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ORIGINAL COMMUNICATION

Exploring *NEK1* genetic variability in Italian amyotrophic lateral sclerosis patients

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Abstract

Background Mutations in *NEK1*, encoding for a serine/threonine kinase which regulates several biological processes, are associated with amyotrophic lateral sclerosis (ALS).

Methods *NEK1* was analysed by amplicon deep sequencing in a cohort of 1016 Italian sporadic and familial ALS patients previously screened for *C9orf72*, *SOD1*, *TARDBP* and *FUS* mutations.

Results We identified 28 rare *NEK1* variants in 29 patients (2.85%) of whom 20/782 were sporadic (2.5%), 6/107 familial (5%) and 3/127 of unknown aetiology (2.3%). Variants were classified as pathogenic (*P*; $n=1$), likely pathogenic (LP; $n=6$ in 7 patients) and of unknown significance (VUS; $n=21$) according the American College of Medical Genetics and Genomics criteria. Notably, 64% of the identified variants (18/28, including 4 LP and 14 VUS) were novel. Among the 29 patients with rare *NEK1* variants, 7 (of whom 5 were familial cases) had additional variants in one of the four main ALS causative genes. Moreover, 23 patients carried the already reported *NEK1* p.Arg261His risk variant (VUS) alone or in addition to *SOD1* mutations ($n=1$) or *C9orf72* repeat expansion ($n=2$) and to the *NEK1* p.Asp128Val variant ($n=1$). Genotype–phenotype correlation analysis showed no significant differences in age at onset or survival in *NEK1* variant carriers, independently on the variant type. No flail arm phenotype, but atypical features, including sensory symptoms, were present in *NEK1* carriers.

Conclusion Our study further expands *NEK1* genetic variability by identifying novel rare variants and confirming ALS oligogenic nature since 19.6% of *NEK1* patients also carried mutations in one of the four main ALS-associated genes.

Keywords ALS · *NEK1* · Oligogenicity · Genetic screening · IPSC

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease caused by the loss of upper and/or lower motor neurons in the motor cortex, brainstem and spinal cord and characterized by a progressive and severe muscular atrophy with death usually occurring for respiratory failure after 3–5 years [1, 2]. Most cases are sporadic (SALS) with no family history, while approximately 10% of forms are inherited (familial ALS, FALS) and characterized by wide genetic heterogeneity with more than 30 causative genes associated so far [3]. Mutations in four major ALS causal genes *(C9orf72*, *SOD1, TARDBP* and *FUS*) account for approximately 60–70% of FALS and 10% of SALS. The hexanucleotide repeat expansion (HRE) in *C9orf72* gene remains the most common genetic cause of both FALS (30–50%) and SALS (5–10%) cases [4].

The association between *NEK1* and ALS disease was first identified by whole exome sequencing [5] and further confirmed in 2016 by two independent studies conducted on large FALS cohorts [6, 7]. In particular, heterozygous *NEK1* loss-of-function (LoF) variants and the missense p.Arg261His variant were significantly associated with the risk of developing the disease in FALS patients [6, 7]. *NEK1* missense variants other than p.Arg261His have a smaller

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risk for ALS as compared to LoF mutations and screenings of other cohorts assessed that *NEK1* variants, including LoF and missense ones, overall account for approximately 3% of ALS cases [8].

NEK1 encodes for a serine/threonine kinase belonging to the highly conserved family of NIMA-related kinases and comprises three different domains: the catalytic domain at the N-terminal, a central coiled-coil domain and then 4 PEST (proline (P), glutamic acid (E), serine (S), and threonine (T))-rich sequences implicated in ubiquitin-mediated protein degradation, interrupted by a second coiled-coil domain [9]. NEK1 is involved in regulating several biological pathways, including cell cycle, DNA damage response, primary cilia formation, and mitochondrial functionality [10].

Since the role of *NEK1* gene in ALS aetiopathogenesis remains to be further established, in this study we analysed the frequency and occurrence of *NEK1* rare variants in a large cohort of 1016 Italian SALS and FALS patients.

