Comprehensive genetic analysis of a Hungarian amyotrophic lateral sclerosis cohort

Ph.D thesis

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2024

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2024
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List of publications

Publications providing the basis of the dissertation:

I. **Nagy, Z. F.**, Pál, M., Salamon, A., Kafui Esi Zodanu, G., Füstös, D., Klivényi, P., & Széll, M. (2022). Re-analysis of the Hungarian amyotrophic lateral sclerosis population and evaluation of novel ALS genetic risk variants. Neurobiology of aging, 116, 1–11. https://doi.org/10.1016/j.neurobiolaging.2022.04.002

II. Tripolszki, K., Gampawar, P., Schmidt, H., **Nagy, Z. F.**, Nagy, D., Klivényi, P., Engelhardt, J. I., & Széll, M. (2019). Comprehensive Genetic Analysis of a Hungarian Amyotrophic Lateral Sclerosis Cohort. *Frontiers in genetics*, *10*, 732. https://doi.org/10.3389/fgene.2019.00732

III. Tripolszki, K., Danis, J., Padhi, A. K., Gomes, J., Bozó, R., **Nagy, Z. F.**, Nagy, D., Klivényi, P., Engelhardt, J. I., & Széll, M. (2019). Angiogenin mutations in Hungarian patients with amyotrophic lateral sclerosis: Clinical, genetic, computational, and functional analyses. Brain and behavior, 9(6), e01293. https://doi.org/10.1002/brb3.1293

List of abbreviations

Gene names are not listed below, their abbreviations are spelled out as they first appear in the text.

ACMG	American College of Medical Genetics and Genomics
ALS	amyotrophic lateral sclerosis
ALS-FRS-R	revised ALS functional rating scale
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
fALS	familial amyotrophic lateral sclerosis
FAM	fluorescein amidite
FDA	U.S. Food and Drug Administration
FTD	frontotemporal dementia
GPCR	G protein-coupled receptors
HUVEC	human umbilical vein endothelial cell
LMN	lower motor neuron
MLPA	Multiplex ligation-dependent probe-amplification
MORN	membrane occupation and recognition nexus
NFL	neurofilament light chain protein
NGS	next generation sequencing
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	percutaneous endoscopic gastrostomy
RNA	ribonucleic acid
sALS	sporadic amyotrophic lateral sclerosis
SMA	spinal muscular atrophy
tRNA	transfer ribonucleic acid
UMN	upper motor neuron
VMD	Visual Molecular Dynamics
VUS	variant of uncertain significance

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Introduction

Amyotrophic lateral sclerosis (ALS) is a rare fatal neurodegenerative disease characterized by the loss of motor neurons in the motor cortex as well as in the brainstem (van Es et al. 2017).

Epidemiology and environmental risk factors of amyotrophic lateral sclerosis

Its incidence in European populations varies between 0.5-3.6/100 000/year, while disease prevalence ranges from 1.1-8.2/100 000 (Alcaz et al. 1996; Joensen 2012). Incidence of ALS cases has been on the rise lately, which may be attributed to more people reaching an older age (Chio, Magnani, and Schiffer 1995). There is a slight male predominance among ALS patients, the typical male:female ratio of ALS is 1.3-1.5. The first symptoms of the disease commonly appear at around 60 years old, and the diagnosis of ALS is usually set up after a year of disease onset (Chiò et al. 2013).

Up to date only smoking cigarettes and ageing have been found to increase susceptibility to ALS (Zhan and Fang 2019; Al-Chalabi and Hardiman 2013). Exposure to heavy metals and pesticides, repeated head trauma and serving in the military have been proposed as environmental risk factors of ALS but the published results are inconclusive (Al-Chalabi and Hardiman 2013).

Symptoms of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis usually presents with weakness confined to voluntary muscles. Muscle wasting, muscle cramps and fasciculations are also characteristic features of ALS. Based on the onset of symptoms limb-onset (around 2/3 of the cases) and bulbar-onset (around 1/3 of the cases) forms of ALS may be differentiated (Masrori and Van Damme 2020). Symptoms of a bulbar onset disease include dysphagia and dysarthria. Muscle weakness commonly spreads to a neighboring region of the body or follows a neuroanatomical pathway. Interestingly, eye movements and sphincter muscles frequently remain unaffected (van Es et al. 2017).

Around half of the ALS patients experience behavioral changes over the course of the disease. These alterations include apathy, executive difficulties, fluency and memory loss (van Es et al. 2017). Cognitive decline often indicates the presence of an underlying genetic variant (Hu et al. 2013). 5-15% of the ALS patients also fulfill the diagnostic criteria for frontotemporal

dementia (FTD), thus ALS and FTD may be considered to fall on a disease spectrum with the two extremes represented by pure ALS and pure FTD (van Es et al. 2017).

Diagnosis of amyotrophic lateral sclerosis

Establishing the diagnosis of ALS may be a lengthy process and it is based on the accurate past medical history and professional physical examination. Alternative diagnoses of diseases which may show a phenotypic overlap must be excluded by relevant examinations (Shefner et al. 2020). Electromyographic examination of an ALS patient shows fibrillations and fasciculations in relaxed muscles as a sign of neurogenic damage (Masrori and Van Damme 2020). Nerve conduction studies may show a decreased response amplitude (de Carvalho et al. 2005). Imaging studies might be also carried out to exclude other conditions such as discopathies or abnormalities of the motor system (Masrori and Van Damme 2020). In later stages of disease there are several imaging markers that may be noted, such as hypointensity of the motor cortex, atrophy of the precentral gyri or cortical thinning of the primary motor cortex and of the corticospinal tract. These irregularities may reflect the decline in motor function (Mazón et al. 2018).

Differential diagnoses to ALS include the identification of "ALS mimicking syndromes" which may mislead clinicians in around 7-8% of cases (Traynor et al. 2000). In a case of ALS presenting with predominantly upper motor neuron (UMN) signs hereditary spastic paraplegia and diseases of the spinal cord and cervical disc protrusion are the most common alternate diagnoses. In cases presenting with a lower motor neuron (LMN) sign dominance peripheral neuropathy, myopathies, Kennedy's disease and myasthenic syndromes should also be considered (Masrori and Van Damme 2020).

ALS diagnostic is a lengthy procedure. Neurofilament light chain protein (NFL) seems a promising biomarker which could speed up the diagnostic procedure (Verde, Otto, and Silani 2021). NFL levels show a more than threefold rise in cerebrospinal fluid of ALS patients compared to patients diagnosed with syndromes mimicking ALS (Forgrave et al. 2019). In addition, measurement of NFL in the serum yielded a similar sensitivity and specificity ratio compared to cerebrospinal fluid NFL analysis (Gaiottino et al. 2013). However, further examinations on NFL as an ALS biomarker are needed to routinely use NFL in everyday clinical practice (Verde, Otto, and Silani 2021).

Until 2021 the El Escorial and the Awaji-shima criteria were used to guide the diagnosis of ALS (Ludolph et al. 2015; Carvalho and Swash 2009) (Table 1.). While the El Escorial criteria solely focuses on the findings of the physical examination, the Awaji-shima criteria

introduces electrophysiological findings as a further base of evidence. In 2022, due to the former two criteria being not easily understandable to patients, the Gold Coast criteria was introduced which defines ALS as a broader entity. According to the Gold Coast criteria ALS is a progressive neurological disease not explained by an alternative diagnosis. ALS must present with UMN and LMN signs in the same body region or with LMN signs in at least two body regions (Turner 2022).

Criteria	Clinically definite ALS	Clinically probable ALS	Clinically possible ALS	Suspected ALS
El Escorial criteria (1994)	UMN and LMN signs in three regions of the body	UMN and LMN signs in at least two regions, with some UMN sign rostral to LMN signs	UMN and LMN signs in only one region, or UMN signs alone in two or more regions, or LMN signs rostral to UMN signs	LMN signs only
Awaji-Shima criteria (2008)	Clinical or electrophysiological evidence of UMN and LMN signs in the bulbar region and at least two spinal regions, or UMN and LMN signs in three spinal regions	Clinical or electrophysiological evidence of UMN and LMN signs in at least two regions, with some UMN signs rostral to LMN signs	Clinical or electrophysiological evidence of UMN and LMN signs alone in two or more regions, or LMN signs rostral to UMN signs	

Table 1. The El Escorial and Awaji-shima criteria for ALS diagnosis

To stratify severity of ALS at the time of diagnosis and to monitor the progression of the disease a questionnaire (ALS Functional Rating Scale Revised) is used. The test contains 12 questions which assess the everyday functions spared by the disease. Questions cover speech disturbances, fine motor function, breathing difficulties etc. The maximum score of 48 points suggests no difficulties in everyday functions. The decline rate of ALS-FRS-R score reflects disease progression and indicates survival time as well (Kimura et al. 2006; Kaufmann et al. 2005).

Treatment of amyotrophic lateral sclerosis

Unfortunately, ALS is currently an incurable disease. Although much effort is directed towards finding an efficient therapy for patients, although the need remains unmet. Currently two molecules are available that moderately increase survival of ALS patients: riluzole and edaravone (Chiò, Mazzini, and Mora 2020). Riluzole was the first FDA approved drug for ALS. The medication prolongs survival of ALS patients by several months and slows down the progression of the disease (Bensimon, Lacomblez, and Meininger 1994). Riluzole is a direct inhibitor of the N-methyl-D-aspartate receptor and kainate type receptor of glutamate. By enhancing the uptake and decreasing the release of glutamate riluzole reduces the glutamate amount in the synaptic cleft and thus fights excitotoxicity (Dorst, Ludolph, and Huebers 2018).

Edavarone earned approval of the FDA in 2017 (Rothstein 2017). This small molecule may act by abolishing lipid peroxides and hydroxyl radical species and thus lowering the levels of oxidative stress (Adriano Chiò, Mazzini, and Mora 2020). Edaravone is administered intravenously and is known to stop the motor decline in ALS patients, although it is questionable in the light of real world evidence whether edaravone really prolongs the survival of patients (Yoshino 2019). Both drugs display relatively good tolerability and a good safety profile with bearable adverse effects (Bensimon, Lacomblez, and Meininger 1994).

Numerous investigational treatments are under development for ALS, several of them are gene-specific therapies, such as antisense oligonucleotides. Tofersen, an antisense oligonucleotide known to reduce the production of *SOD1* protein has already entered the phase 3 clinical trials and has demonstrated promising results. Levels of *SOD1* protein in the cerebrospinal fluid and levels of neurofilament light chain proteins in the plasma have significantly decreased 28 weeks after initiation of intrathecally administered tofersen (Miller et al. 2022). Tofersen is under trial in presymptomatic carriers of *SOD1* mutations which promises to yield even better outcomes than the use of tofersen in symptomatic ALS patients (Benatar et al. 2022).

To provide the best available care for ALS patients a multidisciplinary approach is essential (Masrori and Van Damme 2020). Physical therapy helps to sustain muscle function as long as possible and prevents the formation of contractures (Dal Bello-Haas 2018). Once the patient is at great risk of dysphagia and its complications, implantation of a percutaneous endoscopic gastrostomy (PEG) is recommended. PEG implantation reduces the number of hospitalizations and has been shown to prolong survival of patients (López-Gómez et al. 2021). In case the muscle weakness reaches the respiratory muscles artificial ventilation of the patients may be initiated. Non-invasive ventilation helps with quality of life and extends survival among late-stage ALS patients (Morelot-Panzini, Bruneteau, and Gonzalez-Bermejo 2019). End-of-life care should also involve palliative treatment and hospice care (Karam et al. 2016).

Despite all therapeutic efforts, ALS still has a poor survival rate with the median survival time from disease onset to death ranging from 1.5-4 years (Preux et al. 1996). However, 5-10% of patients experience a slower progression and are still alive more than 10 years after the onset of symptoms (Millul et al. 2005). Negative prognostic factors include bulbar onset of ALS and the emergence of weakness in the respiratory muscles (Bourke et al. 2006; Del Aguila et al. 2003).

Genetic background and pathomechanism of amyotrophic lateral sclerosis

Around 10% of ALS cases show a positive family history (fALS), while the other 90% remains of sporadic etiology (sALS). Currently in almost 70% of familial ALS cases there is an identifiable genetic alteration. However, in sporadic cases the chance of finding the causative genetic variant is slim, only around 10% (Renton, Chiò, and Traynor 2014).

