant association to ALS as well, with Odd's Ratio (OR) of 8.14 (95% CI of 2.23–29.76, $p = .0015$).

3.2. Rare homozygosity was observed in 30% of NAJ-ALS patients

A total of 23 rare *in-silico* deleterious homozygous variants in 22 genes were observed in 13 NAJ-ALS patients (30.2%, Table 3, Supplement Fig. 1, and Supplement Table 4). Each homozygous variant was identified in either one ALS patient, or in 2 related patients. All were classified as VOUS using the ACMG guidelines [21], except *ARMC4*-p.Val295Leu and *ZFYVE26*- p.Ile2029Thr, which were predicted as likely-benign. Two variants were loss-of-function (LoF) variants, one in *CCDC81*, and one in *CCDC40*. Nineteen variants were rare missense variants. Among them, the most deleterious ones (predicted as deleterious by 10 or all 11 algorithms), were variants in *MFN2*, *ACBD5*, *ARCM4*, and *MYO1A* (Table 3, Supplement Table 4). Two variants were in-frame deletion variants (*LZTS3* and *MRPL40*).

Of note is that 19/23 (83%) of the variants were not reported in a homozygous state in gnomAD database in non-neuro cases. Of them, two variants (*RARS*, p.Leu44Ser and MRPL40 c.120_122delAGG) are novel and were not reported in dbSNP and gnomAD databases, although these regions were well covered by NGS at 50X or more.

We identified one patient who was homozygote to *NEK1*-p.Arg261His risk allele. Another patient carried the same risk allele in a heterozygous state. Screening the entire NAJ-ALS patient cohort identified four additional carriers, with allele frequency of 2.6% (7/270) compared to 1.5% in our control group (12/800, OR 1.75, not significant).

In addition to Table 3, we detected one recessive variant, c.1355_1356delCA in *FAM161A* in one of the ALS individuals (data not shown). This variant is a known mutation segregating in NAJ and AJ, causing retinitis pigmentosa type 28. The ALS patient carrying this mutation suffered from blindness, therefore this mutation is likely not related to ALS.

3.3. Multiple damaging variants were detected in individual ALS patients

Of the 13 NAJ-ALS patients who were homozygous to rare deleterious variants, 6 were homozygotes to more than one rare variant (Table 4). A combination of homozygous rare alleles with known pathogenic alleles in known ALS genes was also observed: two patients (patients 7 and 10) carried the *C9orf72* hexanucleotide expansion [16], and patient 2 carried the heterozygous *OPTN* 691_692insAG mutation, known to be a risk factor in ALS in its heterozygous state in this population [5]. Patient 9, who was homozygous for a rare variant in *CARS*, also carried 32 repeats in *ATXN2* (Glutamine expansion), an intermediate number of repeats reported as a risk factor for ALS [22]. In patient 6 we identified a novel splice mutation in *OPTN* (c.780-2A > G), which is predicted to be null, and likely pathogenic/pathogenic mutation. This mutation was not reported in gnomAD or in any other databases, and was also not detected in our 400 ethnically

Table 2
Variant frequencies in all ALS patients of North-Africa-Jewish origin and control groups.

Variant	All NAJ-ALS ($n = 135$)	Controls			p-value	OR (95% CI, p)
		MJ ($n = 400$)	AJ-non-neuro-gnomAD ($n = 3229$)	Total (MJ + AJ, $n = 3629$)		
MFN2, p.R366C, rs369762154 (C > T)						
C/C	133	399	3220	3619		
C/T	1	1	9	10		
T/T	1	0	0	0	0.0359[a]	80.955, (3.28–1996.57, 0.0072)[a]
MAF (T)	0.011	0.00125[b]	0.00139[c]	0.00138	0.01[a]	8.144, (2.23–29.76, 0.0015)[a]

p-values were calculated using Fisher's exact test and odds ratio were calculated using web calculator- www.medcalc.org/calc/odds_ratio
NAJ- North-Africa-Jews; MJ- Moroccan Jews; AJ- Ashkenazi Jews; OR- odds ratio; CI- Confidence interval; MAF- minor allele frequency.
[a] for homozygosity and MAF, comparing All NAJ-ALS to Total controls, since MAF was not significantly different between MJ[b] and AJ[c] controls (Fisher's exact test, $p = 1.0$).

Table 3
Rare predicted-deleterious homozygous variants observed in NAJ-ALS.