Table 1 Clinical and demographic features of the Italian ALS patients included in our cohort

Variable	No. patients (frequency)
Sex	
Male	619 (61%)
Female	397 (39%)
Mean age of onset	58.61 ± 11.05 years (n = 788)
Site of onset	
Bulbar	200 (21%)
Spinal	650 (57%)
Unknown	166 (22%)
Family history	
FALS	107 (11%)
SALS	782 (77%)
Unknown	127 (12%)
Mean survival	31.65 ± 26.38 months (n = 441)
Mutations in the 4 main ALS-related genes[a]	132 (13%)
FALS	53 (40%)
SALS	64 (49%)
Unknown	15 (11%)

[a]The four main ALS causative genes *SOD1*, *TARDBP*, *FUS* and *C9orf72* were tested

Materials and methods

ALS cohort

The diagnosis of ALS was made according to the El Escorial revised criteria [11]. Clinical and demographic features of ALS patients are reported in Table 1. We received approval for this study from the local ethical committees for human studies of the participating Institutions and written informed consent was obtained from all participants (Project DrepALS, REB approval 2018_04_17).

Genetic analyses

Patients' DNA samples were collected and analysed by the two Italian ALS referral centres, IRCCS Istituto Auxologico Italiano and Fondazione IRCCS Istituto Neurologico Carlo Besta. Genomic DNA was extracted from peripheral blood lymphocytes using Wizard Genomic DNA Purification Kit (Promega) or NucleoSpin Blood L Vacuum (Macherey–Nagel using a Freedom Evo Tecan instrument). All participating patients had been tested for the four main ALS causative genes *SOD1*, *TARDBP*, *FUS* and *C9orf72* in the two referral centres. The genetic screening of *SOD1, TARDBP* and *FUS* was performed by amplicon deep sequencing using Sure Select QXT kit (Agilent) or Sanger sequencing. Detection of *C9orf72* HRE was obtained by repeat-primed PCR with the AmplideX PCR/CE *C9ORF72* kit (Asuragen Inc.) [12] or with an optimized assay using FastStart Taq [13].

The entire coding region of *NEK1* gene (34 exons) and the intron/exon boundaries, including at least 20 bp of flanking intronic splicing consensus sequences, were analysed with amplicon deep sequencing (sequences of oligo pairs are reported in Supplementary Table S1). Sequencing of enriched libraries was performed using an Illumina MiSeq platform with paired-end approach. All the identified nucleotide changes were confirmed by sequencing an independent PCR product on a newly extracted DNA sample using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on 3130 or 3500 ABI Prism Genetic Analysers (Applied Biosystems). Nucleotide numbering of *NEK1* gene variants reflects cDNA numbering with + 1 corresponding to the A of the ATG translation initiation codon of the GenBank reference sequence NM_001199397.

Bioinformatic analyses

Data analysis was performed using: (a) MiSeq Reporter software (Illumina) or FastQC (https://qubeshub.org), BWA and GATK (https://gatk.broadinstitute.org) HaplotypeCaller for quality control, alignment against hg19 reference genome and variant calling, respectively; (b) VariantStudio software (Illumina) or SnpEff (https://pcingola.github.io/SnpEff), public variation databases dbSNP137 (https://www.ncbi.nlm.nih.gov/projects/SNP), NHLBI Exome Sequencing Project 6500 (http://evs.gs.washington.edu/EVS), 1000 Genomes project (http://www.internationalgenome.org/1000-genomes-browsers/), Exome Aggregation Consortium (ExAC) and Human Gene Mutation Pro Database (http://

www.hgmd.cf.ac.uk/ac/index.php) and an in-house database for variant annotation; c) CLC Genomics Workbench software (CLCbio, Qiagen) or GATK DepthOfCoverage for coverage analysis.

Variants were filtered based on a minor allele frequency (MAF) < 1% in the population databases (dbSNP137, NHLBI, SP6500, 1000 Genomes project, and ExAC). The clinical significance of the identified rare variants was assessed according to the American College of Medical Genetics and Genomics (ACMG) guidelines [14]. In *silico* prediction of intronic variants on splicing was performed using four splice site prediction tools: Maximum Entropy Scan (MaxEntScan) [15], Human Splicing Finder (HSF) [16], NN Splice [17] and Gene Splicer [18].

Statistical analyses

Clinical data from ALS patients were analysed using R (version 4.3.2). The Kruskal–Wallis rank sum test was performed to compare the age at onset (AAO) between patients with and without *NEK1* variants. Survival analysis between the same groups was performed using the Kaplan–Meier method. Variants identified in *NEK1* gene were visualized with the Lolliplot function from the Bioconductor package trackViewer.

Results

Identification of *NEK1* variants in Italian ALS patients

A cohort of 1016 Italian ALS patients (619 males and 397 females), including 107 FALS (11%), was screened for variants in *NEK1* gene. The main clinical features of the ALS cohort are detailed in Table 1. All patients were preliminarily tested for the four main ALS causative genes (*C9orf72, SOD1, TARDBP, FUS*) and pathogenic variants were identified in 132 subjects (13% of the whole cohort), including both FALS and SALS cases (Table 1).