The first gene to be associated with ALS was superoxide dismutase 1 (*SOD1*) in 1993 by a linkage analysis study (Rosen et al. 1993). *SOD1* variants may be identified in around 12% of familial ALS cases and in 1% of sporadic ALS patients (Renton, Chiò, and Traynor 2014). The *SOD1* gene encodes the Cu/Zn superoxide dismutase enzyme which is responsible for converting reactive superoxide species into oxygen and hydrogen peroxide (Eleutherio et al. 2021).

In 2011 came a breakthrough in ALS genetics: an intronic GGGGCC hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72)* gene was detected in ALS patients by two independent research groups (Renton et al. 2012; DeJesus-Hernandez et al. 2012). This repeat expansion is the most frequently noted genetic cause of ALS. The *C9orf72* hexanucleotide repeat expansion may be identified in around 40% of familial ALS cases. In sporadic ALS cases the frequency of the hexanucleotide repeat expansion varies from population to population: while in Finnish sALS patients it is identified in more than 21%, in Italian sALS patients only around 4%. (Majounie et al. 2012). Normal length alleles contain up to 23 repeats, while fully expanded alleles range from 30 to more than 1000 hexanucleotide units (DeJesus-Hernandez et al. 2012). Bulbar onset and cognitive decline seem to be more common among *C9orf72* repeat expansion carriers (Chiò et al. 2012).

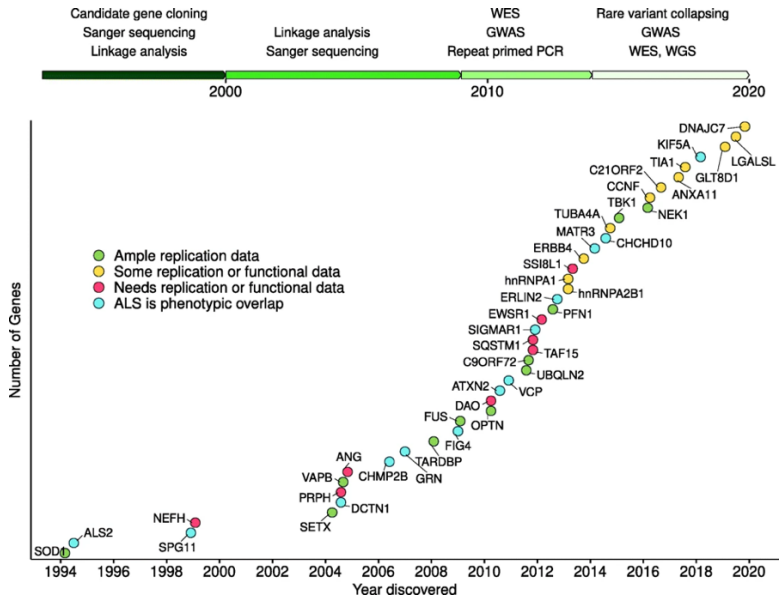

Figure 1. The most frequently identified ALS associated genes in a timeline noting the year of discovery. Colors show the stability of evidence connecting the gene to ALS (Gregory et al. 2020).

The application of next-generation sequencing methods in the last 20 years has led to a boom in the number of ALS genes (Figure 1) (Gregory et al. 2020). Currently there are more than 130 genes linked to ALS in the most comprehensive database (https://alsod.ac.uk). Out of these genes there are around 20 genes that have a well-established causal relationship with ALS and the other more than 100 genes may act as genetic risk factors or clinical modifiers of the disease. With the technical development in recent years more and more large-scale ALS genetic studies are being published. Vast sample repositories and consortia have been called to life to help coordinate the efforts to further expand the knowledge on the genetic background of ALS (Gregory et al. 2020).

The pathomechanism of amyotrophic lateral sclerosis is not fully explored yet and most likely multiple pathological processes are involved (Figure 2) (Van Damme, Robberecht, and Van Den Bosch 2017; van Es et al. 2017). Among others the pathological role of mitochondrial disturbances, defects of the axonal transport mechanisms, excitotoxicity, oxidative stress, inflammation, RNA metabolism dysregulations, RNA toxicity and compromised protein degradation have been proposed as pathological processes behind the disease. Results of extensive research also shows that a final common pathway could bind these various processes together (Nguyen, Thombre, and Wang 2019).

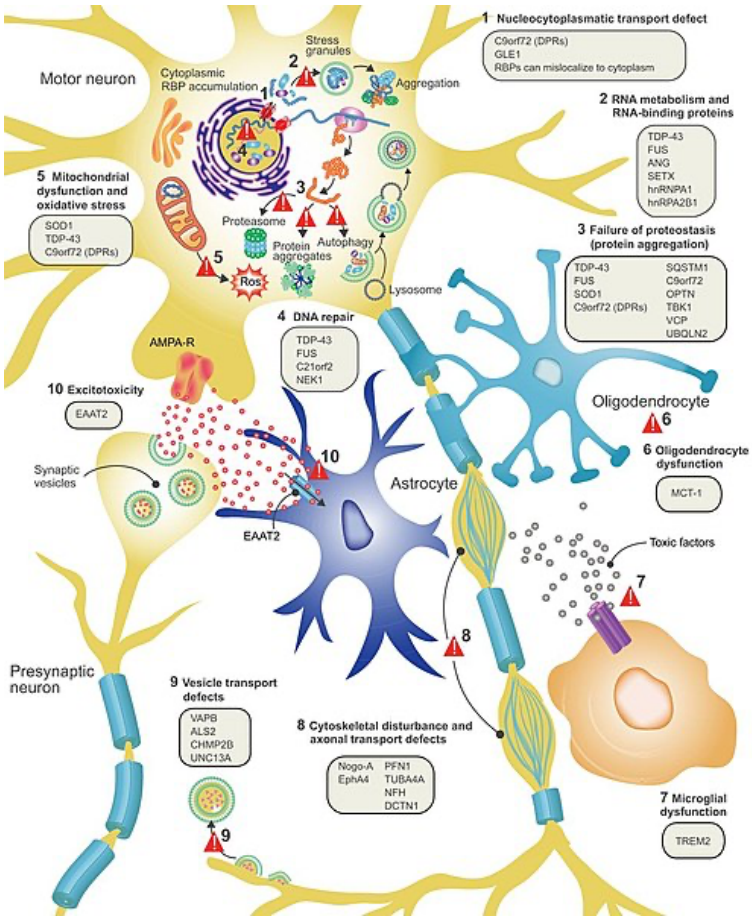

Figure 2. Pathological processes identified behind ALS

Several ALS-related proteins (for example: *SOD1, TDP-43, C9orf72*) can enter mitochondria and disrupt normal functioning, with increased formation of reactive oxygen species (ROS) consequently. *SOD1* encodes an antioxidant enzyme, which eliminates free radicals produced as a byproduct of catabolic processes, thus has a cytoprotective effect. Recent studies have identified the enzyme also as an RNA binding protein and as a regulator of transcription (Eleutherio et al. 2021). It would be logical, that the loss of enzymatic activity and thus the enhanced stress level of the cells would lead to cellular harm. However, *SOD1* variants do not cause ALS by the above-described loss-of-function mechanism; the proposed pathway for *SOD1* variants is rather a gain-of-function: toxic SOD1 aggregates are formed which have increased stability. The stable and misfolded protein aggregates resist the physiological protein turnover and the protein overload is the factor driving the cellular stress and the subsequent cell death.

The TAR DNA-binding protein (TDP-43 or *TARDBP*) is involved in the most prominent ALS associated pathways, such as RNA metabolism as an RNA binding protein and the molecule is involved in the biogenesis and processing of RNA molecules. Furthermore, the

defects of the protein are associated with mitochondrial dysfunction, defects of protein homeostasis and DNA repair (Bhardwaj et al. 2013). TDP-43 positive inclusion bodies often appear in the brain of those ALS patients who do not carry either a *SOD1* or a *FUS* variant (Hasegawa et al. 2008). These aggregates localize to the cytoplasm and contain ubiquitinated and hyperphosphorylated TDP-43 proteins which are resistant to normal breakdown processes (De Boer et al. 2021). TDP-43 aggregates may disturb multiple essential intracellular processes such as RNA metabolism (Tsuiji et al. 2013; De Boer et al. 2021). The aggregates also contribute to the mislocalization of nucleoporins and other transport proteins and disturb the usual nucleocytoplasmic transport routes and generate functional defects of the mitochondria (Chou et al. 2018; Zuo et al. 2021). This leads to the accumulation of TDP-43 in the cytoplasm where it cannot fulfill its role (Arai et al. 2006).

The FUS RNA-binding or fused-in-sarcoma protein, encoded by the FUS RNA-binding protein (*FUS)* gene, is also a key player of several different pathways. It is an RNA binding protein, involved in protein aggregation, alteration of DNA repair processes defect of RNA metabolism, in regulation of transcription, RNA splicing and transport and DNA repair in response to damage (Yamaguchi and Takanashi 2016). Evidence shows that experimental overexpression of FUS leads to the formation of cytoplasmic inclusions which is cytotoxic (Mitchell et al. 2013).

Another pathophysiological factor behind ALS is linked to the hexanucleotide repeat expansion of the *C9orf72* gene. The physiological function of the encoded protein is not fully understood yet. It is thought to behave as a trafficking protein and may be involved in RNA binding and RNA processing. Furthermore, it has a role as a regulator of GTPase enzymes (Pang and Hu 2021). It was also proven to be a regulator of endosomal trafficking and may act as a Rab guanine nucleotide exchange factor (Farg et al. 2014). The repeat expansion thought to act by a gain-of-function, and also by a loss-of-function mechanism (Walsh et al. 2015). Three non-exclusive potent pathological process have been pinpointed in association with the repeat expansion of the gene: haploinsufficiency, RNA toxicity and sequestering as well as the aggregation of faulty dipeptide repeat proteins (Walsh et al. 2015). Haploinsufficiency means that the level of wild type protein product translated from only one allele is not sufficient for normal operation of the cell. Haploinsufficiency might not be a highly prominent pathological feature of *C9orf72* repeat expansion since steady levels of *C9orf72* mRNA have been found in ALS patients (Sareen et al. 2013). If the C9orf72 protein is unable to process the RNAs in a physiological manner, some RNA molecules may become sequestered. The sequestered RNAs could include essential molecules which are unable to fulfill their role then and this mechanism

leads to altered expression of various genes (Cooper-Knock et al. 2014). The expanded intronic region may be translated into dipeptides which could form hairpin like structures and aggregate. The dipeptide repeat proteins may overload the cell and thus lead to degenerative processes (Mizielinska et al. 2014).

As depicted above, the molecular pathways that have been sofar implicated seem heterogeneous and complex, from protein aggregation and defects in multiple key intraneuronal processes, to dysfunction of glial cells. These above-mentioned pathological pathways may all contribute to the process of neurodegeneration.

Aims

In my thesis I aim to summarize the genetic examinations carried out by our research group to explore the complex genetic architecture of the Hungarian amyotrophic lateral sclerosis population.

In our project we aimed to thoroughly characterize from a genetic point of view the patients diagnosed with amyotrophic lateral sclerosis between 2008 and 2021 at the Department of Neurology, University of Szeged. Firstly, we intended to carry out the mutational screening of the major ALS genes in all patients. Secondly, in a subset of patients we targeted genes that have an ambiguous relationship to ALS. Lastly, in the same subset of patients we investigated genes that are related to conditions which might be a differential diagnosis to ALS.

Furthermore, we aimed to establish genotype-phenotype relationships and explore the proposed oligogenic background of the disease in Hungarian ALS patients.

Patients and methods

Patients

183 unrelated patients of Hungarian origin diagnosed with ALS were recruited for our study. The frequency of the detected variants was checked in an age and sex matched control group of 204 healthy individuals. Epidemiological data can be seen in Table 2. Senior neurologist clinicians from the Department of Neurology of the University of Szeged enrolled the patients into our study. All patients fulfilled the revised Awaji-Shima and El Escorial criteria (Carvalho and Swash 2009; Ludolph et al. 2015). Our study was performed in accordance with the Helsinki Declaration and its later amendments and was approved by the Ethical Board of the University of Szeged under the 553/2013 number. A written informed consent document was signed by all participating patients.

Number of patients recruited for the current study		183
Male:Female (ratio)		73:110 (0.66)
Mean age at onset in years (SD in years)		62.99 (10.12)
Minimum age at onset (years)		36
Maximum age at onset (years)		86
Number of healthy age and sex matched control individuals		204

Table 2. Demographical data concerning the enrolled ALS patients.

