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NEK1 genetic variability in a Belgian cohort of ALS and ALS-FTD patients

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ABSTRACT

We evaluated the genetic impact of the amyotrophic lateral sclerosis (ALS) risk gene never in mitosis gene a–related kinase 1 (*NEK1*) in a Belgian cohort of 278 patients with ALS (n = 245) or ALS with frontotemporal dementia (ALS-FTD, n = 33) and 609 control individuals. We identified 2 ALS patients carrying a loss-of-function (LOF) mutation, p.Leu854Tyrfs*2 and p.Tyr871Valfs*17, that was absent in the control group. A third LOF variant p.Ser1036* was present in 2 sibs with familial ALS but also in an unrelated control person. Missense variants were common in both patients (3.6%) and controls (3.0%). The missense variant, p.Arg261His, which was previously associated with ALS risk, was detected with a minor allele frequency of 0.90% in patients compared to 0.33% in controls. Taken together, *NEK1* LOF variants accounted for 1.1% of patients, although interpretation of pathogenicity and penetrance is complicated by the observation of occasional LOF variants in unaffected individuals (0.16%). Furthermore, enrichment of additional ALS gene mutations was observed in *NEK1* carriers, suggestive of a "second hit" model were *NEK1* variants may modify disease presentation of driving mutations.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by adult-onset progressive motor neuron loss. A positive family history is reported in 5%–10% of all ALS cases. Mutations in more than 20 genes have been associated with these inherited forms, of which, pathogenic expansions of the chromosome 9 open reading frame 72 (*C9orf72*) repeat are the most common genetic cause. Ample clinical, genetic, and pathologic evidence have linked ALS and frontotemporal dementia (FTD) as partners of a disease continuum in which both phenotypes may present in the same individual or within families, referred to as ALS-FTD or FTD-ALS.

Recently, gene-burden analyses in large-scale whole-exome sequencing studies demonstrated significant enrichment of never in mitosis gene a–related kinase 1 (*NEK1*) heterozygous loss-of-function (LOF) variants in ALS (Brenner et al., 2016; Cirulli et al., 2015; Kenna et al., 2016). Follow-up in replication cohorts confirmed disease association of *NEK1* LOF variants and identified an additional role for the p.Arg261His missense variant in disease susceptibility (Kenna et al., 2016). In these studies, the frequency of *NEK1* LOF variants ranged from 0.70% to 1.2% for both familial and sporadic ALS patients versus 0.12% to 0.17% for control individuals. To date, a total of 33 *NEK1* LOF variants have been reported including 10 that were also observed in unaffected controls

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(Black et al., 2017; Brenner et al., 2016; Cirulli et al., 2015; Kenna et al., 2016). Reduced penetrance was also demonstrated in an unaffected sib of ALS patients with the p.Ser1036* variant (Brenner et al., 2016).

NEK1 is a member of the highly conserved never in mitosis gene a kinase family. It is a serine/threonine kinase involved in cell-cycle regulation, ciliogenesis, mitochondrial membrane permeability, and DNA damage repair (Chen et al., 2009; Pelegrini et al., 2010; Shalom et al., 2008). Interestingly, in DNA damage repair, NEK1 was shown to interact with C21orf2, which was also recently associated to ALS risk (Fang et al., 2015; van Rheenen et al., 2016).

In the present study, we examined the impact of *NEK1* in the genetic etiology of 278 Belgian unrelated patients suffering from pure ALS (n = 245) or ALS with concomitant FTD (n = 33).