The genetic screening of the entire coding region of *NEK1* by amplicon deep sequencing identified 28 different rare variants in heterozygous state in 29/1016 (2.85%) unrelated ALS patients. These variants included 5 deletions/insertions in 5 patients, 2 nonsense mutations in 3 patients, 20 missense variants in 20 patients and 1 splicing variant in 1 patient (Table 2). The novel c.2974 + 2 T > C splice variant was consistently predicted to alter the canonical splice site by three out of four different in silico tools (Supplementary Table S3). Seven of the identified variants (7/28; 25%) were LoF, classified as pathogenic/likely pathogenic, while one in-frame deletion (1/28) and all the missense variants (20/28) were classified as VUS (variants of unknown significance) (Table 2). LoF and missense variants were identified both in FALS (6/107; 5%) and in SALS (20/782; 2.5%) cases as well as in patients for whom we do not have information about their family history (3/127; 2.3%) (Tables 1 and 3).

The most of the identified variants (18/28; 64%) were novel, among which 4 were LoF (Table 2). Novel and previously reported mutations were distributed along the entire *NEK1* coding sequence, showing no mutational hot spots (Fig. 1).

Among the 29 patients harbouring rare variants in *NEK1*, we found 7 patients, mostly FALS (5/7), carrying additional variants in the four main ALS causative genes: 2 cases with *TARDBP* variants (one in heterozygous state, p.Gly294 Val and one in compound heterozygosity, p.Ala382 Thr and p.Gly295Ser); 2 patients with variants in *FUS* gene (p.Arg521His and p.Asp5Gly); 3 patients carrying *C9orf72* HRE (Table 3). In these double mutant patients, the *NEK1* variants were VUS, with the exceptions of the nonsense variant p.Ser1036* and the splicing variant c.2974 + 2 T > C, which were both identified together with the *C9orf72* HRE (Table 3).

In addition, in our screening we found that 23 (2.2%) patients carried the p.Arg261His variant (rs200161705) (Table 2), previously reported to be a risk factor for ALS [6, 7, 19]. Among these patients, one carried also the *NEK1* p.Asp128 Val (Table 3) and 3 a concomitant mutation in one of the four main ALS-related genes (Supplementary Table S2). Among these 3 latter ones, 2 patients had *SOD1* mutations (one with p.Ile150 Thr in heterozygous state and the other one with p.Asp91 Ala in homozygous state) and one patient carried a *C9orf72* HRE. The clinical features of the 23 FALS and SALS patients with the risk p.Arg261His variant are all reported in Supplementary Table S2.

Collectively, we found that, among the 51 *NEK1*-positive patients identified by our screening, 10 of them (19.6%) also carried an additional variant in one of the four main ALS causative genes (Table 3 and Supplementary Table S2).

Clinical features of the ALS patients carrying *NEK1* LoF variants

According to the literature data, the *NEK1* LoF mutations are strongly associated with ALS, while most missense variants represent risk factors or VUS [7, 8]. In our cohort, the 7 *NEK1* LoF variants identified in 8 ALS patients included 4 frameshift (p.Lys332Ilefs*18, p.Ile633 Thrfs*4, p.Ile633 Asnfs*28, and p.Ile1237 Argfs*17), 2 nonsense (p.Arg161* and p.Ser1036*, the latter one in 2 patients), and the splicing c.2974 + 2 T > C (Table 2). The main demographic and clinical features of the patients harbouring LoF variants in *NEK1* are reported in Table 3. The median age at onset was 52.7 years (IQR 45.0–59.0)