One patient had a family history of ALS. Two more patients reported a late-onset neuromuscular disease, mimicking ALS in their family. Four patients stated the occurrence of additional neurodegenerative condition (Alzheimer's or Parkinson's disease) in their family history.

Methods

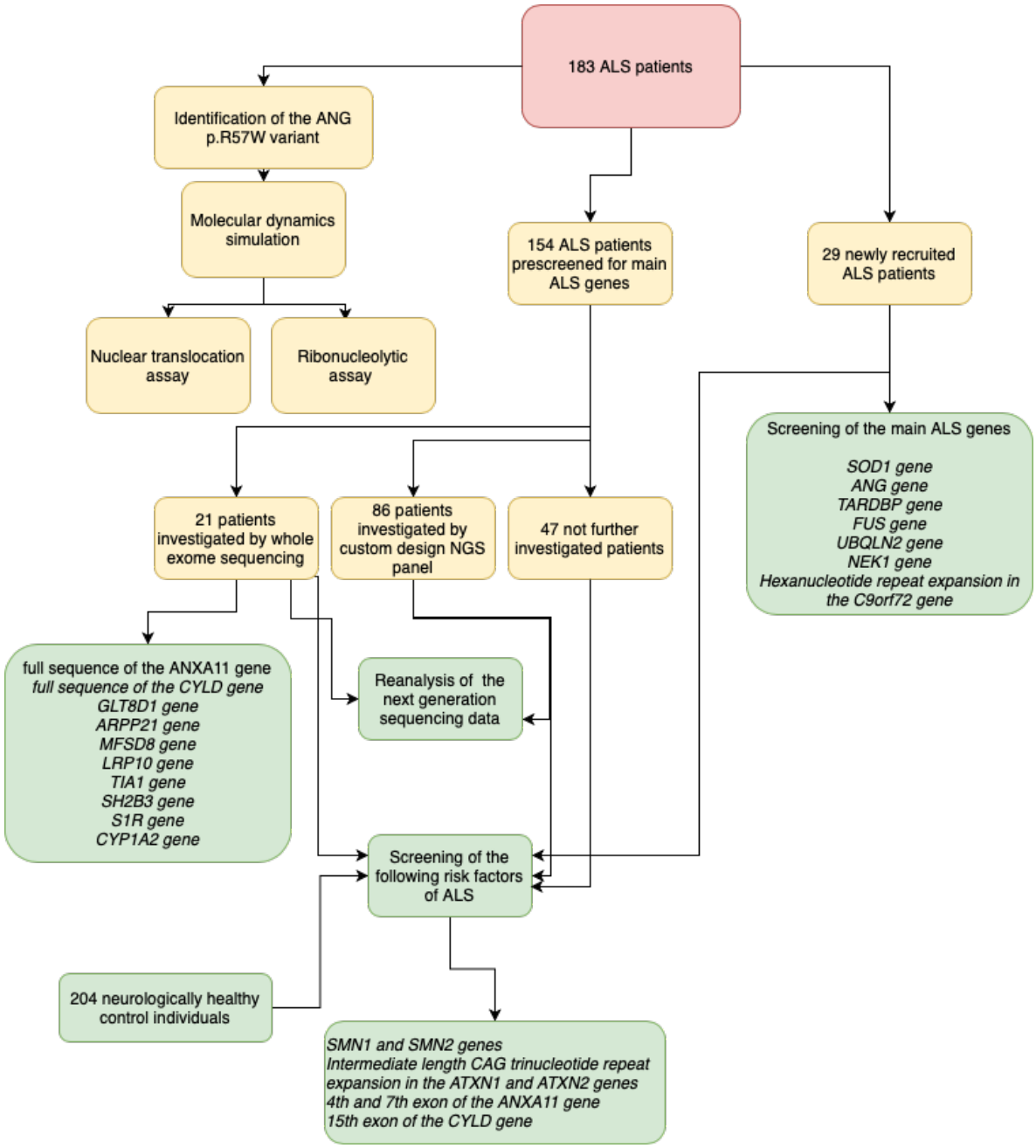

Figure 3.: Workflow of our study.

DNA extraction

Genomic DNA extraction was performed from peripheral blood samples of ALS patients with a commercially available DNA extraction kit (DNeasy® Blood & Tissue Kit, QIAGEN, Hilden, Germany), according to the manufacturer's protocol.

Next generation sequencing

Next generation sequencing techniques were used to assess 107 patients prescreened for variants in the most important ALS genes. Whole exome sequencing was carried out in 21 ALS patients, whilst a custom design gene panel was applied in 86 further patients on an Illumina NextSeq 500 sequencer. A mean on-target coverage of 189x and 71x were reached on the panel sequencing and on the WES, respectively.

The custom design panel contained 294 genes which were selected based on a thorough literature search. These 294 genes were divided into 3 sets: “Major ALS genes set” (35 genes) (Table 3), “Candidate or minor ALS genes set” (99 genes) (Table 4) and “Further neurodegenerative and/or neuromuscular diseases gene set” (160 genes) (Table 5).

Variants with a read depth under 25 reads were validated by bidirectional Sanger sequencing.

ANG	*DAO*	*GLE1*	*PFN1*	*TAF15*
ALS2	*DCTN1*	*GRN*	*SETX*	*TARDBP*
ATXN2	*ELP3*	*HNRNPA1*	*SIGMAR1*	*TBK1*
C9orf72	*ERBB4*	*MATR3*	*SOD1*	*TUBA4A*
CCNF	*EWSR1*	*NEFH*	*SPG11*	*UBQLN2*
CHCHD10	*FIG4*	*NEK1*	*SQSTM1*	*VAPB*
CHMP2B	*FUS*	*OPTN*	*SS18L1*	*VCP*

Table 3.: The “Major ALS gene set” of the used custom designed gene panel

AGT	*CCS*	*CST3*	*EPB41L1*	*KIF5A*	*NAIP*	*PON3*	*SELL*	*SYNE1*
ALAD	*CDH13*	*CX3CR1*	*EPHA4*	*KIFAP3*	*NETO1*	*PRPH*	*SEMA6A*	*SYT9*
ANXA11	*CDH22*	*CYP2D6*	*FEZF2*	*LGALSL*	*NIPA1*	*PSEN1*	*SLC1A2*	*TREM2*
APEX1	*CHGB*	*DIAPH3*	*FGGY*	*LIF*	*NT5C1A*	*PVR*	*SLC39A11*	*TRPM7*
APOE	*CNTF*	*DISC1*	*GARS*	*LIPC*	*OGG1*	*RAMP3*	*SMN1*	*UNC13A*
AR	*CNTN4*	*DNMT3A*	*GRB14*	*LMNB1*	*OMA1*	*RBMS1*	*SMN2*	*VDR*
ARHGEF28	*CNTN6*	*DOC2B*	*HEXA*	*LOX*	*PARK7*	*RNASE2*	*SNCG*	*VEGFA*
B4GALT6	*CRIM1*	*DPP6*	*HFE*	*LUM*	*PCP4*	*RNF19A*	*SOD2*	*VPS54*
BCL11B	*CRLF3*	*DYNC1H1*	*HNRNPA2B1*	*MAOB*	*PLEKHG5*	*SARM1*	*SOX5*	*ZFP64*
BCL6	*CRYM*	*EFEMP1*	*ITPR2*	*MAPT*	*PON1*	*SCFD1*	*SPG7*	*ZNF512B*
C21orf2	*CSNK1G3*	*ENAH*	*KDR*	*MOBP*	*PON2*	*SCN7A*	*SUSD1*	*ZNF746*

Table 4.: The “Candidate or minor ALS genes set” of the used custom designed gene panel

AARS	*BAG3*	*CPT1C*	*EGR2*	*GMPPB*	*KCNC3*	*MTMR2*	*PNKP*	*SBF1*	*TIA1*
ACTA1	*BICD2*	*CRYAB*	*EIF4G1*	*GNB4*	*KCND3*	*MUSK*	*PNPLA2*	*SBF2*	*TMEM240*
AFG3L2	*BSCL2*	*CYP2U1*	*ELOVL4*	*GNE*	*KIF1A*	*MYH7*	*PNPLA6*	*SCN11A*	*TPM2*
AGRN	*C19orf12*	*DCAF8*	*ELOVL5*	*GRID2*	*KIF1B*	*MYOT*	*POLG*	*SEPT9*	*TPM3*
ALDH18A1	*CA8*	*DDHD1*	*ERLIN1*	*HADHB*	*KIF1C*	*NDRG1*	*POLG2*	*SH3TC2*	*TPP1*
ANO5	*CACNA1A*	*DES*	*FBLN5*	*HNRNPDL*	*LAMA2*	*NEFL*	*POMGNT1*	*SIL1*	*TRIM2*
AP4E1	*CAMTA1*	*DHTKD1*	*FGD4*	*HSPB1*	*LDB3*	*NKX2-1*	*POMT2*	*SLC18A3*	*TRIM32*
AP4M1	*CAPN1*	*DMD*	*FGF14*	*HSPB3*	*LITAF*	*NPC1*	*PRKCG*	*SLC33A1*	*TRPC3*
AP4S1	*CCDC88C*	*DMPK*	*FKTN*	*HSPB8*	*LMNA*	*NPC2*	*PRPS1*	*SPAST*	*TRPV4*
AP5Z1	*CHRNE*	*DNAJB2*	*FLNC*	*HSPD1*	*LRRK2*	*PCNA*	*PRX*	*SPTBN2*	*TSEN54*
APTX	*CLN5*	*DNAJC13*	*GAN*	*IGHMBP2*	*LRSAM1*	*PDYN*	*RAB7A*	*STUB1*	*TTBK2*
ATG5	*COL13A1*	*DNM2*	*GBE1*	*INF2*	*MARS*	*PEX10*	*REEP1*	*SYNE2*	*TTN*
ATL1	*COL6A2*	*DRP2*	*GDAP1*	*ITPR1*	*MED25*	*PIK3R5*	*REEP2*	*SYNJ1*	*TTPA*
ATM	*COL6A3*	*DST*	*GIGYF2*	*KARS*	*MFN2*	*PLEC*	*RTN2*	*TAF1*	*YARS*
ATXN7	*COX10*	*DSTYK*	*GJB1*	*KBTBD13*	*MORC2*	*PLP1*	*RYR1*	*TFG*	*ZFYVE26*
B3GALNT2	*COX20*	*EEF2*	*GJC2*	*KCNA2*	*MPZ*	*PMP22*	*SACS*	*TGM6*	*ZNF9*

Table 5.: The “Further neurodegenerative and/or neuromuscular diseases gene set” of the used custom designed gene panel

Sanger sequencing

Sanger sequencing was used to assess the 6 following major ALS genes: *SOD1, TARDBP, ANG, FUS, UBQLN2* and *NEK1*. Target specific primers were designed using the NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) program and then used in a polymerase chain reaction (PCR) to amplify the exons and the splice sites at each end of the exons. Three mutational hotspots were also examined by Sanger sequencing: exon 4 and exon 7 of the *ANXA11* gene and exon 15 in the *CYLD* gene. Sequencing was performed on an ABI 3100 sequencer and compared then to the reference gene sequences utilizing NCBI Nucleotide BLAST software.

Analysis of the hexanucleotide repeat expansion in the *C9orf72* gene

The G_4C_2 sequence found in the intronic region of the *C9orf72* gene was investigated by long read repeat primed PCR method. The AmplideX PCR/CE *C9orf72* Assay (Asuragen, Inc.) kit was used according to the modified protocol reported by Suh et al (Suh, Grando, and Van Deerlin 2018).

Fragment length analysis

The exact length of the repeat containing regions was determined by fragment length analysis. The CAG repeat containing regions of the *ATXN1* and *ATXN2* genes were amplified by polymerase chain reactions using specific 6-FAM fluorophore marked primers. Fluorescently labeled DNA fragments were separated by capillary electrophoresis and sized by comparison to an internal standard.

Multiplex ligation-dependent probe-amplification assay

Copy number variation of the *SMN1* and *SMN2* genes were analyzed by multiplex ligation-dependent probe-amplification assay (MLPA). SALSA MLPA SMA carrier probemix P060 kit was used in accordance with the manufacturer's protocol (MRC Holland).

Variant calling, filtering and interpretation

Burrows–Wheeler Aligner was used to line up the paired-end reads to the hg19 Human Reference Genome. Variant calling was executed by Genome Analysis Toolkit software and the Annovar tool was used for variant annotation (Wang, Li, and Hakonarson 2010).