2. Materials and methods

2.1. Study cohort

The Belgian patient cohort screened in this study consisted of 278 index patients with clinical diagnosis of ALS (n = 245, mean onset age: 58 ± 11.6 years) and FTD together with ALS (ALS-FTD, n = 33, mean onset age: 62.3 ± 9.4 years). The patient cohort was recruited in the framework of the Belgian Neurology consortium, a multicenter collaboration of dementia expertise centers in Belgium. Clinical diagnosis of ALS and ALS-FTD was based on established international diagnostic criteria (Brooks et al., 2000; de Carvalho et al., 2011; Neary et al., 1998; Rascovsky et al., 2011; Schrooten et al., 2011). A positive family history was defined by at least one first- or second-degree relative with motor neuron disease or dementia and was present in 19% of ALS and 42% of ALS-FTD patients. All patients were genetically profiled for the most common genes associated with the ALS/FTD spectrum (*TBK1*, *SOD1*, *VCP*, *TARDBP*, *FUS*, *GRN*, *MAPT*, *CHCHD10*, and *TUBA4A*), Alzheimer's disease (*APP*, *PSEN1*, *PSEN2*), prion disease (*PRNP*), and Parkinson's disease (*LRRK2*, *PARK2*, *SNCA*) by massive parallel or Sanger sequencing. The *C9orf72* repeat was typed by repeat-primed polymerase chain reaction (PCR) and fragment length analysis, as described by Gijselinck et al. (2012).

We also included 609 unrelated geographically matched control individuals (mean age at inclusion: 70 ± 10 years), who were negative for a personal or family history of neurodegenerative disease and had a Mini-Mental State Examination or Montreal Cognitive Assessment score >25 (Folstein et al., 1975; Nasreddine et al., 2005).

2.2. Ethical assurances

All research participants or their legal representatives signed informed consent for participation in clinical and genetic research. Clinical study protocols and informed consent forms were approved by the local medical ethics committees of the collaborating medical centers in Belgium. Genetic study protocols and informed consent forms were approved by the ethics committees of the University Hospital of Antwerp and the University of Antwerp, Belgium.

2.3. NEK1 *genetic analysis*

A MASTR assay (Multiplex Amplification of Specific Targets for Resequencing technology, www.multiplicom.com) was designed for the multiplex amplification of all 34 coding exons and intron-exon boundaries of *NEK1* (relative to NM.001199397.1; NP_001186326.1). Briefly, primers were designed using the multiplex PCR software (Multiplicom, Niel, Belgium) (Goossens et al., 2009). Specific target regions were amplified using multiplex PCR, followed by purification of the equimolar pooled amplicons using Agencourt AMPureXP beads (Beckman Coulter, CA, USA). Individual barcodes (Illumina Nextera XT) were incorporated in a universal PCR step prior to sample pooling. Libraries were sequenced on an Illumina MiSeq platform using v2 reagent kit with a paired-end read length of 250 bp (Illumina, San Diego, CA, USA). The Burrows-Wheeler Aligner tool was used to align and map reads against the human reference GRCh37 (hg19) (Li and Durbin, 2009). All 34 *NEK1* coding exons were successfully sequenced with 0% dropout and average coverage of 1747 ± 425×, ranging from 120× (exon 23) to 6665× (exon 21). Variant calling and annotation were carried out by the Genome Analysis Toolkit, version 2.2, in combination with GenomeComb (http://genomecomb.sourceforge.net/) (McKenna et al., 2010; Reumers et al., 2012). Identified variants were subsequently filtered using 1000 Genome Project database (http://www.1000genomes.org), dbSNP version 147 (http://www.ncbi.nlm.nih.gov/projects/SNP), Exome Variant Server (http://evs.gs.washington.edu/EVS/), and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/). Raw reads of rare variants were manually checked using the integrative genomics viewer (Broad Institute, Cambridge, MA, USA). Predicted protein-altering variants, defined as nonsense, splice site, initiation codon, indel, and missense variants with a minor allele frequency (MAF) ≤1% were selected. Selected variants were confirmed by Sanger sequencing on genomic DNA and Combined Annotation Dependent Depletion (CADD) scores were computed for functional annotation (http://cadd.gs.washington.edu/score) (Kircher et al., 2014). Fisher's exact tests were applied for variant frequency comparisons between patients and controls in SPSS 24.0 (IBM SPSS Inc, Chicago, IL, USA). Post hoc power analysis was used to evaluate the statistical power of our study cohort based on reported mutation frequencies of *NEK1* LOF variants and the *NEK1* p.Arg261His variant (Kenna et al., 2016). A binomial test was used to investigate whether the frequency of ALS patients with multiple mutations was higher in the *NEK1*-positive group than expected by chance (as described by van Blitterswijk et al. (2012)) based on the overall mutation frequency of 9 ALS genes (*SOD1*, *TARDBP*, *VCP*, *FUS*, *TBK1*, *C9orf92*, *CHCHD10*, *TUBA4A*, and *NEK1*) in the total patient cohort (57/278 or 21%) (Gijselinck et al., 2012; Gijselinck et al., 2015; Perrone et al., 2017; van der Zee et al., 2017). The following formula was used: pbinom(7,278,**0.047***0.21, lower.tail=FALSE, log.p=FALSE), where 7 is the number of additional mutation carriers in the *NEK1*-positive group versus tot total patient cohort of 278, 0.047 is the frequency of *NEK1* rare variants in 278 patients (13/278), and 0.21 is the total frequency of pathogenic mutation carriers (57/278). Both post hoc and binomial test were performed in R software 3.2.3.