Table 2 List of the *NEK1* gene variants identified in our Italian ALS cohort

HGVS_C	HGVS_P	SNP identifier	Genetic effect	ACMG classification	Patient count	Allele frequency (gnomAD v4.1)	References
LoF variants							
c.481 C > T	p.Arg161*	–	nonsense	P	1	0.00005171	[7]
c.995_996 delinsT	p.Lys332Ilefs*18	–	frameshift	LP	1	–	–
c.1898 del	p.Ile633 Thrfs*4	rs1242400046	frameshift	LP	1	0.00000062	–
c.1897 dup	p.Ile633 Asnfs*28	rs1461849431	frameshift	LP	1	0.00000062	[20]
c.2974 + 2 T > C	–	–	splicing	LP	1	–	
c.3107 C > G	p.Ser1036*	rs199947197	nonsense	LP	2	0.00020823	[6, 7, 19, 21]
c.3710_3713 del	p.Ile1237 Argfs*17	rs1416401611	frameshift	LP	1	0.00000251	–
Missense and other rare variants							
c.292G > A	p.Val98Ile	rs115005766	missense	VUS	1	0.00004773	–
c.338G > A	p.Cys113 Tyr	–	missense	VUS	1	–	–
c.380G > A	p.Arg127Gln	rs1312619422	missense	VUS	1	0.00000440	[23]
c.383 A > T	p.Asp128 Val	–	missense	VUS	1	–	[23]
c.734 A > G	p.Asp245Gly	rs756066992	missense	VUS	1	0.00000186	[23]
c.1080G > C	p.Glu360 Asp	rs765591688	missense	VUS	1	0.00004179	–
c.1217 A > C	p.Glu406 Ala	–	missense	VUS	1	–	–
c.1450 C > T	p.Arg484 Cys	rs183378326	missense	VUS	1	0.00000558	–
c.1535 C > T	p.Ala512 Val	rs771824152	missense	VUS	1	0.00005392	[33]
c.1942 A > G	p.Lys648Glu	rs371562840	missense	VUS	1	0.00005406	[7, 23, 28, 34]
c.1970_1972 del	p.Glu657 del	–	inframe deletion	VUS	1	0.00000124	–
c.1989 A > T	p.Lys663 Asn	–	missense	VUS	1	–	–
c.2053G > A	p.Gly685 Arg	rs866331832	missense	VUS	1	0.00000869	–
c.2137G > T	p.Val713Leu	rs199827465	missense	VUS	1	0.00000560	–
c.2353 C > T	p.Arg785 Cys	rs374639577	missense	VUS	1	0.00001058	–
c.2500 A > T	p.Ser834 Cys	rs187263822	missense	VUS	1	0.00001864	–
c.2585G > T	p.Ser862Ile	–	missense	VUS	1	–	–
c.2651 T > C	p.Ile884 Thr	–	missense	VUS	1	0.00000440	–
c.2701 A > G	p.Lys901Glu	–	missense	VUS	1	–	–
c.3287 C > G	p.Thr1096Ser	rs371901834	missense	VUS	1	0.00000249	[7]
c.3295G > C	p.Asp1099His	rs765205496	missense	VUS	1	0.00001429	[7]
Risk factor							
c.782G > A	p.Arg261His	rs200161705	missense	VUS	23	0.00265460	[6, 7, 19]

Human Genome Variation Society (HGVS)_C, transcript reference sequence based on HGVS nomenclature, HGVS_P, protein reference sequence based on HGVS nomenclature

P pathogenic, *LP* likely pathogenic, *VUS* variant of unknown significance

and the median survival time after disease onset was 28.8 months (IQR 12.0–46.5). Except for one patient presenting dysarthria and dysphagia at onset (A899) and for 2 patients (A802 and SLA2132) for whom full clinical data were not available, all the other 5 patients showed a classical phenotype with spinal onset, mostly in the lower limbs (Table 3). During disease progression, 4 (A418, A560, SLA2016, SLA2327) out of 5 patients developed bulbar involvement and one (A560) also cognitive decline. Two patients reported atypical clinical features, including positive sensory symptoms in SLA2016 and slow saccades in A560, respectively.

Specifically, 3 out of the 4 frameshift variants (p.Lys332Ilefs*18, p.Ile633 Thrfs*4 and p.Ile1237 Argfs*17) were identified as novel variants and predicted to generate truncated proteins. The p.Ile1237 Argfs*17 in patient SLA2132 is located at the C-terminal of the NEK1 protein, where no functional domains are found (Fig. 1). Interestingly, the novel frameshift p.Ile633 Thrfs*4, identified in patient A802, starts from the same amino acid as the p.Ile633 Asnfs*28 found in patient SLA2016 (Table 3), which has been already reported as pathogenic [20]. Patient SLA2016 exhibited an early onset, slowly progressive ALS complicated by sensory symptoms