Variants matching the following criteria were further evaluated: variants with a 10+ read depth per base, variants with an alternative allele frequency over 30%, variants with a minor allele frequency of below 0.1% in the most relevant population genetics databases, exonic or variants located in close vicinity of a splice site, non-synonymous variants.

The detected variants were assessed based on the minor allele frequency, checked in comprehensive population genetic databases, such as the non-Finnish European ancestry database of gnomAD (https://gnomad.broadinstitute.org). Several predictive programs and genomic variance prioritization tools were put to use in assessing the significance and the possible consequences of a variant, such as SIFT, PolyPhen2, MutationTaster or SpliceAI. To aid the interpretation of variants the Franklin bioinformatic online tool (https://franklin.genoox.com) and VarSome (https://varsome.com) (Kopanos et al. 2019) were also used.

During variant interpretation we adhered to the 2015 guidelines of the American College of Medical Genetics and Genomics (Richards et al. 2015). Variant classification was updated in the fall of 2022 to check whether the classification of the variants have changed since the

2019 initial analysis. Those variants that have been reported in the literature as causal in connection with ALS have been categorized as pathogenic.

In silico modelling and computational simulation

To confirm the supposed pathogenicity of the novel *ANG* variant identified by our group we designed and conducted in silico and in vitro studies. As a starter protein structure of the human angiogenin protein was used from the Protein Data Bank (PDB) (Leonidas et al. 1999). The in-silico p.R57W mutant was created while keeping the wild type secondary protein structure. Hydrogens were connected to the molecule after detaching the CIT cofactor and crystallographic waters with the help of the Xleap tool of AMBER 14. Figures were created by PyMOL and visual molecular dynamics (VMD). VolArea plug-in was implemented for calculating the solvent-accessible surface area of the nuclear localization signal (Humphrey, Dalke, and Schulten 1996; Ribeiro et al. 2013).

Ribonucleolytic assay

Ribonucleolytic activity of wild type and p.R57W mutant angiogenin proteins was measured on yeast tRNA substrate. The wild type and the p.R57W mutant angiogenin proteins were incubated for 2 hours at 37°C in the presence of the yeast tRNA substrate and the supernatant absorbance was measured at 260 nm. The tRNA substrate was used in different concentrations. All measurements were repeated three times for accuracy. Mutant enzyme activity was calculated related to the wild type angiogenin's ribonucleolytic activity.

Nuclear translocation analysis

Human umbilical vein endothelial cells (HUVEC) were incubated with 1 μg/ml of wild type and with 1 μg/ml p.R57W angiogenin protein for 30 minutes and then immunostained for angiogenin. For visualization, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) fluorescent stain. Nuclear translocation assay was performed by enumerating the average angiogenin staining intensity level in the nucleus and in the cytoplasm of cells in three fields of view.

Statistical analysis

Descriptive and comparative statistical methods were utilized to characterize our dataset. GraphPad Prism version 8.0.0 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com) and RStudio software (RStudio Team 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/) were used to carry out Student's t-tests, Fisher's exact test and χ^2 statistics and to determine an odds ratio. The level of significance was ascertained as $p < 0.05$.

Results

In our large-scale genetic examinations, which were carried out to explore the complex genetic architecture of the Hungarian amyotrophic lateral sclerosis population a total of 106 variants of interest were uncovered in 70 genes by coalescing Sanger sequencing, next-generation sequencing techniques and repeat sizing.

Genetic variants identified in the main ALS genes

The most commonly identified genetic alterations in the European ALS population are found in the *SOD1*, *ANG, FUS, TARDBP, UBQLN2* and *NEK1* genes and the *C9orf72* repeat expansion plays a pivotal role as well. In our study the *SOD1*, *ANG, FUS, TARDBP, UBQLN2* and *NEK1* genes were investigated in all the 186 patients by Sanger sequencing. The presence of the intronic GGGGCC hexanucleotide repeat expansion of the *C9orf72* were screened in 136 patients. Unfortunately, the rest of the samples did not have sufficient quality to perform the *C9orf72* hexanucleotide assessment. Presumably causative variants were identified in 29 cases (Table 6).

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)	Number of patients	Reference
ANG	c.3G>T	p. M1I	-	0	likely pathogenic	2	Tripolszki et al., 2019a
ANG	c.169C>T	p.R57W	rs749308574	0.0018%	VUS	1	Tripolszki et al., 2019a
ANG	c.379G>A	p V127I	rs553513710	0.0009%	VUS	1	Tripolszki et al., 2019a
NEK1	c.749A>G	p.N250S	rs368762503	0.0018%	VUS	1	Tripolszki et al., 2019b
SOD1	c.43G>A	p.V15M	rs1568807400	0	pathogenic	1	Tripolszki et al., 2017a
SOD1	c.272A>C	p.D91A	rs80265967	0.0743%	likely pathogenic	1	Tripolszki et al., 2017a
SOD1	c.275_276del	p.K92HfsTer9	-	0	pathogenic	1	Tripolszki et al., 2017a
SOD1	c.435G>C	p.L145F	rs1482760341	0.0035%	pathogenic	2	Tripolszki et al., 2017a
SOD1	c.435G>C	p.L145F	rs1482760341	0.0035%	pathogenic	1	Nagy et al., 2022
C9orf72	hexanucleotide repeat expansion	-	-	0	pathogenic	10	Tripolszki et al., 2017a, Tripolszki et al., 2019b
C9orf72	hexanucleotide repeat expansion	-	-	0	pathogenic	3	Nagy et al., 2022

Table 6. Variants identified in the main ALS genes in the cohort of 186 patients.

The most causative variants were identified in *SOD1* gene, from which only one was identified in the 29 lastly recruited patients. This *SOD1* c.435G>C; p.L145F variant has already been found in two previously investigated Hungarian ALS patients, thus, it is the most common causative variant in our cohort. It is a missense variant, which affects the final exon of the gene. It was identified in heterozygous form, which is in good agreement with the fact that the disease caused by this variant follows autosomal dominant inheritance.

The causative intronic GGGGCC repeat expansion in the *C9orf72* gene was identified in 13 patients, which is 9.56% (13/136). From the 29 lastly recruited patients 3 carried it. Normal repeat length was defined as 28 or less hexanucleotide units. The expanded allele encompassed more than 145 repeats in each case and all identified carriers harboured the expansion in a heterozygous form.

Re-analysis of the major ALS gene set variants

A set of 35 major ALS genes were investigated in 107 patients by NGS analysis, in 21 patients by whole exome sequencing and in 86 patients by a custom designed NGS panel. From the investigated 35 gwes NGS analysis revealed probable causative variants in 16 genes (Figure 4).

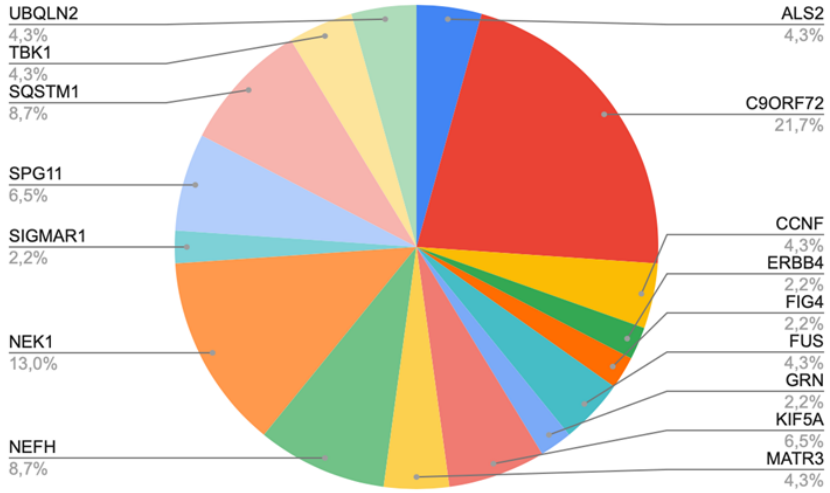

Figure 4. Distribution of the identified variants of the "Major ALS gene set". Only pathogenic, likely pathogenic and variants of uncertain significance are included in the figure.

At least one variant of interest was identified in 43% (46/107) of the 107 examined patients. According to the ACMG classification 1 pathogenic, 4 likely pathogenic variants and 22 VUS were uncovered originally (Table 7). Thanks to incredible advances in NGS technology new decision support variant classification softwares are available, which are great help in the variant classification. The previously identified variants were reanalyzed with two of these softwares, VarSome and Franklin (https://varsome.com, https://franklin.genoox.com/clinical-db/home).

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)	Detection method
ALS2	c.3529G>T	p.G1177X	rs386134180	0	pathogenic	WES
ALS2	c.4496G>A	p.R1499H	rs566436589	0.0018%	VUS	Panel
C9orf72	c.1292G>A	p.R431Q	-	0.0074%	VUS	Repeat primed PCR
CCNF	c.1714C>T	p.R572W	rs199743115	0	VUS	Panel
CCNF	c.316C>G	p.L106V	rs990719669	0.0046%	VUS	Panel
ERBB4	c.268G>T	p.A90S	rs201678258	0.0078%	VUS	Panel
FIG4	c.2095C>T	p.R699C	rs764799053	0.007%	VUS	Panel
GRN	c.1003T>C	p.C335R	-	0	VUS	Panel
KIF5A	c.1735G>A	p.A579T	rs760135493	0.0018%	VUS	Panel
MATR3	c.31C>T	p.P11S	rs995345187	0	VUS	WES
MATR3	c.824G>A	p.S275N	-	0	VUS	Panel
NEFH	c.1013C>T	p.T338I	rs774252076	0.0035%	VUS, leaning pathogenic	WES, panel
NEFH	c.443G>C	p.R148P	-	0	VUS	Panel
NEFH	c.1514C>T	p.P505L	rs1414968372	0.0009%	VUS	Panel
NEK1	c.782G>A	p.R261H	rs200161705	0.3903%	benign	WES, panel
NEK1	c.749A>G	p.N250S	rs368762503	0.0018%	VUS	Panel
SIGMAR1	c.125T>G	p.I42R	rs1206984068	0	VUS	Panel
SPG11	c.6101G>A	p.R2034Q	rs750101301	0.0018%	VUS	WES
SPG11	c.6352C>G	p.L2118V	rs766851227	0.0018%	VUS	Panel
SPG11	c.6009G>T	p.E2003D	-	0	VUS	Panel
SQSTM1	c.1175C>T	p.P392L	rs104893941	0.1344%	likely pathogenic	Panel
SQSTM1	c.1165G>C	p.E389Q	rs1391182750	0	likely pathogenic	Panel
SQSTM1	c.1178G>A	p.R393Q	rs200551825	0.0008%	VUS	Panel
TBK1	c.1888_1890del	p.K631del	-	0	VUS, leaning pathogenic	Panel
TBK1	c.1190T>C	p.I397T	rs755069538	0.0149%	VUS	Panel
UBQLN2	c.1174A>G	p.M392V	rs1384003425	0.0012%	likely pathogenic	Panel
UBQLN2	c.252A>T	p.Q84H	-	0	VUS	Panel

Table 7. The identified variants of the “Major ALS gene set” in the 107 patients analyzed by NGS analysis.

In three cases the reanalysis suggested changes in the classification. The *NEK1*:c.782G>A, p.R261H variant has been recategorized from VUS to benign due to its high allele frequency in the gnomAD healthy databases. Two additional VUS, the *NEFH*: c.1013C>T, p.T338I and the *TBK1*: c.1888_1890del, p.K631del are still VUS, although now they are leaning pathogenic (Table 7, highlighted in green), because their deleteriousness is supported by several aggregative predictive algorithms. Although the *TBK1*: c.1888_1890del, p.K631del small deletion affecting the 631st amino acid of the TBK1 protein does not lead to a frameshift, it is missing from the healthy population in gnomAD. The submission of the above mentioned 3 recategorized variants into ClinVar has certainly catalyzed their recategorization (https://www.ncbi.nlm.nih.gov/clinvar/).

Identification of genetic variants in candidate or minor ALS genes

A set of 99 candidate or minor ALS genes was investigated by NGS analysis. The analysis identified 32 variants in 25 genes (Table 8).