2.4. Nonsense-mediated mRNA decay experiments

Lymphoblast cell lines of carriers of predicted LOF variants were incubated with 150 μg/mL cycloheximide (Sigma St. Louis, MO, USA) at 37 °C for 4 hours to inhibit nonsense-mediated mRNA decay. As a negative control, cells were treated with equal volumes of dimethyl sulfoxide. Total RNA was extracted from cells using the RiboPure kit (Ambion, Life Technologies, Carlsbad, CA, USA) followed by a DNase treatment (TURBODNase Kit, Ambion, Life Technologies). First-strand complementary DNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies) using both oligo(dT) and random hexamer primers. Exon 28 (p.Tyr871Valfs*17) and exon 31 (p.Ser1036*) of *NEK1* were amplified by flanking primers in exon 27–29 and exon 30–32, respectively. PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) on an AB3730 DNA Analyzer (Life Technologies). Sequences were analyzed and visually inspected by novoSNP (Weckx et al., 2005).

3. Results

We conducted full exonic resequencing of *NEK1* in Belgian patients and control individuals. We identified 17 unique *NEK1* variants counting 7 variants (2 LOF and 5 missense) present in 13/278 patients (4.7%) and 14 variants (1 LOF, 12 missense, and 1 amino acid deletion) in 20/609 control individuals (3.3%; Table 1).

3.1. NEK1 *loss-of-function variants*

Three variants introduced a premature termination codon, c.2560delC (p.Leu854Tyrfs*2), c.2609insA (p.Tyr871Valfs*17), and p.3107C>G (p.Ser1036*), of which the p.Leu854Tyrfs*2 is reported for the first time in this study (Black et al., 2017; Brenner et al., 2016; Kenna et al., 2016). The generated premature termination codons are located in the acidic C-terminal region of the protein and would result, if translated, in loss of one of the 2 nuclear export signals (Fig. 1). Lymphoblast cell lines from the p.Tyr871Valfs*17 and p.Ser1036* carriers were available for transcript analysis and complementary DNA sequencing demonstrated near complete loss of the mutated transcript by nonsense-mediated mRNA decay, confirming loss of function by loss of transcript for these variants (Fig. 2).

The 2 frameshift variants, p.Leu854Tyrfs*2 and p.Tyr871-Valfs*17, were each present in 1 isolated ALS patient and absent from controls (Table 2). In both patients (ages at onset/inclusion of 62 and 61 years, respectively), previous mutation screening excluded mutations in *C9orf72*, *TBK1*, *SOD1*, *VCP*, *FUS*, *TARDBP*, *GRN*, and *MAPT*. The nonsense variant, p.Ser1036*, was found in 1 familial ALS patient (onset age: 47 years) and her affected sib (onset age: 52 years and age at death: 60 years), who both also harbored a *C9orf72* repeat expansion and *TUBA4A* p.Thr381Met variant (Gijselinck et al., 2012; Perrone et al., 2017). Yet, this p.Ser1036* nonsense variant was also observed in 1 control individual aged 54 years (Table 2).