Table 3 Clinical features of *NEK1* variant carriers

DNA code	Sex	Site of onset	Age of onset (years)	Survival (months)	Phenotype*	Family history	*NEK1* variants	Other variants in ALS-related genes
LoF variants								
SLA3154	M	S-LL	59	n.a	Classic	SALS	*NEK1* p.Arg161*	
A418	M	S-LL	53	17.64	Classic	SALS	*NEK1* p.Lys332Ilefs*18	
A802	M	n.a	n.a	n.a	n.a	SALS	*NEK1* p.Ile633 Thrfs*4	
SLA2016	F	S-LL	34	n.a	Classic	SALS	*NEK1* p.Ile633 Asnfs*28	
A560	F	S-UL	45	33.18	Classic	FALS	*NEK1* c.2974 + 2 T > C	*C9orf72* HRE
SLA2434	M	S-LL	68	n.a	Classic	n.a	*NEK1* p.Ser1036*	
A899	F	B	50	27	Bulbar	FALS	*NEK1* p.Ser1036*	*C9orf72* HRE
SLA2132	F	n.a	59	n.a	n.a	n.a	*NEK1* p.Ile1237 Argfs*17	
Missense and other rare variants								
A1053	F	S-LL	71	26.02	Classic	SALS	*NEK1* p.Val98Ile	
A678	F	S-UL	66	7.46	Classic	SALS	*NEK1* p.Cys113 Tyr	*FUS* p.Asp5Gly
SLA2018	F	S-LL	77	n.a	Flail leg	SALS	*NEK1* p.Arg127Gln	
A949	M	S-UL	53	35.81	Classic	SALS	*NEK1* p.Asp128 Val *NEK1* p.Arg261His	
A469	M	B	60	38.4	Bulbar	SALS	*NEK1* p.Asp245Gly	
SLA0768	M	n.a	76	n.a	n.a	FALS	*NEK1* p.Glu360 Asp	*TARDBP* p.Gly294 Val
SLA1979	F	S-UL	50	n.a	Classic	SALS	*NEK1* p.Glu406 Ala	
A1036	M	S-LL	n.a	n.a	n.a	n.a	*NEK1* p.Arg484 Cys	
SLA1984	M	S-UL	54	n.a	Classic	SALS	*NEK1* p.Ala512 Val	
A1150	F	B	54	28.68	Bulbar	FALS	*NEK1* p.Lys648Glu	
SLA2327	M	S-LL	67	n.a	Classic + FTD	SALS	*NEK1* p.Glu657 del	
SLA1831	F	S-UL	49	n.a	Classic	SALS	*NEK1* p.Lys663 Asn	
SLA1986	M	B	73	n.a	Bulbar	SALS	*NEK1* p.Gly685 Arg	
A817	M	B	55	32.78	Bulbar	SALS	*NEK1* p.Val713Leu	
SLA1832	M	S-LL	63	n.a	Classic	SALS	*NEK1* p.Arg785 Cys	
SLA2378	F	S-LL	25	n.a	Classic	SALS	*NEK1* p.Ser834 Cys	
A928	F	S-LL	75	18.95	Classic	SALS	*NEK1* p.Ser862Ile	
SLA2283	F	S-UL	49	n.a	Classic	FALS	*NEK1* p.Ile884 Thr	*FUS* p.Arg521His
A831	M	S-UL	62	11.89	Classic	SALS	*NEK1* p.Lys901Glu	
A726	M	B	47	24.38	Bulbar	FALS	*NEK1* p.Thr1096Ser	*C9orf72* HRE
A1142	M	S-UL	60	n.a	Classic	SALS	*NEK1* p.Asp1099His	*TARDBP* p.Gly295Ser; *TARDBP* p.Ala382 Thr

M male, *F* female, *S* spinal onset, *S-LL* spinal low limb, *S-UL* spinal upper limb, *B* bulbar onset, *n.a.* not available data, *SALS* sporadic amyotrophic lateral sclerosis, *FALS* familial amyotrophic lateral sclerosis, *FTD* frontotemporal dementia, *HRE* hexanucleotide repeat expansion

*Phenotype classification (classic, bulbar, flail leg) according to [35]

including paraesthesia and tingling in the right lower limb, later evolving into widespread pain in the feet and hands. An EMG performed at age 46 showed a motor axonopathy with chronic neurogenic changes in the 4 limbs. At 47 years of age, she received a diagnosis of spinal onset ALS, further complicated by bulbar signs (Table 3). Conversely, the male ALS patient previously described in the literature with the same *NEK1* p.Ile633 Asnfs*28 variant had a later disease onset at age 54 with left-hand weakness, rapidly progressing within 6 months and leading to respiratory failure and death at 55 years of age [20].