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)	Detection method
CCS	c.490-1G>A	-	rs1199737977	0.0008%	VUS, leaning pathogenic	WES
CDH13	c.713A>G	p.N238S	rs757294994	0.0047%	VUS	Panel
CDH22	c.352G>A	p.D118N	rs372007812	0.0016%	VUS	Panel
CNTN6	c.2222G>C	p.R741P	rs754797179	0.0009%	VUS	Panel
DIAPH3	c.1445G>A	p.R482Q	rs762394580	0.0009%	VUS	WES
DIAPH3	c.3554C>G	p.A1185G	-	0	VUS	Panel
DISC1	c.1055C>G	p.P352R	rs750722558	0.0018%	VUS	WES
DISC1	c.1163T>C	p.L388P	rs1196077554	0	VUS	Panel
DPP6	c.1424A>G	p.D475G	-	0	VUS	Panel
DYNC1H1	c.7748C>T	p.T2583I	-	0	VUS	WES
DYNC1H1	c.12868G>C	p.G4290R	-	0	VUS	WES
DYNC1H1	c.11221C>T	p.R3741C	-	0	VUS	Panel
EPB41L1	c.1123C>T	p.R375W	rs758635781	0	VUS	Panel
EPHA4	c.281C>T	p.A94V	rs1347356714	0.0009%	VUS	Panel
FEZF2	c.675G>T	p.K225N	-	0	VUS	Panel
GRB14	c.1007C>T	p.A336V	rs756507556	0.0039%	VUS	Panel
GRB14	c.386C>T	p.T129M	rs141568578	0.00264%	VUS	Panel
HEXA	c.1073+1G>C	-	rs76173977	0	pathogenic	Panel
KDR	c.1595C>T	p.A532V	rs147066083	0.017%	VUS	Panel
KIFAP3	c.1043A>G	p.K348R	rs1203689157	0.0065%	VUS	Panel
PLEKHG5	c.1037A>C	p.H415P	-	0	VUS	Panel
PLEKHG5	c.773C>T	p.P327L	rs910474236	0	VUS, leaning pathogenic	Panel
PON2	c.705T>G	p.Y235X	-	0.0065%	VUS, leaning pathogenic	Panel
PON3	c.916A>T	p.I306F	rs201661676	0.0139%	VUS	Panel
SCFD1	c.608C>T	p.T203I	rs1459319965	0	VUS	Panel
SCN7A	c.1585A>G	p.M529V	rs1462705963	0	VUS	Panel
SPG7	c.1235C>T	p.A412V	rs746668495	0.0023%	VUS, leaning pathogenic	Panel
SPG7	c.1813G>C	p.G605R	-	0	likely pathogenic	Panel
SUSD1	c.1135C>T	p.R379C	rs778163116	0.0053%	VUS	Panel
VPS54	c.1078A>G	p.T360A	rs200574098	0.011%	VUS	Panel

Table 8. The identified variants of the "Candidate or minor ALS genes set" in the 107 patients analyzed by NGS analysis.

Each variant was identified in only one patient. Out of these 32 variants one is classified as pathogenic, another one as likely pathogenic, 4 as VUS-s that have a classification leaning in the pathogenic direction, and 26 as VUS. The reanalysis by VarSome and Franklin software has not changed any of the original classifications.

Identification of genetic variants in genes associated with neurodegenerative or neuromuscular diseases different from ALS

Additional 160 neurodegenerative or neuromuscular disease associated genes were also investigated in the NGS study. From this "Further neurodegenerative and/or neuromuscular diseases gene set" 45 variants in 29 genes were revealed (Table 9).

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)	Detection method
AFG3L2	c.499G>A	p.G167R	rs1234429070	0	VUS	Panel
ALDH18A1	c.2308G>A	p.V770M	rs369153920	0.0031%	VUS	Panel
ATM	c.2755A>G	p.R919G	-	0	VUS	Panel
ATM	c.8717T>C	p.V2906A	rs730881328	0.0009%	VUS, leaning pathogenic	Panel
BSCL2	c.641A>G	p.H214R	rs544020840	0.0018%	VUS	WES
CACNA1A	c.6517G>T	p.D2173Y	rs370289732	0	VUS	Panel
CACNA1A	c.3256G>T	p.G1087C	-	0	VUS	Panel
FLNC	c.3428C>T	p.S1143L	rs756192123	0.0018%	VUS, leaning pathogenic	WES
GBE1	c.1193A>G	p.H398R	rs755004170	0.0009%	VUS	WES
GBE1	c.496C>T	p.R166C	rs376546162	0.0039%	VUS	WES
GJB1	c.688C>T	p.R230C	rs587781246	0	VUS	WES
INF2	c.2489G>T	p.G830V	rs377340315	0.0303%	VUS	WES
KBTBD13	c.1112G>C	p.C371S	rs1019923438	0	VUS	Panel
KBTBD13	c.239A>T	p.Q80L	-	0	VUS	Panel
KBTBD13	c.436C>T	p.R146C	-	0	VUS	Panel
KBTBD13	c.1322T>A	p.L441Q	rs1005611902	0	VUS	Panel
KCNA2	c.1351T>C	p.S451P	-	0	VUS	Panel
KIF1B	c.3099A>C	p.K987N	-	0	VUS	WES
KIF1B	c.5408G>A	p.R1757Q	rs1010276300	0.0008%	VUS	WES
KIF1B	c.2942G>T	p.R981L	-	0	VUS	WES
KIF1B	c.1210A>G	p.S404G	-	0	VUS	Panel
KIF1B	c.2707C>T	p.R903C	-	0	VUS	Panel
LDB3	c.1786G>A	p.V601I	rs727503130	0.0044%	VUS	Panel
LMNA	c.1520G>A	p.S395N	-	0	VUS, leaning pathogenic	Panel
MORC2	c.1753C>T	p.R523C	rs548292999	0.0023%	VUS	WES
MTMR2	c.408A>C	p.E136D	-	0	VUS	Panel
MTMR2	c.256G>C	p.D86H	-	0	VUS	Panel
NDRG1	c.498C>A	p.N166K	-	0	VUS	Panel
NPC1	c.2959A>C	p.K987Q	rs969498640	0.0023%	VUS, leaning pathogenic	Panel
PNKP	c.287T>G	p.V96G	-	0	VUS	WES
POLG	c.2243G>C	p.W748S	rs113994097	0.0907%	pathogenic	Panel
POLG	c.1570C>G	p.P524A	rs577476988	0.0062%	VUS	WES, Panel
POLG	c.391T>C	p.Y131H	rs562847013	0.0371%	VUS	Panel
SACS	c.6706C>T	p.P2236S	-	0	VUS	Panel
SACS	c.6278G>A	p.R2093H	rs150018812	0.0016%	VUS	Panel
SACS	c.12491T>C	p.I4164T	-	0	VUS	Panel
SBF1	c.3274G>A	p.E1092K	rs371576175	0.0024%	VUS	Panel
SBF2	c.2527A>G	p.M843V	rs1228700161	0.0009%	VUS	WES
SEPT9	c.1405G>A	p.G469S	rs1039884847	0	VUS	Panel
SH3TC2	c.1973G>A	p.R658H	rs138040787	0.014%	pathogenic	Panel
SPTBN2	c.4396G>A	p.V1466M	rs763193925	0.0046%	VUS	Panel
SPTBN2	c.944C>T	p.S315L	rs1373892107	0	VUS	Panel
TPM2	c.356C>T	p.A119V	rs757873408	0.0009%	VUS, leaning pathogenic	Panel
TRPC3	c.868G>A	p.A290T	-	0	VUS	WES
WASHC5	c.2644T>G	p.F882V	rs779756399	0.0039%	VUS	WES

Table 9. The NGS identified variants of genes associated with neurodegenerative and/or neuromuscular diseases different from ALS

The reanalysis by VarSome and Franklin software has not changed any of the classifications: two variants are considered pathogenic, 6 are VUS-s which have an ACMG classification that leans towards pathogenicity and 37 VUS. All variants were identified in a heterozygous state.

By reanalyzing the whole exome sequencing data obtained in 2018 we were able to evaluate the genes that have not been associated with ALS back then. Based on the literature nine new genes are associated with ALS since 2018 (Table 10).

Gene	Reference
ANXA11	Smith et al., 2017
ARPP21	Li et al., 2020
CYLD	Dobson-Stone et al., 2020
CYP1A2	Siokas et al., 2021
GLT8D1	Cooper-Knock et al., 2019
MFSD8	Huang et al., 2021
S1R	Couly et al., 2020
SH2B3	Lahut et al., 2012
TIA1	Mackenzie et al., 2017

Table 10. Novel genes associated with ALS in the last 5 years, and analyzed in our study.

Analyzing the sequence results from the NGS studies for these genes 4 variants were detected in 4 different genes (Table 11). All variants were found in different patients in heterozygous state except for the truncating variant in the *MFSD8* gene which was observed in homozygous form.

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)
ANXA11	c.1105G>A	p.E369K	rs34414015	0.000016	benign
ARPP21	c.1724T>C	p.V521A	-	0	VUS
GLT8D1	c.13A>G	p.K5E	-	0	VUS
MFSD8	c.910C>T	p.Q304X	-	0	likely pathogenic

Table 11. Variants identified by NGS in genes newly associated with ALS

No relevant variants were identified in *TIA1, CYLD, SH2B3, S1R* and in *CYP1A2* genes.

Functional studies of the *ANG* R57W variant

A total of 3 *ANG* gene variants were identified in 4 patients from the 183 ALS patients (2.19%) (Table 12). Two patients carried the c.3G>T, p.M1I variant, and the c.379G>A, p.V127I and the c.169C>T, p.R57W variants were detected in one patient.

Gene	Nucleotide change	Amino acid change	dbSNP	MAF in non-Finnish European (gnomAD)	Pathogenicity (ACMG)	Number of patients
ANG	c.3G>T	p. M1I	-	0	likely pathogenic	2
ANG	c.169C>T	p.R57W	rs749308574	0.0018%	VUS	1
ANG	c.379G>A	p V127I	rs553513710	0.0009%	VUS	1

Table 12. Variants identified in the *ANG* gene within the framework of our project.

The c.3G>T, p. M1I variant affects the transcriptional starting methionine and changes it to valine. The variant is located in the signal peptide region that is cleaved from the mature protein product (Strydom et al. 1985). Prediction tools categorize it as a likely pathogenic variant with a possible start loss effect.

The c.379G>A, p.V127I variant is predicted as VUS. The 127th amino acid position is highly conserved, thus variants affecting the position are noteworthy.

The c.169C>T, p.R57W variant is predicted as VUS. To understand the underling mechanism by which the c.169C>T, p.R57W alteration causes the possible phenotype computational simulations, ribonucleolytic assays and nuclear translocation assays were performed.

The molecular dynamics simulation studies were conducted via *in silico* modelling. Although more structural fluctuations were observed in the first 15 nanoseconds after the emergence in case of the c.169C>T, p.R57W variant than in case of the wild type protein, eventually steadiness was reached, therefore we conclude that the mutation does not alter the stability of the protein (Figure 5).

During a 100 ns all-atom simulation study of the c.169C>T, p.R57W variant, a catalytic site amino acid, the histidine in the 114th position showed a conformational switch. This conformational change implicates a reduced ribonucleolytic activity.

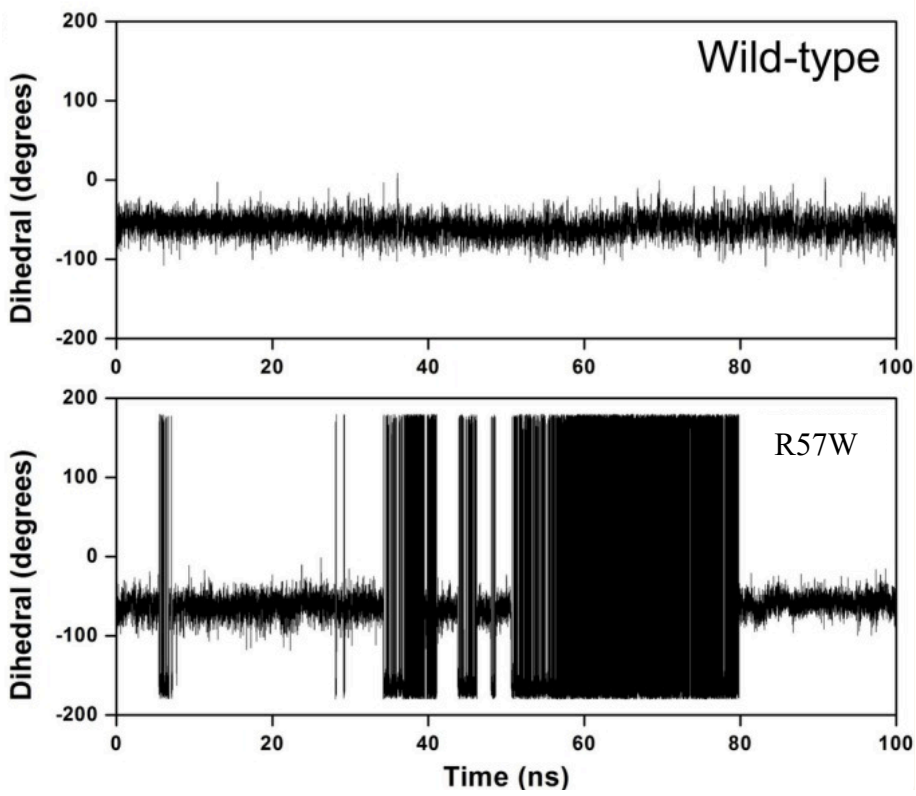

Figure 5. Catalytic residue His114 changed its conformation from native −80º during the simulations, indicating its role in probable loss of ribonucleolytic activity, whereas wild-type ANG maintained a stable His114 conformation

As an endorsement of the *in silico* results a ribonucleolytic activity assay was performed. In this study the mutant protein could only exhibit 57.4% of the wild type protein's ribonucleolytic activity (Figure 6). This means that the mutant angiogenin protein has a lower activity than the wild type one.