Gene	Genomic location (hg19)	Effect (Sequence ontology)	Non-neuro gnomAD allele frequency (number homozygotes/ total individuals)	Number of algorithms with deleterious score (out of 11)[a]	Filtered-in GO from AmiGO2
MFN2	1:12,067,224	Missense (c.1987C > T, p.Arg663Cys)	0.00015 (**0**/ 114,661)	11	Mitochondria, Autophagy, Cytoskeleton
HTT	4:3,188,417	Missense (c.4960C > T, p.Arg1654Trp)	0.00035 (**0**/ 113758)	7	Autophagy, Cytoskeleton
NEK1	4:170,506,525	Missense (c.782G > A, p.Arg261His)	0.00225 (**1**/ 113342)	8	Cytoskeleton
PPARGC1B	5:149,212,298	Missense (c.662C > A, p.Thr221Asn)	0.00148 (**0**/ 113758)	8	RNA-binding, Mitochondria
RARS	5:167,915,692	Missense (c.131 T > C, p.Leu44Ser)	Not reported	9	RNA-binding, Mitochondria
AIF1L	9:133,989,973	Missense (c.248 T > C, p.Met83Thr)	0.00207 (**1**/ 114688)	8	Cytoskeleton
ACBD5	10:27,499,809	Missense (c.1138G > A, p.Gly380Arg)	0.00014 (**0**/ 104066)	10	Autophagy
ARMC4	10:28,149,726	Missense (c.2849G > C, p.Cys950Ser)	0.000005 (**0**/ 104050)	11	Cytoskeleton
ARMC4	10:28,270,448	Missense (c.883G > C, p.Val295Leu)	0.00142 (**1**/ 85757)	7	Cytoskeleton
CARS	11:3,028,171	Missense (c.2087 T > A, p.Leu696His)	0.00009 (**0**/ 103942)	8	RNA-binding
CCDC81	11:86,126,322	Loss of function (c.1657_1658insG, p.Asp554fs)	0.00011 (**0**/ 114641)	11	Cytoskeleton
ATM	11:108,186,610	Missense (c.6067G > A, Gly2023Arg)	0.00138 (**0**/ 114669)	8	Autophagy, Cytoskeleton
MYO1A	12:57,435,044	Missense (c.1193A > G, p.Asn398Ser)	0.00001 (**0**/ 104058)	11	Cytoskeleton
PIP4K2C	12:57,985,163	Missense (c.91A > C, p.Lys31Gln)	0.00006 (**0**/ 103792)	6	Autophagy
FGD6	12:95,535,177	Missense (c.2824A > G, p.Arg942Gly)	0.00001 (**0**/ 103987)	8	Cytoskeleton
CFAP54	12:97,150,305	Missense (c.7910C > G, p.Ala2637Gly)	0.00002 (**0**/ 101169)	8	Cytoskeleton
ZFYVE26	14:68,229,462	Missense (c.6086 T > C, p.Ile2029Thr)	0.0013 (**0**/ 114684)	6	Cytoskeleton
MTHFSD	16:86,588,254	Missense (c.120 T > G, p.Phe40Leu)	0.00002 (**0**/ 102701)	8	RNA-binding
CCDC40	17:78,063,948	Loss of function (NM_017950.3:c.2832 + 265_2832 + 296delCACGTGCACGGACACACGCAGGACACAACAAG)	0.00012 (**1**/ 66195)	11	Cytoskeleton
ARHGAP33	19:36,269,475	Missense (c.380C > T, p.Pro127Leu)	0.00028 (**0**/ 114567)	6	Cytoskeleton
LZTS3	20:3,145,385	Deletion (c.1588_1599delGAGGTGGGGCGG, p.Glu576_Arg579del)	0.00002 (**0**/ 55350)	11	Cytoskeleton
MRPL40	22:19,420,854	Deletion (c.120_122delAGG, p.Trp40Cys, Glu41del)[b]	Not reported	7[b]	RNA-binding, Mitochondria
ZNF74	22:20,760,975	Missense (c.1652C > G, p.Ser551Cys)	0.00001 (**0**/ 103552)	7	RNA-binding, Cytoskeleton

[a] The list of algorithms appears in the Methods section. No-scores were counted as deleterious.
[b] This individual is also homozygote T/T at position 19,420,857 (reference allele is A), resulting in p.Trp40Cys and Glu41del changes. The prediction score is only for the missense change.

matched controls (Goldstein *et al*, data not shown). Altogether, 9 of the 13 ALS patients who carried homozygous deleterious variants, carried also pathogenic/likely-pathogenic ($n = 5$) or VOUS ($n = 4$) variants in known ALS genes. The clinical picture in all these patients was of classical ALS, with upper and lower motor neuron signs and characteristic progression. Two patients had fronto-temporal dysfunction (patients 3 and 10, Table 4). The age at disease onset ranged from 36 to 63 years (mean 51.0) and tracheostomy-free survival was 20–110 months (mean 42.0), except one patient who is alive and stable for more than 140 months after disease onset (patient 2, Table 4).