Both the nonsense variants p.Arg161* (in patient SLA3154) and p.Ser1036* (in patients A899 and SLA2434) had been previously described in the two original discovery studies [6, 7] and in other independent cohorts [19, 21]. Patient SLA3154 first showed hands tremor and muscle cramps in lower limbs at age 59 (Table 3) and then developed progressive difficulties in manual activities and writing.

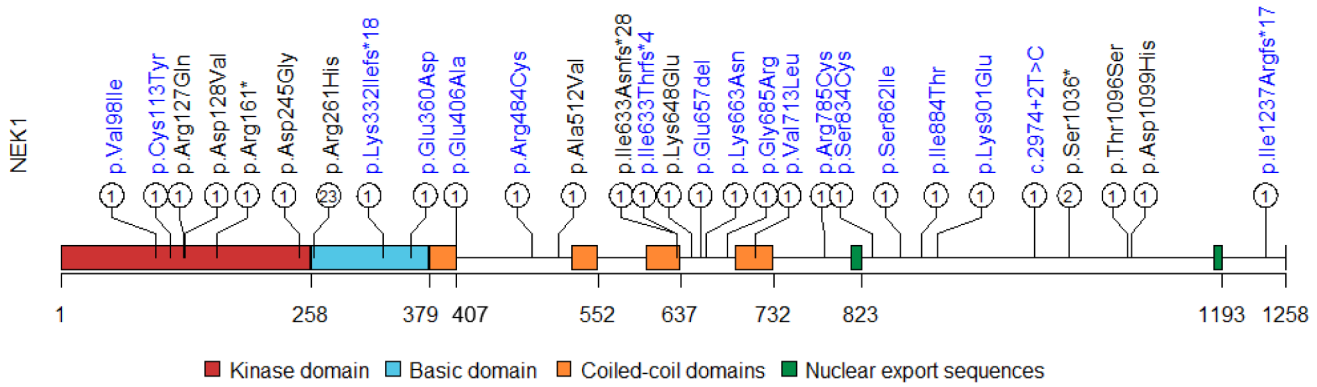

Fig. 1 Schematic representation of the NEK1 protein structure where coloured boxes represent functional protein domains. All the identified variants in our ALS cohort are reported in black if they were previously reported or in blue if they are novel. The number in each lollipop indicates the count of cases in our cohort with the respective variant

At 61 years of age, he received the diagnosis of a classical form of spinal ALS. After 5 years from the onset, he was still able to walk without support for short distances and he did not present any bulbar sign. As regards the nonsense variant p.Ser1036*, the 68-year-old SLA2434 male presented a motor neuron disease characterized by spinal onset with fasciculations at upper limbs and to a lesser extent in the proximal area of the lower limbs, with no bulbar involvement six months after the onset. Conversely, the female patient A899 is a FALS with bulbar onset with dysarthria and dysphagia starting at the age of 50 (Table 3). Notably, the patient also carried the *C9orf72* HRE. The possible biological effects of this oligogenic condition were recently investigated by our group using motor neurons differentiated from her reprogrammed induced pluripotent stem cells (iPSCs) [22].

The c.2974 + 2 T > C variant, predicted to affect splicing (Supplementary Table S3), was identified in A560, a female patient with FALS presenting with spinal onset at the age of 45. Interestingly, this patient also carried the pathogenic *C9orf72* HRE (Table 3).

Genotype–phenotype correlations in ALS patients carrying *NEK1* variants

To further investigate possible genotype–phenotype correlations in ALS patients carrying *NEK1* variants, we first analysed and compared age at onset (AAO) in patients stratified according to the type of *NEK1* variant (LoF, missense and the risk p.Arg261His) and without *NEK1* variants for whom AAO data were available ($n = 788$). Our analysis revealed no significant differences among all these groups (Kruskal–Wallis chi-squared = 2.9245, p value = 0.4034) (Fig. 2A). Even when merging all *NEK1*-positive patients in a single group or considering only the risk p.Arg261His variant separately, the AAO did not differ in these *NEK1*