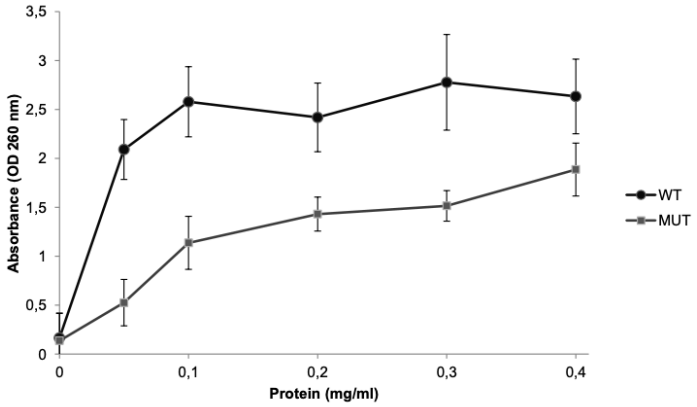

Figure 6. Comparison of the measured enzymatic activity of the wild type (WT) and the mutant(MUT) angiogenin protein.

Additional results from the 100 ns simulations of the c.169C>T, p.R57W mutant protein show that the most crucial amino acids forming the nuclear localization signal, 55RRW57 go through a different folding process which makes the nuclear localization signal less accessible

by solvents (Figure 7). A closed off structure may be observed in case of the mutant at both examined timepoints while the wild type protein displays a loose and open conformation at both timepoints.

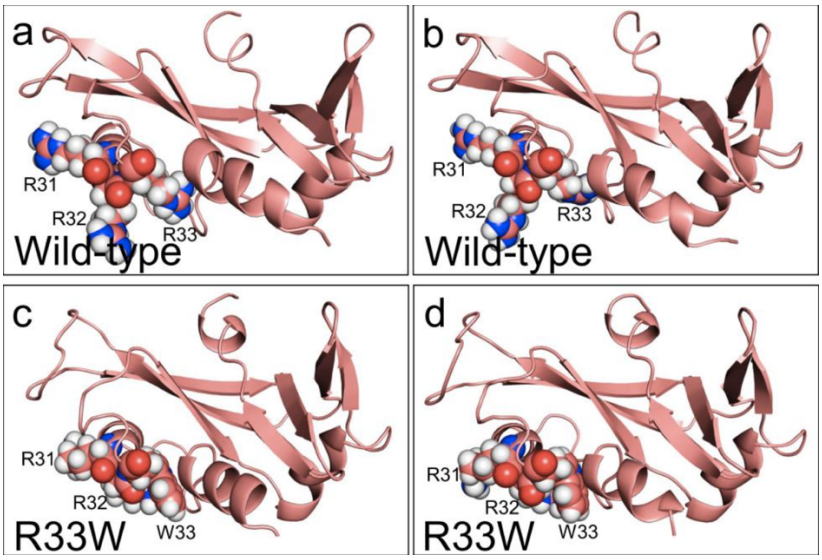

Figure 7. The local folding of the nuclear localization signal in case of the wild type angiogenin (a and b) and in case of the variant (c and d). Pictures were taken at two different timepoints (a and c, b and d, respectively). Arginine residues are labelled as the former nomenclature of the ANG protein as R31, R32 and R33 which are currently named R55, R56, R57 respectively.

As a validation of the above results, a nuclear translocation assay was carried out using human umbilical vein endothelial cell (HUVEC) cultures. HUVECs were incubated with 1 µg/ml of wild type and with 1 µg/ml p.R57W angiogenin protein for 30 minutes and then immunostained for angiogenin. Nuclear translocation assay was performed by enumerating the average angiogenin staining intensity level in the nucleus and in the cytoplasm of cells in three fields of view. Cells treated with wild type angiogenin showed a nuclear/cytoplasmic staining in 24/34 cells per field of view, whilst cells treated with the c.169C>T, p.R57W mutant protein exhibited a nuclear/cytoplasmic staining in 14/37 cells per field of view (Figure 8). Thus, a statistically significant difference could be observed between the nuclear translocation ability of the wild type and mutant protein ($p=0.001$). This confirms our hypothesis that the c.169C>T, p.R57W mutation impairs the ability of the protein to undergo nuclear translocation.

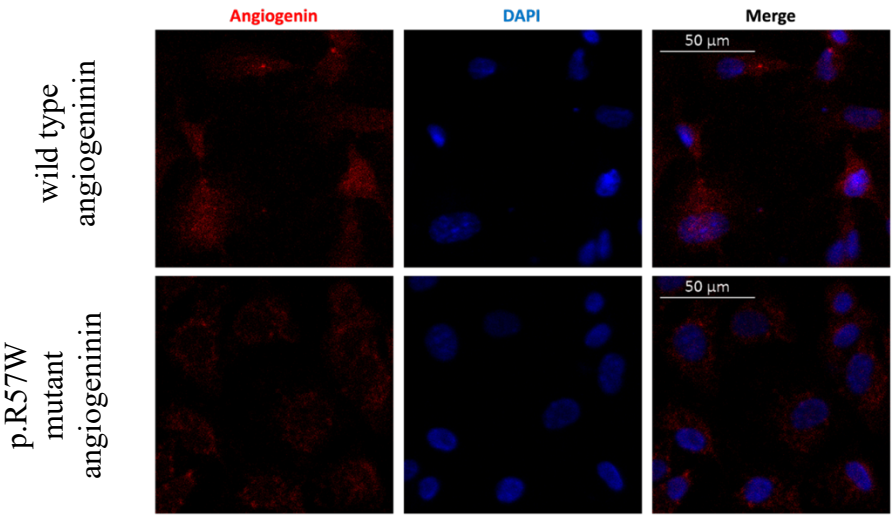

Figure 8. The c.169C>T, p.R57W mutation impairs the ability of the angiogenin protein to undergo nuclear translocation. Cells treated with wild type angiogenin showed a nuclear/cytoplasmic staining, while cells treated with wild mutant angiogenin protein showed a higher degree of cytoplasmic staining. The DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue).

Furthermore, an overall decrease in staining uptake could be observed in case of the cells treated with mutant angiogenin which suggests that not only the ribonucleolytic activity and the nuclear translocation ability of the protein was damaged, but also the cellular recognition and internalization have decreased.

Screening of ALS genetic risk factors

Potential genetic risk factors, based on exhaustive search of the scientific literature, were also screened. Genes that have been reported from ALS cohort studies and genes labelled as ‘moderate evidence’ or ‘tenuous’ in the ALS Online Database were examined.

Repeat expansion of the *ATXN1* gene

The intermediate length repeat expansion in the *ATXN1* gene was assessed in 182 ALS patients and 178 healthy control individuals. Normal alleles contain 6-31 repeats. Repeats longer than 39 units confer a risk of spinocerebellar ataxia 1. In our study the cut-off number ≥32 repeats was used as recommended by Conforti et al (Conforti et al. 2012). The distribution of different alleles among the ALS patient cohort and the healthy control group are similar (Figure 9).

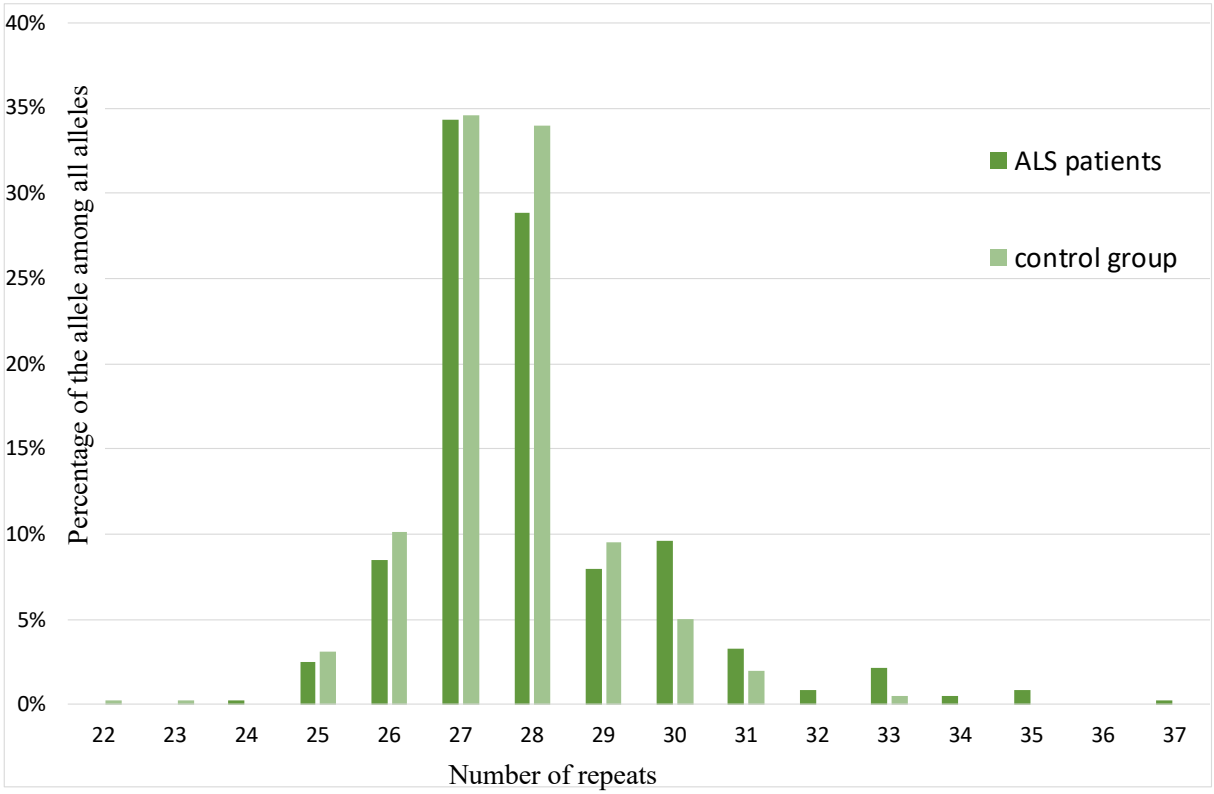

Figure 9. Distribution of *ATXN1* alleles by length in ALS patients and in healthy control individuals

Repeat length in case of ALS patients ranged from 24-37 units, while among control individuals from 22-33 units (Figure 9). On average, ALS patients carried 28.03 CAG repeats, while healthy control individuals bore a mean repeat length of 27.62 units (p=0.0014, 95% confidence interval (CI): 0.16–0.6589). 8.79% (16/182) of the ALS patients proved to carry an intermediate length allele (32-39 repeats) in heterozygous form while only 1.12% (2/178) of the healthy control individuals were found to carry an intermediate length repeat expansion in a heterozygous state. The difference reached the level of significance and an odds ratio of 8.482 (95% CI: 2.052-37.56) could be determined.

Repeat expansion of the *ATXN2* gene

The CAG trinucleotide repeat expansion in the *ATXN2* gene was assessed in 153 ALS patients and 195 healthy control individuals. Normal *ATXN2* allele were defined as less than 24 CAG units, intermediate length alleles contained 24-33 trinucleotide repeats while fully expanded alleles encompassed more than 33 repeats.