In terms of risk association, our cohort with 1.1% (3/278) LOF variants in patients and 0.16% (1/609) in control individuals was enriched for LOF variants in patients but did not reach statistical significance compared to the Belgian control cohort ($p = 0.095$, Fisher's exact test). When comparing to the frequency of heterozygous LOF variants in the ExAC European Non-Finnish data set (39/55,109 alleles, http://exac.broadinstitute.org), however, a significant *p*-value of 0.001 was calculated.

3.2. NEK1 *missense variants*

The majority of identified variants were missense variants (Table 1). In patients, 4 missense variants were identified in 10/278 (3.6%) patients (Table 2). Of those, only one c.694C>T (p.Arg232Cys) was absent from controls and found in an ALS-FTD patient (age at inclusion: 45 years) with previously identified *C9orf72* mutation. The p.Arg232Cys substitution maps to the kinase domain of the protein and is classified amongst the top 0.1% most deleterious variants in the human genome (CADD score: 34). Yet, most of the other missense variants present in controls also reached CADD scores >20.

The most common variant was the c.782G>A (p.Arg261His; CADD score: 34), reported to be associated with ALS risk (Kenna et al., 2016). In our study, we identified the p.Arg261His variant in 5/278 patients (all ALS) and 4/609 controls, giving MAFs of 0.90% in the patient group compared to 0.33% in the control group ($p = 0.150$, Fisher's exact test). Comparison to the ExAC European Non-Finnish data set (MAF: 0.37%) showed the same trend but did not reach significance ($p = 0.062$). Another 9 singleton missense variants and 1 single-amino acid deletion, c.2206-2209delTCA (p.Ser735del), were detected in controls only.

3.3. NEK1 *polygenic carriers*

Additional ALS gene mutations were present in 7 out of 13 *NEK1* patient carriers (Table 2).

Table 1
Rare coding variants identified in the *NEK1* gene

Exon	Genomic position	cDNA	Predicted protein	CADD score	dbSNP147-MAF (%)	ExAC-MAF (%) (allele count)	ALS, n = 245	ALS-FTD, n = 33	Controls, n = 609
Patient-only variants									
Exon10	g.170506613G>A	c.694C>T	p.Arg232Cys	34	-	0.001 (1/119910)		1	
Exon27	g.170384421delG	c.2560delC	p.Leu854Tyrfs*2	24.3	-	-	1		
Exon28	g.170359388insT	c.2609insA	p.Tyr871Valfs*17	25	-	-	1		
Variants present in both patients and controls									
Exon10	g.170506525C>T	c.782G>A	p.Arg261His	34	rs200161705 (0.226)	0.232 (276/118,776)	5		4
Exon13	g.170483347C>T	c.1021G>A	p.Ala341Thr	24.2	rs189186475 (0.112)	0.112 (27/24,036)	1		2
Exon26	g.170398474A>C	c.2235T>G	p.Asn745Lys	28.6	rs34324114 (0.575)	0.618 (298/48,232)	2	1	3
Exon31	g.170345819G>C	c.3107C>G	p.Ser1036*	37	rs199947197 (0.012)	0.012 (14/120,612)	1		1
Controls-only variants									
Exon3	g.170523673T>C	c.109A>G	p.Ile37Val	25.3	-	-			1
Exon6	g.170511956A>C	c.317T>G	p.Leu106Trp	27.2	-	0.002 (1/49,184)			1
Exon6	g.170511887A>C	c.386T>G	p.Ile129Ser	31	-	-			1
Exon10	g.170506672A>C	c.635T>G	p.Val212Gly	26.6	-	-			1
Exon21	g.170429415T>C	c.1817A>G	p.Lys606Arg	12	-	-			1
Exon24	g.170400556C>T	c.2137G>A	p.Val713Met	18.8	rs199827465 (0.082)	0.084 (10/120,434)			1
Exon25	g.170398590G>C	c.2198C>G	p.Ala733Gly	26.1	-	-			1
Exon25	g.170398587A>T	c.2201T>A	p.Ile734Asn	24.2	-	-			1
Exon25	g.170398582-170398585delTGA	c.2206-2209delTCA	p.Ser735del	22.4	-	-			1
Exon31	g.170345733T>C	c.3193A>G	p.Thr1065Ala	23.5	rs371084271 (0.021)	0.019 (22/115,810)			1