4. Discussion

A combination of homozygosity analysis, very rare frequency, and deleterious functional prediction scores in genes annotated to ALS-associated cellular pathways identified 23 rare homozygous variants in 22 genes in our cohort of ALS patients of North-Africa Jewish origin.

We showed that the *MFN2* variant c.1987C > T (p. R663C) is significantly associated with ALS in our cohort. To the best of our knowledge this is the first time that a mutation in *MFN2* is reported as associated with a clinical picture of ALS, though a Charcot-Marie-disease (CMT) case with heterozygous *MFN2* mutation was reported to develop ALS at later stage [23]. Although *MFN2* mutations are known to cause CMT type 2A [24], our patient had many features different

Table 4
ALS patients with multiple variants.

Patient #	Family history	Genes and variants identified with rare homozygous alleles[a]			Genes associated with ALS		Patient's clinical features		
		Variant 1	Variant 2	Additional Variants	Variation	Pathogenicity determination[b]	AAO (years)	Survival (months)	Dementia
1	Yes[c]	*MTHFSD* (p.Phe40Leu)	*ZFYVE26* (p.Ile2029Thr)	–	*KIF5A* (p.Thr976Ile)	VOUS	63	51	No
2	Yes[c]	*MTHFSD* (p.Phe40Leu)	*ZFYVE26* (p.Ile2029Thr)	*ATM* (p.Gly2023Arg), *FGD6* (p.Arg942Gly)	*OPTN* (p.Asp128fs)	Pathogenic	62	Stable at 140	No
3	Yes	*MFN2* (p.Arg663Cys)	*ACBD5* (p.Gly380Arg)	*ARMC4* (p.Cys950Ser); *CCDC40* (LoF); *CFAP54* (p.Ala2637Gly); *ARMC4* (p.Val295Leu);	–	–	36	20	FTD
4	No	*MYO1A* (p.Asn398Ser)	–	–	*NEK1*(p.Thr814Ala)	VOUS	42	36	No
5	No	*LZTS3* (p.Glu530_Arg533del)	–	–	*SQSTM1* (p.Ala33Val)	VOUS	45	54	No
6	No	*PPARGC1B* (p.Thr221Asn)	–	–	*OPTN* (c.780-2A > G)	Likely pathogenic risk allele	49	27	No
7	No	*RARS* (p.Leu44Ser)	*AIF1L* (p.Met83Thr)	–	*C9orf72* hexanucleotide expansion	Pathogenic	52	24	No
8	No	*HTT* (p.Arg1654Trp)	*PIP4K2C* (p.Lys31Gln)	–	*CCNF* (p.Phe197Leu)	VOUS	52	110	No
9	No	*CARS* (p.Leu696His)	–	–	ATXN2 (32 repeats)	Pathogenic risk allele	53	20	No
10	No	*NEK1*(Arg261His)	*ARHGAP33* (p.Pro127Leu)	–	*C9orf72* hexanucleotide expansion	Pathogenic	56	36	FTD

[a] The homozygous variations are reported in our paper. The variants within each individual were ordered based on their deleterious scores and connection to ALS.
[b] The pathogenicity was determined [21]. Variants with benign or likely benign scores were not included.
[c] Siblings.

from the CMT2 phenotype, such as asymmetric weakness, brisk reflexes, fasciculations, preserved sensation, frontal behavior, and a very rapid progression, altogether, supporting the clinical diagnosis of ALS.

Of interest, this variant was also reported in the ProjectMinE database (http://databrowser.projectmine.com), with 1 carrier in ALS group and none in control. It is absent from HGMD (http://www.hgmd.cf.ac.uk) but missense mutations in nearby residues (K662E, F665S, L673P) have been reported in association with Charcot-Marie-Tooth 2A, supporting the functional importance of this region of the protein. In spite of its rarity, the deleterious functional prediction by all algorithms, and the recent identification of this variant in a pediatric patient with an atypical phenotype including acute neurological failure [25], this variant is currently classified as a variant of uncertain significance (VOUS) by using the joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) [21]. However, our results suggest that this variant might be a risk allele associated with ALS, and likely pathogenic in its homozygote state.