The repeat range was 20-33 repeats in case of ALS patients and 18-32 repeats for the healthy control group (Figure 10). The mean repeat number of the *ATXN2* gene among ALS patients was 23.28 CAG trinucleotide units, whilst control patients carried 23.11 repeats in

average. The difference between the groups reached the level of significance (p= 0.0409, 95% CI: 0.007215 – 0.3395).

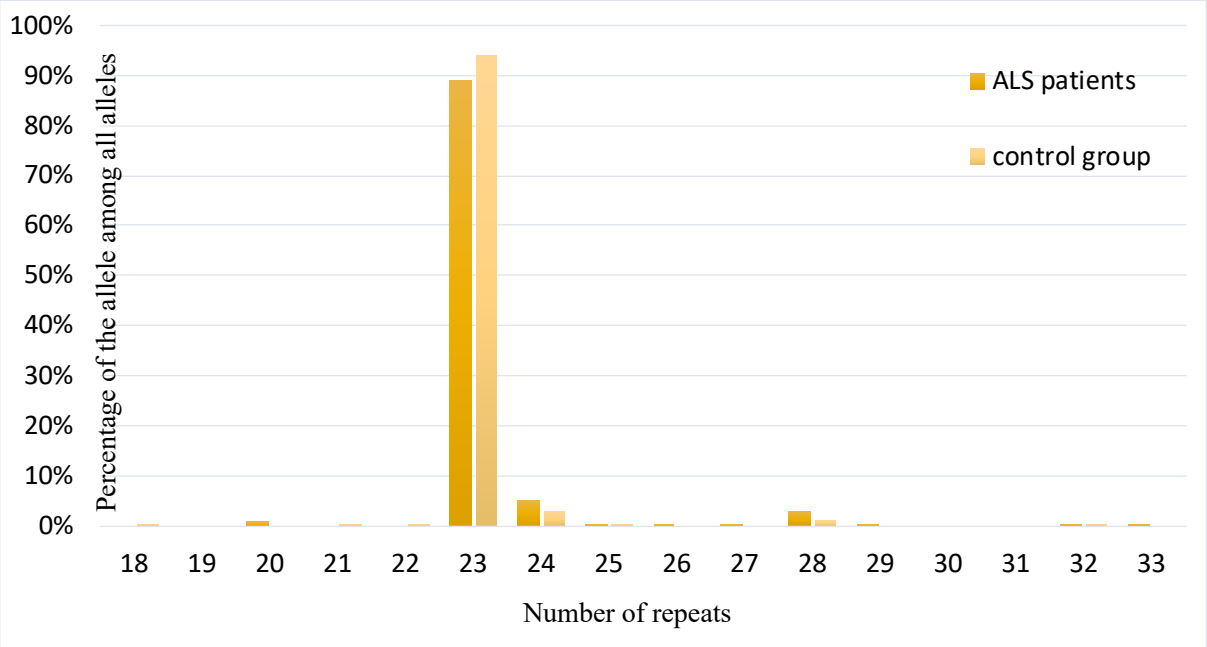

Figure 10. Distribution of *ATXN2* alleles by length among ALS patients and healthy control individuals

18.3% (26/153) ALS patients were found to carry an intermediate length repeat expansion, while this rate was 9.23% (18/195) among control individuals. Only ALS patients were observed to carry the intermediate length repeat expansion in a homozygous form (3 patients, one with a genotype off 24/24 and two patients represented 28/28 repeats), the repeat expansion was only identified in a heterozygous state among control individuals. Statistically, carrying an intermediate length allele confers a 2.203-fold risk for developing ALS. (95% CI: 1.196–4.098)

Copy number variations of the *SMN1* and *SMN2* genes

The copy number variations of the *SMN1* and *SMN2* genes were studied in 148 ALS patients and in 148 control individuals by multiplex ligation-dependent probe-amplification assay (MLPA). Duplications of the *SMN1* gene were present in 6 ALS patients and in 7 control patients (6/148, 4.05% and 7/148, 4.73% respectively).

Homozygous deletions of the *SMN2* pseudogene were observed in 7 ALS patients and in 10 control individuals (4.73% and 6.76%, respectively).

Discussion

In our study we performed a thorough genetic characterization of 183 Hungarian amyotrophic lateral sclerosis patients, diagnosed between 2008 and 2021 at the Department of Neurology, University of Szeged. By following a stepwise working algorithm, utilizing Sanger sequencing, multiplex ligation-dependent probe-amplification assay, next-generation sequencing methods and fragment length analysis we screened the major and the minor ALS genes, genes associated with other neurodegenerative and neuromuscular diseases and additionally provided information on the genetic risk factors of ALS.

Our work provides the first comprehensive genetic study of ALS patients of Hungarian origin and we report the genetic landscape and architecture of these patients.

This work is the continuation and expansion of the previously published articles by our research group on this topic (Tripolszki, Török, et al. 2017; Tripolszki, Csányi, et al. 2017).

C9orf72 repeat expansion and *SOD1* mutations are the most common genetic alterations in Hungarian ALS patients

The main ALS genes (*SOD1*, *ANG, FUS, TARDBP, UBQLN2* and *NEK1* genes and the *C9orf72* repeat expansion) were investigated in every recruited patient since the involvement of these genes is established as the most common ones in European populations.

Based on the literature, *SOD1* variants may account for 12–23% of the familial ALS forms and 1% of the sporadic cases (Andersen 2006; Yilmaz et al. 2023). Identifying *SOD1* mutation carriers is key in the treatment of ALS patients, since patients with *SOD1* variants may be eligible for antisense oligonucleotide-based therapy which is already available in Hungary, too.

In our study altogether four different *SOD1* variants were identified in 6 patients from the investigated 183. Three recurrent missense variants (c.43G>A, p.V15M, c.272A>C, p.D91A and c.435G>C, p.L145F) and a novel frameshift variant (c.275_276del, p.K92RfsTer9) leading to premature termination have been detected. The c.435G>C; p.L145F variant is the most causative variant in our cohort as it was identified in three cases, twice in the first tested 66 patients (Tripolszki, Csányi, et al. 2017) and ones in the 29 lastly recruited patients. This missense variant affecting the last, fifth exon of the gene. It is described as a mutational hot

spot (Wahiduzzaman et al. 2021). This variant is very rarely identified in the healthy non-Finnish European population, its allele frequency is 0.0035% in the gnomAD database. Functional studies have confirmed the damaging effect of the variant (Gal et al. 2016).

It was identified in heterozygous form in our patients, which is in good agreement with its autosomal dominant inheritance. All three carrier patients were female. One of them reported a family history of ALS (Tripolszki, Csányi, et al. 2017). The c.435G>C, p.L145F variant is thought to stem from the Istro-Rumanian peninsula or Serbia (Corcia et al. 2011). Although none of our patients were of Balkan origin, based on the geographical proximity a common ancestor may not be excluded. Studies associate the variant with a later onset of disease and a slower progression (Ferrera et al. 2003), which fits our lastly diagnosed patient's phenotype. Both the later onset and the moderately progressive disease can be attributed to the mild decrease of the enzymatic activity of the superoxide dismutase 1 enzyme (Masè et al. 2001). A non-specific cognitive decline has been also described in the literature. However, in our lastly diagnosed patient neuropsychological tests are not suggestive of cognitive impairment. Early respiratory involvement has been also noted in connection with the variant but in the case of our lastly diagnosed patient there is no data pointing to early respiratory involvement (Corcia et al. 2011).

The most recent patient (diagnosed in 2021) carrying the c.435G>C, p.L145F variant has presented at the neurologist at the age of 70, 8 months after the onset of symptoms. She started noticing the weakness of her left lower limb and gradually the weakness spread to all other limbs. Her symptoms originated predominantly from LMN damage, but UMN and pseudobulbar signs could also be observed. Her family history was negative for ALS and diseases alike. Despite being treated with riluzole, her state slowly deteriorated and she died due to the complications of progressive respiratory insufficiency 24 months after the onset of symptoms.

Sparse data is available on the other two Hungarian patients carrying the c.435G>C, p.L145F variant. One was a 29-year-old female at the onset of the disease. Her paternal grandmother had a similar disease. Her symptoms started with lower limb weakness and progressed with UMN signs as well. She passed away 4 years after the onset of the disease. The other patient was a 46-year-old female with a negative family history for ALS. Her comorbidities included lumbar polydiscopathy. She showed signs of LMN, UMN and bulbar involvement and died 3 years after the onset of symptoms (Tripolszki, Csányi, et al. 2017).

Our solved six cases are a 3.28% (6/183) diagnostic rate for the *SOD1* gene. The larger fraction of apparently sporadic ALS patients with an identified *SOD1* variant in our study may

be attributed to the possible miscategorization of familial cases as sporadic due to the lack of information about the ancestors.

According to a 2014 publication (Renton, Chiò, and Traynor 2014b), among ALS patients of European origin, 40% of familial ALS cases and around 7% of sporadic ALS cases may be explained by the repeat expansion of the *C9orf72* gene. Lower frequencies have been reported in Italy, where 5.1% of sporadic ALS cases were positive for the repeat expansion, and in a Flanders-Belgian cohort, where 3.81% of sporadic ALS patients were carriers of the expansion (Ratti et al. 2012; van der Zee et al. 2013). *C9orf72* hexanucleotide repeat expansion was detected in 9.56% (13/136) of the examined Hungarian patients. In our Hungarian sporadic ALS patients group a larger fraction of patients carry the *C9orf72* repeat expansion, which again may be caused by the possible miscategorization of familial cases as sporadic due to the lack of information about the ancestors.

The mean age of the *C9orf72* repeat expansion positive patients was 60.22 years, which is a bit lower than the mean age at onset of the whole patient cohort (62.99 years). Most patients presented at a neurologist's office in less than a year after the onset of symptoms (mean time to diagnosis from symptoms onset: 9.62 months). At the initial examination all patients had LMN symptoms and 92.31% (12/13) of them displayed UMN signs as well. Bulbar symptoms were also common, 69.23% (9/13) had experienced bulbar impairment as well, which is consistent with the literature (Cooper-Knock, Shaw, and Kirby 2014).

We identified 3 *C9orf72* repeat expansion positive patients carrying additional relevant variants in another major ALS gene. One patient harbored the *SQSTM1* c.1178G>A, p.R393Q variant, another carried the *NEK1* c.749A>G, p.N250S variant, and the third patient proved positive for the intermediate length repeat expansion in the *ATXN1* gene. This finding supports the hypothesis that ALS is rather an oligogenic disease and more than one potent genetic variant may be identified in one patient (Blitterswijk et al. 2012).

Re-analysis of the panel sequencing data re-categorizes variants and detects rare damaging ALS associated gene variants

In our project we re-analyzed the whole exome and panel sequencing data and recategorized the variants that have earned an updated ACMG classification in the elapsed 5 years. It important to periodically re-assess the NGS data and keep re-classifying the VUSs to decide on the real significance of the variants. With time additional data emerges which might

necessitate the reconsideration of significance. This becomes easier with time as large cross-ethnic healthy subject databases are becoming available.

From the investigated 35 major ALS genes additional variants of interest were detected in 16 genes. One pathogenic and three likely pathogenic variants and 21 VUSs were identified.

One pathogenic variant was identified in the Alsin Rho Guanine Nucleotide Exchange Factor (*ALS2*) gene. The ALS2 protein functions as a guanine nucleotide exchange factor for the small GTPase RAB5. The ALS2 protein co-localizes with the RAB5 protein on early endosomal compartments, and functions as a modulator for endosomal dynamics. *ALS2* gene variants when found in homozygous form or in compound heterozygous state may cause infantile-onset ascending spastic paralysis, while heterozygous variants are associated with an adult-onset motor neuron disease (Sztriha et al. 2009). The uncovered c.3529G>T, p.G1177Ter known pathogenic stop gain variant localizes to the Membrane Occupation and Recognition Nexus (MORN 6) repeat motif. MORN motifs have a highly conserved sequence (Sajko et al. 2020). Each MORN repeat sequence begins with a GxG amino acid series, in which the second glycine (in our case the 1177th amino acid) must be the smallest amino acid possible because any larger amino acid would not be able to lodge the following highly conserved and structurally bulky amino acid residues. Thus, the repeat region would not be able to form the desired architecture (Sajko et al. 2020). The truncated protein would not be able to oligomerize and to perform its Rab5-guanine exchange factor activity (Hadano et al. 2007).