gDNA numbering according to the human reference sequence (Genome Reference Consortium Human Build 37/human genome 19, GRCh37/hg19); cDNA numbering according to the cDNA reference sequence NM_001199397.1; protein numbering relative to the NEK1 isoform NP_ 001186326.1.
Key: ALS, amyotrophic lateral sclerosis; CADD, Combined Annotation Dependent Depletion (http://cadd.gs.washington.edu) (Kircher et al., 2014); cDNA, complementary DNA; dbSNP147, the Single Nucleotide Polymorphism Database version 147; ExAC, Exome Aggregation Consortium (http://exac.broadinstitute.org/); FTD, frontotemporal dementia; MAF, minor allele frequency (%); *NEK1*, never in mitosis gene a–related kinase 1.

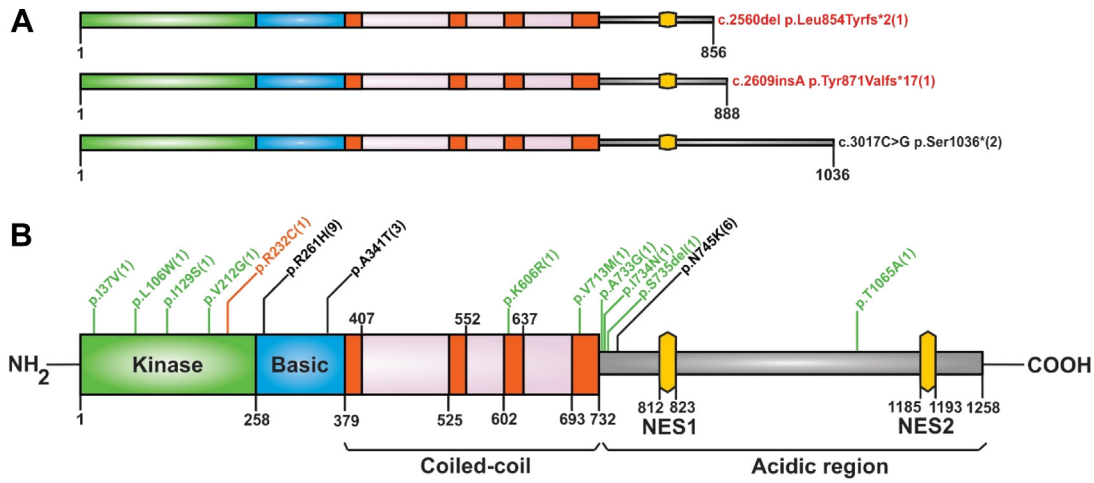

Fig. 1. Schematic representation of *NEK1* protein structure with identified variants in the Belgian ALS, ALS-FTD, and control cohort. (A) *NEK1* LOF variants with predicted premature termination. (B) Predicted structural and functional domains of *NEK1* with identified coding variations (NM_001199397.1). Kinase domain (1-258), basic domain (258-379), coiled-coil domains (379-732), and 2 nuclear export sequences 1 (NES1) and 2 (NES2) are shown. For (A), coding variants identified in ALS patients only are in red, in both patients and controls in black. For (B), variants identified in ALS-FTD patients only are in orange, in controls only in green, and in patients and controls in black. The numbers between brackets refers to the number of carriers. Abbreviations: ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; LOF, loss-of-function; *NEK1*, never in mitosis gene a–related kinase 1. Figure adapted from Brenner et al. (2016).