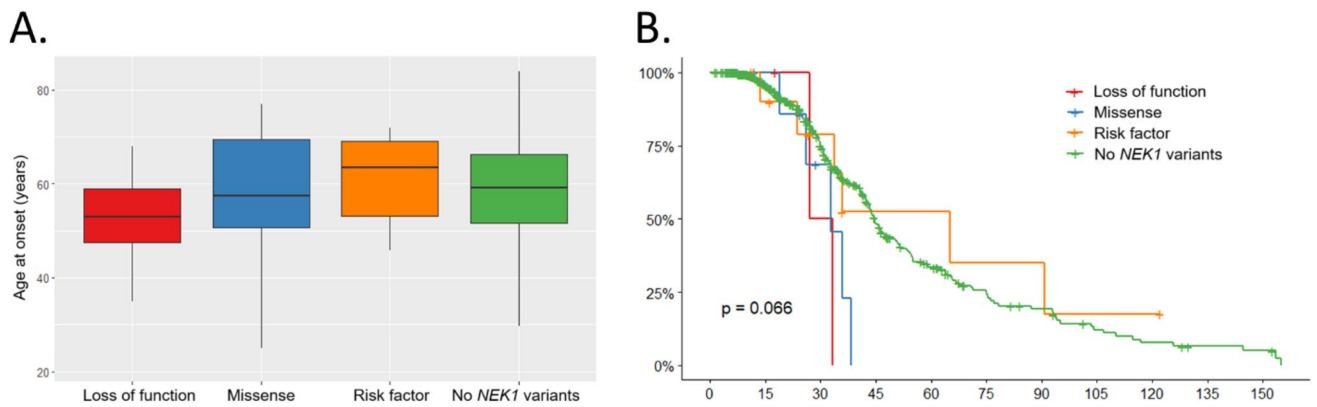

Fig. 2 Genotype–phenotype correlations for **a** age at onset among patients with *NEK1* LoF ($n = 7$), missense ($n = 18$), the risk p.Arg261His ($n = 20$) variants and without any *NEK1* variant ($n = 743$) (Kruskal–Wallis chi-squared = 2.9245, p value = 0.4034) and for **b** survival among patients with *NEK1* LoF ($n = 3$), missense ($n = 9$), the risk p.Arg261His variant ($n = 11$) and without *NEK1* variants ($n = 418$) (Log-rank p value = 0.066)

carriers as compared to ALS patients with no *NEK1* variants (Supplementary Figure S1).

Similarly, we found no significant differences in survival after disease onset among the distinct groups (Log-rank p value = 0.066) (Fig. 2B), even when grouping together all the *NEK1*-positive patients (data not shown), likely due to the limited survival data available for the *NEK1* rare variants' carriers (n = 441) (Table 3 and Supplementary Table S2).

Unlike the previous data from an independent cohort of Italian ALS patients [23], we found no evidence of recurrent flail arm phenotype in our *NEK1*-positive patients, independently on the variant type considered (LoF or missense/risk) (Table 3).

Discussion

Homozygous or compound heterozygous variants in *NEK1* gene are causative of a group of human ciliopathies with skeletal defects, namely short-rib thoracic dystrophy (SRTD) and axial spondylometaphyseal dysplasia (SMD) [21, 24–26], as well as of juvenile retinitis pigmentosa [27]. On the other hand, heterozygous *NEK1* LoF variants, together with the missense p.Arg261His variant, have been associated with a significant increased risk of developing ALS [8]. The role of the other rare *NEK1* missense variants in ALS aetiopathogenesis is not very clear but, although at a lesser extent as compared to LoF mutations, it is more and more evident that they also contribute to disease risk [8].

In this study, we performed a genetic screening of *NEK1* within the largest Italian ALS cohort to date, comprising 1016 patients, and identified LoF and missense rare variants in both FALS and SALS cases. Collectively, the prevalence of rare *NEK1* variants in our cohort is 2.85%, in line with previously described ALS cohorts [5–8, 19, 28]. More specifically, we observed a 0.8% frequency of *NEK1* LoF variants, which is consistent with the 0.9% frequency found in a meta-analysis of 8603 ALS cases from 8 studies [8] and in two other Italian ALS cohorts described so far (0.4 and 1.1%) [23, 28]. For the risk p.Arg261His variant, we observed a frequency of 2.3% in our cohort, in line with the other two Italian studies which reported frequencies between 0.6 and 3.7%. Likewise, for all the missense *NEK1* variants, including the risk one, our ALS cohort exhibited a frequency of 4.2%, compared to a range of 3.7–6% in the other Italian studies [23, 28] and to 2.3% in the overall cohorts screened so far [8].

Importantly, our analysis identified a great number (18/28; 64%) of novel rare variants, consisting of both LoF and missense ones, further contributing to the definition of the genetic variability of *NEK1* in ALS. We observed no mutational hot spots across the *NEK1* gene, neither a preferential distribution of the LoF variants in specific functional domains of the protein as previously suggested [8]. Indeed, we identified LoF variants in the basic domain at the N-terminal, in the coiled-coil central domain as well as in the C-terminal region with no functional or structural domains. Of interest, we identified two different frameshift LoF mutations (one novel and one already described in literature [20]) starting at the same 633 amino acid position within the coiled-coil domain. As regards the missense variants found in our study and so far in other cohorts, functional studies will be necessary to better assess their role at biological level given their relatively lower risk associated with the disease compared to the LoF mutations [8].