MFN2 encoded protein is a critical regulator of mitochondrial fusion/fission dynamics. Several new studies discovered tight connections of MFN2 to ALS cellular pathways. Knocking-down TDP-43 in HEK293T cells led to a significant reduction in MFN2 protein, and interaction between TDP-43 and MFN2 was shown by immunoprecipitation in cortical human brain tissue [26]. Moreover, in SOD1-ALS mice model it was shown that MFN2 acts as a dominant suppressor of neuromuscular synaptic loss, by taking a role in nutrients transport to neuromuscular synapses via mitochondria transport mechanism [27].

The ALS patient carrying the *MFN2* homozygous variant in our study was also homozygous to other very deleterious variants, including in the *ACBD5* and *CCDC40*, genes that share ontology of autophagy and cytoskeleton with *MFN2*. Such rare homozygous findings in one individual patient may suggest an explanation for her very early age at onset (AAO = 36) and rapid clinical deterioration.

Although all genes were filtered-in for their ontology to one of the 5 pathways affected in ALS, some genes showed more substantial supporting evidence for their potential role in ALS, either by their function, or by published experimental evidence. The expression levels of *MFN2*, *ARMC4*, *ATM*, *CFAP54* and *MTHFSD* were all reported as down-regulated in ALS patients [28–31], while *LZTS3* showed up-regulation [28]; *MRPL40* is a mitochondrial membrane protein that contributes to the maintenance and operation of the mitochondrial network; *MTHFSD* was recently identified as a protein localized to stress granules upon stress [29]; and both *ACBD5* and *PPARGC1B* are involved in lipid metabolism by peroxisome, while *ACBD5* acts also as the peroxisome receptor for pexophagy.

Two rare homozygous variants were detected in two aminoacyl-tRNA synthetases. *RARS* catalyzes the aminoacylation of arginine, and *CARS* of cysteine. Both variants were scored as very deleterious, and although there is no evidence for a direct connection to ALS, other amino acid tRNA catalyzers *(YARS* and *GARS)* were reported as causing CMT with unusual phenotypes [32].

NEK1- p.Arg261His is a susceptibility variant for ALS, and is enriched in our ALS cohort. Interestingly, the homozygote ALS patient for this variant also carries the *C9orf72* hexanucleotide repeat expansion. Such *NEK1*-carrier individuals with oligogenic mutations in ALS-genes, and more specifically with *C9orf72* repeat expansion, were already published [33,34]. A model in which a second mutation or risk allele may be needed for disease presentation is especially appealing with evidence of *C9orf72* mutation causing DNA damage [35,36] and the role of *NEK1* in DNA repair.

In this study we identified potential risk recessive alleles to ALS. These alleles were scored as deleterious by functional prediction algorithms and are extremely rare. Each was identified in only one ALS patient, or in two related ones. Therefore, these variants' pathogenicity or association to ALS is conditioned on further replication studies, as well as functional studies. Our results can be used as a comparison reference in future studies, building evidence in the field of ALS genetics, for the involvement of new genes in the disease. Our finding of *NEK1* risk allele is an example of the determination of this gene as risk to ALS, receiving the support from many different research groups and populations.

In summary, by using a combination of homozygosity analysis, together with *in-silico* functionality and rarity, we have identified a homozygous *in-silico* deleterious variant in *MFN2* associated with ALS, and enrichment of *NEK1*- p.Arg261His variant, together with other rare variants. Functional assays are warranted to establish the pathogenicity of the rare homozygous variants described here. The multiple variants sharing the same ALS related cellular pathways suggest a complex genetic architecture and emphasize the heterogeneity and intricacy of ALS genetics.

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Disclosure of interest

Orly Goldstein reports no disclosure; Merav Kedmi reports no disclosure; Mali Gana-Weisz reports no disclosure; Shir Twito reports no disclosure; Beatrice Nefussy reports no disclosure; Batel Vainer reports no disclosure; Omri Nayshool reports no disclosure; Avi Orr-Urtreger receives research support from ALS Association and from Kahn and Adelis Foundations; Vivian E Drory receives research support from ALS Association and from Adelis Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2019.05.006.

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