The p.Ser1036* nonsense variant was detected in a familial ALS patient with previously reported *C9orf72* repeat expansion and *TUBA4A* p.Thr38Met variant (Perrone et al., 2017).

Three of the p.Arg261His patients carried a *C9orf72* repeat expansion and 1 patient carried a *TARDBP* p.Tyr374* nonsense mutation (Daoud et al., 2009). A *C9orf72* expansion was also present in the ALS-FTD patient with patient-only *NEK1* p.Arg232Cys variant. One of the 3 patients with a *NEK1* p.Asn745Lys variant harbored an additional *SOD1* p.Ile114Thr mutation (Brown et al., 2012).

Taken together, the frequency of oligogenic carriers in the *NEK1*-positive group of 54% (7/13) was significantly higher than what would be expected by chance based on the observed mutation frequency in the total patient cohort of 21% (57/278; $p = 0.007$).

4. Discussion

We explored the genetic contribution of *NEK1* in a Belgian cohort of 278 ALS and ALS-FTD patients. We identified 3 LOF variants, of which one was novel (p.Leu854Tyrfs*2), giving a frequency of 1.1%, comparable to recent reports (Black et al., 2017; Brenner et al., 2016; Cirulli et al., 2015; Kenna et al., 2016). So far, cosegregation of *NEK1* LOF variants has only been demonstrated for one variant (p.Arg550*) in one affected first-degree relative (Kenna et al., 2016). In the present study, we identified the p.Ser1036* nonsense variant in a familial ALS patient (onset age: 47 years) and confirmed the same variant in her deceased brother (onset age: 52 years and age at death: 60 years) who had suffered from ALS and cognitive problems. Remarkably, in the same sibship, we previously described the co-occurrence of a *C9orf72* repeat expansion and *TUBA4A* p.Thr381Met variant, so both affected sibs carried the *NEK1*, *C9orf72* and *TUBA4A* variants (Gijselinck et al., 2012). Yet, the p.Ser1036* nonsense variant was also present in one of the Belgian controls. In line with this, this variant has been observed by others in 0.39%–0.57% of ALS patients and 0.06 of controls, as well as in an asymptomatic aged sib of a familial ALS index patient (Brenner et al., 2016; Kenna et al., 2016). In total, 10 LOF variants were identified in control individuals, including the present study (Brenner et al., 2016; Kenna et al., 2016). These multiple observations indicate reduced penetrance for heterozygous *NEK1* LOF variants, complicating genetic counseling in carriers. Although our observations were comparable to previous studies, we could not confirm a significant enrichment of *NEK1* LOF variants in patients