We also found in 2 patients the p.Ser1036*, which was first described by Brenner et al. [6] as a variant with reduced penetrance due to its relatively high frequency in population databases and its presence also in one unaffected sibling of an ALS patient. The same nonsense variant was also reported in a Belgian ALS cohort [19], where it was found in one healthy control and in two affected siblings who also harbored a *C9orf72* HRE and the *TUBA4A* p.Thr381Met variant [29, 30]. Interestingly, the A899 patient in our cohort also had a pathogenic *C9orf72* HRE, supporting the oligogenic hypothesis and the reduced penetrance of the *NEK1* p.S1036* LoF variant, which maps in the distal C-terminal region of NEK1 protein where no functional domains are present.

The presence of mutations in more than one causative gene is a common condition in ALS [31] and has been already described for *NEK1* variants in two other independent Italian studies [23, 28]. In our cohort, which was preliminarily screened for the four main ALS genes *C9orf72, SOD1, TARDBP* and *FUS*, we identified 10 patients with a double mutation accounting for 19% of all *NEK1* carriers and about 1% of all ALS cases. However, it is likely that the oligogenic condition in *NEK1* carriers and, in general, in our ALS cohort is underestimated because we only tested the four main genes and not all the causative ones. No clear genotype–phenotype correlation is observed in ALS patients with *NEK1* variants and mutations in one of the four main ALS causative genes. On the other hand, synergistic effects have been observed at biological level in iPSC-derived motor neurons obtained from the double mutant patient A899 [22]. By studying the response to DNA damage and primary cilium formation, we recently found that the presence of the *NEK1* p.S1036* LoF variant worsens DNA damage repair which is already defective when *C9orf72* HRE is present, confirming the previous literature data similarly conducted on mutant *NEK1* iPSC-motor neurons [32]. Conversely, primary cilium formation seems not to be affected in a synergistic manner by the presence of both *NEK1* LoF and *C9orf72* HRE [22], suggesting that a specific and complex interplay

between these two genes occurs in iPSC-derived motor neurons and in different biological pathways. We speculate that this is likely to be reflected also in patients' brain where more intricate or compensatory mechanisms may occur to regulate motor neuron cell homeostasis, resulting in a nonmanifest effect of this oligogenic condition at clinical level.

When we specifically evaluated possible genotype–phenotype correlations in *NEK1* carriers, we failed to observe significant differences in clinical parameters, such as age at onset and survival, when compared to the other ALS patients. The results did not differ if we considered *NEK1* LoF or missense variants grouped together or separately to account for distinct biological effects, likely associated to the type of mutation. However, we cannot exclude that the scarce availability of clinical data of the *NEK1* carriers might represent a bias for such statistical analyses. Moreover, the previous observation of a higher frequency of flail arm phenotype in *NEK1* carriers of Italian descent [23] was not confirmed in our ALS cohort. We instead reported atypical features, including sensory symptoms, in 2/8 (25%) carriers of LoF variants, which might support the decision of screening *NEK1* gene in selected patients.

In conclusion, the present study further contributes to define *NEK1* genetic variability by identifying novel rare variants in the biggest cohort of Italian ALS patients screened so far, highlighting that genotype–phenotype associations based on the nature of the *NEK1* genetic variants (Lof or missense) are not evident, likely due to their reduced penetrance and to conditions of oligogenicity, and confirming the complex genetic architecture of ALS disease.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00415-025-13153-6.

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Data availability Pseudo-anonymized datasets analysed for this study are archived on Zenodo (https://zenodo.org/records/14742377) and available upon reasonable request.

Declarations

Conflict of interest V.S. received compensation for consulting services and/or speaking activities from AveXis, Cytokinetics, Italfarmaco, Liquidweb S.r.l., Novartis Pharma AG, Amylyx Pharmaceuticals, Biogen, and Zambon Biotech SA. He is in the Editorial Board of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, European Neurology, American Journal of Neurodegenerative Diseases, Frontiers in Neurology, and Exploration of Neuroprotective Therapy.

Ethical approval Approval for this study was obtained from the ethical committees of the participating Institutions (REB approval 2018_04_17) and studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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