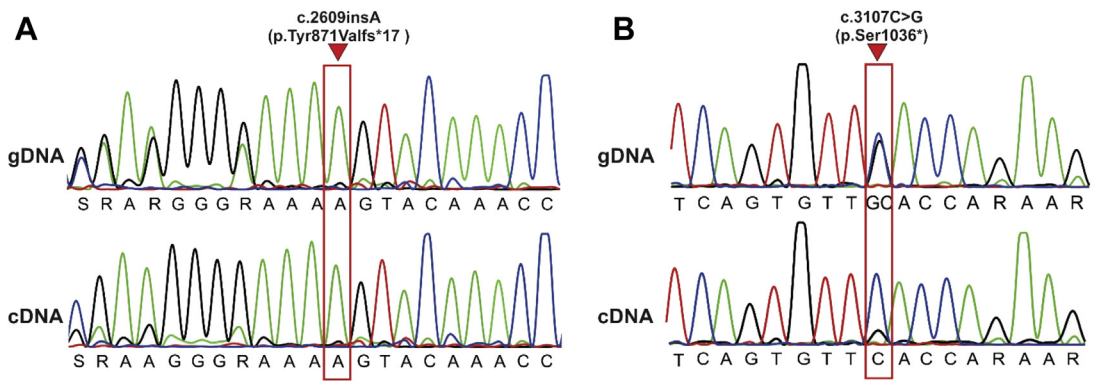

Fig. 2. cDNA transcript analysis in *NEK1* LOF carriers. Expression of the mutant transcripts in lymphoblast cell lines of carriers with the c.2609insA (p.Tyr871Valfs*17) (A) and the c.3107C>G (p.Ser1036*) (B) mutation. Sequence traces flanking the respective mutations on genomic DNA (gDNA) extracted from blood and complementary DNA (cDNA) isolated from lymphoblast cells, showing strong, but not complete, reduction of the mutant transcript for both mutations. Abbreviations: LOF, loss-of-function; *NEK1*, never in mitosis gene a–related kinase 1.

Table 2
Clinical characteristics of *NEK1* mutation carriers

Patients	*NEK1* mutation	Gender	Family history	Age (y)	Age at death (y)	Disease duration (y)	Clinical diagnosis	Site of onset	Mutations
Carriers of patient-only variants									
DR682.1	p.Arg232Cys	Female		AAI: 45	46	1[c]	ALS-FTD		*C9orf72*
DR1320.1	p.Leu854Tyrfs*2	Female		AAO: 62	67	5	ALS	Spinal	
DR1327.1	p.Tyr871Valfs*17	Male		AAI: 61			ALS		
Carriers of variants present in both patients and controls									
DR665.1	p.Arg261His	Male		AAI: 43	45	2[c]	ALS		*C9orf72*
DR668.1	p.Arg261His	Male		AAI: 63	63	1[c]	ALS		*C9orf72*
DR1319.1	p.Arg261His	Female		AAO: 54	58	4	ALS	Bulbar	
DR1110.1	p.Arg261His	Female	+	AAO: 47	51[a]	4[b]	ALS	Spinal	*TARDBP* p.Tyr374[a]
DR1322.1	p.Arg261His	Male		AAI: 67			ALS		*C9orf72*
DR1323.1	p.Ala341Thr	Female		AAO: 54	58[a]	4[b]	ALS	Bulbar	
DR1324.1	p.Asn745Lys	Female	+	AAI: 64	66	2[c]	ALS + D		*SOD1* p.Ile114Thr
DR1325.1	p.Asn745Lys	Female		AAO: 57	61	4	ALS	Spinal	
DR1326.1	p.Asn745Lys	Male		AAO: 76			ALS	Spinal	
DR519.1	p.Ser1036*	Female	+	AAO: 47	57	1	ALS	Spinal	*C9orf72*, *TUBA4A* p.Thr38Met

Amino acid positions are relative to NP_ 001186326.1. Family history: Patients with a positive family history, based on first- or second-degree relatives with symptoms of motor neuron disease or dementia, are indicated by "+." Age: AAO = age at onset, AAI = age at inclusion, D = dementia unspecified; *C9orf72*: pathogenic repeat expansion mutation in *C9orf72*.
Key: ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; *NEK1*, never in mitosis gene a–related kinase 1.
[a] Age at last evaluation, alive.
[b] Duration from onset till last evaluation.
[c] Duration from inclusion till death.

compared to controls in our Belgian cohort, likely due to the low prevalence of these variants, combined with the limited sample size of our in-house control cohort (power to detect significant association was only 46%). When comparing our cohort to the public ExAC European data set however, association of LOF variants became clearly significant ($p = 0.001$). Available biomaterials facilitated transcript analysis of the p.Tyr871Valfs*17 and the p.Ser1036* variant and demonstrated a strong but not complete loss of mutant transcript (Fig. 2). This is in line with studies on short-rib polydactyly syndrome type II, caused by homozygous *NEK1* LOF variants, where affected children exhibit 64% reduction but not complete absence of *NEK1* mRNA levels, and their unaffected heterozygous parents display 30%–40% reduction (Kenna et al., 2016; Thiel et al., 2011). Our data, together with previous findings, therefore support the hypothesis of haploinsufficiency of *NEK1* in ALS (Black et al., 2017; Brenner et al., 2016; Cirulli et al., 2015; Kenna et al., 2016).

In addition to LOF variants, we identified another 13 missense variants present in comparable fractions of patients (10/278 or 3.6%) and controls (18/609 or 3.0%) and 1 single-amino acid deletion in 1 control. Notably, one of the missense variants, p.Arg232Cys identified in an ALS-FTD patient, was absent from our in-house control set and reported only once in the ExAC database. The p.Arg232Cys is located in the kinase domain of NEK1 and affects a highly conserved Arg-residue. Consequently, CADD score evaluation classifies this variant as amongst the top 0.1% most deleterious variants in the genome (CADD score: 34). However, this patient with early disease onset of 45 years also carried a previously identified *C9orf72* repeat expansion mutation. Furthermore, 4 similar missense variants in the kinase domain with CADD scores >25 were present in controls. The *NEK1* p.Arg261His had been demonstrated to significantly increase ALS risk. In the present study, we identified p.Arg261His in 5/278 (1.8%) patients and 4/609 (0.66%) control individuals, showing a trend toward, but not significant, enrichment in patients (power = 28%). During the course of the study, we identified an additional affected p.Arg261His carrier, careful re-evaluation of this patient's medical records revised his diagnosis from possible ALS to inclusion-body myopathy, and was therefore excluded from the study. Yet, this observation is of interest as it may indicate a possible relationship between *NEK1* and the complex clinical spectrum of ALS, FTD, inclusion-body myopathy (and Paget's disease of bone), comparable to missense mutations in the *VCP* gene.

As we are uncovering more genes in ALS, carriers of multiple ALS-associated mutations become apparent (van Blitterswijk et al., 2012). This was also evident from our study where we observed additional ALS gene mutations in 54% of *NEK1* carriers. Strikingly, this was significantly more than what would be observed by chance based on the individual mutation frequencies of the known ALS genes that had an overall frequency of 21% in the total patient cohort. This enrichment of pathogenic ALS mutations in *NEK1* variant carriers may fit into the "2-hit" or "multiple-hit" hypothesis that disease presentation results from accumulation of mutations (with variable penetrance). In that context, it is important to note that *C9orf72* and *SOD1* are known to be associated with reduced penetrance and wide ranges in disease onset. So although they may be the driver of the disease, the additional burden of a *NEK1* variant may be needed for disease presentation or may accelerate it. An earlier disease onset was suggestive for such disease-modifying effect in the p.Arg261His patient with *TARDBP* p.Tyr374* nonsense mutation. This *TARDBP* mutation was previously described in an ALS patient with onset age of 63 years and predicted to generate a truncated TDP-43 protein that abolishes interaction of the C-terminal region of TDP-43 with heterogeneous nuclear ribonucleoproteins (Daoud et al., 2009). In the present study, the *TARDBP* p.Tyr374* mutation was detected in an ALS patient with >15 years earlier onset age of 47 years, possibly due to the additional burden of the *NEK1* p.Arg261His risk variant, although more observations are needed to substantiate this claim. The most striking example of oligogenic carriers was the *NEK1* p.Ser1036* nonsense variant that we identified in 2 affected sibs with family history of ALS and cognitive impairment that also segregated a *C9orf72* repeat expansion and *TUBA4A* p.Thr38Met variant with early disease onsets of 47 and 52 years (Perrone et al., 2017). In this family, the *C9orf72* expansion is likely the most penetrant mutation, but a synergistic effect of *NEK1* and *TUBA4A* on the phenotype cannot be excluded.

In conclusion, we observed rare *NEK1* coding variants in 4.7% of patients. Of these, LOF variants accounted for about 1% of patients, in line with previous reports, although interpretation of

pathogenicity and penetrance is complicated by the observation of occasional LOF variants in asymptomatic carriers. Furthermore, we observed excess concurring pathogenic mutations in *NEK1* carriers, supporting the proposed notion of an oligogenic model for ALS.

Disclosure statement

PVD holds a senior clinical investigatorship of the Research Foundation Flanders (FWO). The other authors have no conflicts of interest to disclose.

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