Two likely pathogenic and one VUS variants were identified in the Ubiquitin binding protein p62 *(SQSTM1)* gene. All three revealed variants localize to the ubiquitin binding domain. The ubiquitin binding domain is a conserved site of the protein involved in binding of other polyubiquitinated molecules and directing them towards degradation (Seibenhener et al. 2004). Disruption of the ubiquitin binding domain may disturb the physiological breakdown of polyubiquitinated substrates. *SQSTM1* variants have been first described in ALS patients in 2011 (Fecto et al. 2011). Before that *SQSTM1* gene variants have been described in Paget disease of the bone (Laurin et al. 2001). Surprisingly, the patient carrying the c.1165G>C, p.E389Q variant also reported to have been diagnosed with Paget disease of the bone before.

The NIMA-related kinase 1 (*NEK1)* gene was recently identified as an ALS candidate gene based on gene-burden studies thanks to the support from the viral online ALS Ice Bucket Challenge (Cirulli et al. 2015). In our study two *NEK1* missense variants have been observed:

the c.782G>A, p.R261H and the c.749A>G, p.N250S variants. Both variants affect the protein kinase domain. The p.R261H variant is a known ALS susceptibility factor (Kenna et al. 2016). We identified the p.R261H variant in 3.68% (5/107) of ALS patients and in 1.08% (4/370) of healthy control individuals, while a larger study involving ALS patients from all over Europe noted a *NEK1* variant carriership frequency of 1.13% and 0.6% among patients and control individuals respectively (Brenner et al. 2016). *NEK1* variants seem to be more common among Hungarian ALS patients than in other ethnicities. However, the exact cause of it remains unsolved. As *NEK1* loss of function variants are associated with an incomplete penetrance, thus their elevated frequency among healthy individuals does not exclude the disease-causing potential (Nguyen et al. 2018).

A likely pathogenic and a VUS variant were identified in the Ubiquilin 2 (*UBQLN2*) gene which encodes an ubiquitin-like protein. The novel c.252A>T, p.Q84H VUS variant affects the ubiquitin-like domain, which is located towards the N-terminal of the protein. This domain is responsible for inhibiting GPCR mediated endocytosis and for coupling the protein with other parts of the proteosome (Zhang et al. 2014). The identified likely pathogenic c.1174A>G, p.M392V variant was also reported by a Chinese research group (Huang, Shen, and Fan 2017). Their patient also presented as the classical ALS phenotype which is consistent with our findings. Previously, a different variant affecting the same amino acid position (p.M392I) has been reported pathogenic (Özoğuz et al. 2015). The reported patient had a rapidly progressive juvenile onset disease.

Three different missense variants were observed in the Neurofilament Heavy Chain *(NEFH)* gene. Variants in the *NEFH* gene have been associated with the development of ALS since 1994 (Figlewicz et al. 1994). In our cohort two patients carried the c.1013C>T, p.T338I, one person carried the c.443G>C, p.R148P and one the c.1514C>T, p.P505L variant. The c.1013C>T, p.T338I and c.443G>C, p.R148P variants are located inside the intermediate filament rod domain. The rod domains are essential in dimerization processes (Chernyatina et al. 2012). The alteration of these sequences might result in loss of dimerization activity.

We identified a novel deletion c.1888_1890del, p.K631del and a recurrent missense variant, c.1190T>C, p.I397T in the TANK binding kinase 1 (*TBK1)* gene. The protein encoded by this gene mediates NFKB activation in response to certain growth factors. The role of *TBK1* variants has been implied in ALS-FTD and also in pure forms of FTD (Freischmidt et al. 2015;

Lamb et al. 2019). The patient carrying the *TBK1* c.1888_1890del, p.K631del variant was 37 years old at disease onset. Signs of FTD were also noted in her case. The frequency of the c.1190T>C, p.I397T variant is extremely low in the gnomAD population database. However, in contrast to other known pathogenic *TBK1* variants, the c.1190T>C, p.I397T variant does not decrease but rather increases the phosphorylation activity of the protein. Moreover, it does not seem to alter the structure and stability of the protein. The suggested classification of the variant is VUS, although the pathogenicity of it is debatable (Pozzi et al. 2017).

Interestingly, in our study 5.61% (6/107) patients were observed to carry more than one relevant variant in major ALS genes. Two patients carrying an expanded allele on the *C9orf72* locus and one *SQSTM1* or one *NEK1* variant are described above. Co-occurrence of different variants have been noted by others as well in ALS patients (Blitterswijk et al. 2012).

Variants detected in candidate or minor ALS genes presented with the classical ALS phenotype

Thirty-two variants in 25 candidate or minor ALS genes were detected in 25 patients. We cannot exclude the possibility that several of these variants contributed to the development of ALS or ALS like phenotypes. However, in some cases the association between the disease and the detected variant is tenuous, since the mode of inheritance does not fit the zygosity of the variant.

The Hexosaminidase A *(HEXA)* gene is associated with Tay-Sachs disease which follows an autosomal recessive hereditary pattern (Okada et al. 1971). In the literature a *HEXA* variant was described in a male patient diagnosed with ALS at the age of 16. His disease progressed into dementia, and he also showed ataxic symptoms as previously reported by Mitsumoto et al (Mitsumoto et al. 1985). Based on enzyme activity assays and large-scale genetic studies it was concluded that *HEXA* variants are not associated with ALS but the two diseases may share a phenotypic overlap (Drory et al. 2003). The c.1073+1G>C splice variant of the *HEXA* gene was uncovered in heterozygous form in one patient in our study. The variant uncovered in our patient is likely not disease-causing since our patient showed the classic ALS symptoms and had a later disease onset.

Two variants were revealed in the paraplegin *SPG7* gene: the c.1813G>C, p.G605R likely pathogenic missense variant and the c.1235C>T, p.A412V variant of uncertain significance. All pathogenic *SPG7* variants described to date affect the highly conserved AAA+

homology ATPase domain (Osmanovic et al. 2020). Interestingly, the less plausible variant, the c.1235C>T, p.A412V localizes to this domain. Although *SPG7* variants were identified in ALS patients in a large German cohort, they do not seem to have a causal relationship to ALS but rather help the differentiation of spastic paraplegias from ALS (Krüger et al. 2016).

In one of the patients, the c.490-1G>A splice variant of the copper chaperone for superoxide dismutase gene (*CCS*) was detected. It can be interesting as overexpression of the *CCS* gene combined with a *SOD1* mutation results in a more rapid curse of disease in animal models (Son et al. 2007). In two small studies no relevant *CCS* variants were revealed, thus it may be a rare genetic factor contributing to ALS (Orlacchio et al. 2000; Silahtaroglu et al. 2002).

Pleckstrin Homology And RhoGEF Domain Containing G5 *(PLEKHG5)* gene variants have been associated with juvenile onset and slowly progressing LMN disease (Maystadt et al. 2007). A missense variant, c.773C>T, p. P327L was uncovered in the *PLEKHG5* gene. A *PLEKHG5* variant was found to segregate with a pathogenic *TARDBP* variant, which raises the possibility of the two genes being in a functional connection (Goldstein et al. 2020). The phenotype of our ALS patient does not fit to the previously described literature data; thus, it is unlikely to be disease-causing one in our case.

A VUS variant - c.705T>G, p.Y235X - of the Paraoxonase 2 (*PON2)* gene was also detected in our study. Paraoxonases possess antioxidant properties and are involved in cholesterol and lipoprotein metabolism (Reichert, Levy, and Bydlowski 2021). The link between abnormal lipid metabolism and neurodegeneration is well established for a while, which could explain the potential role of the variant in the pathomechanism of ALS (Hall and Harcup 1969).

Genetically based differential diagnosis to ALS offered by next generation sequencing

The third gene set of our analysis included 160 genes that are associated with neurodegenerative and neuromuscular diseases that can mimic ALS in their clinical picture. In this gene set 45 variants in 29 genes were revealed. However, many of the findings are likely to be not disease-causing in our patients, since the clinical picture and inheritance pattern do not always match the expected phenotype based on the genetic alteration.

Mitochondrial defects may also contribute to the pathomechanism of ALS (Dupuis et al. 2011). We have identified a variant in the catalytic subunit of mitochondrial DNA

polymerase encoded by the DNA Polymerase Gamma, Catalytic Subunit (*POLG)* gene. Some of the adult onset *POLG* related diseases include progressive external ophthalmoplegia, sensory ataxia, neuropathy, dysarthria and atypical parkinsonism (Rahman and Copeland 2019). Although the phenotype spectrum associated with *POLG* variants is rather wide, the phenotype of our patient does not exactly fit the *POLG* associated disease spectrum.

Homozygous or compound heterozygous variants of the SH3 domain and tetratricopeptide repeats 2 *(SH3TC2)* gene are known to cause Charcot–Marie–Tooth neuropathy type 4C (Laššuthová et al. 2011). The gene product is thought to be an adapter or docking molecule. We identified the c.1973G>A, p.R658H variant of the gene in heterozygous form. A Turkish study also identified an ALS pedigree carrying a heterozygous *SH3TC2* variant (c.1568T>C, p.M523T). The Turkish proband was in her 5th decade of life and had a lower limb onset of ALS (Kotan et al. 2020). Future examinations are needed to clarify the possible link between heterozygous *SH3TC2* variants and ALS.

Variants of the N-terminal actin binding domain of the Filamin-C (*FLNC)* gene may be identified in patients with distal myopathy type 4, while variants affecting the filamin C rod and dimerization domain account for myofibrillar myopathy type 5 (Duff et al. 2011). The c.3428C>T, p.S1143L variant detected in one of our patients localizes to the filamin 9 repeat domain which guides dimerization processes (Lamsoul, Dupré, and Lutz 2020). *FLNC* variants were observed in abundance in frontotemporal dementia (FTD) patients, one of the described patients displayed the FTD-ALS phenotype (Janssens et al. 2015). Since ALS and FTD are located on the same phenotypic spectrum we may expect a growing number of studies linking ALS and *FLNC* variants.

Lamin A *(LMNA)* gene variants are linked to autosomal dominant Emery-Dreifuss muscular dystrophy, Dunningan type partial familial lipodystrophy and cardiomyopathies (Bonne et al. 2000; Peters et al. 1998). Specific variants of each region of the protein are likely to contribute to the development of different phenotypes. An *LMNA* variant was reported to coexist with variants in major ALS genes *SETX* and *FUS* in a patient diagnosed with lipodystrophy and ALS (Volkening et al. 2021). Our identified variant is the c.1520G>A, p.S395N. In wild type lamin A the 395th amino acid position undergoes a posttranslational modification and becomes a phosphoserine. Phosphorylation may play a role in the modulation of its lamin associations (Kochin et al. 2014). Due to the serine to asparagine change it cannot be modified, thus we suppose its deleterious effect.

Based on the literature, nine novel genes have been linked to ALS since 2018 when our first analysis of the next-generation sequencing (NGS) data took place. Taking advantage of the possibility to re-analyze NGS data we were able to evaluate the ALS associated novel genes. Four variants in 4 different genes were detected. All variants were found in heterozygous state except for the truncating variant in the *MFSD8* gene which was observed in a homozygous form.

In the literature an Annexin A11 (*ANXA11*) gene variant (c.119A>G, p.D40G) is reported to segregate with ALS (Smith et al. 2017). Although we detected an other variant (c.1105G>A, p.E369K) we believe it is causative as it is close to the C-terminal domain. C-terminal domain variants of the encoded protein damage the calcium homeostasis and calcium metabolism may be a key player in common pathway of neurodegeneration (Patai et al. 2017; Nahm et al. 2020).

Variants of the major facilitator superfamily domain containing 8 (*MFSD8)* gene have been linked to frontotemporal dementia in 2019 (Geier et al. 2019). Later the association was confirmed in ALS patients as well (Huang et al. 2021). The encoded protein is a putative lysosomal membrane transporter (Siintola et al. 2007). The identified c.910C>T, p.Q304X nonsense variant of the *MFSD8* gene results in a truncated protein, thus we suppose it is not able to fulfill its function.

The Glycosyltransferase 8 Domain Containing 1 *(GLT8D1)* gene is responsible for coding a glycosyltransferase protein. Although the specific function of the coded protein has not been determined yet, it was proven that both wild type and mutant GLT8D1 protein localize to the Golgi network. ALS associated variants of the gene localize near the substrate binding domain and impair the glycosyltransferase activity of the protein. (Cooper-Knock et al. 2019). Since the c.13A>G, p.K5E variant detected in our patient disrupts the Golgi localization signal, we expect the protein to mislocalize and thus damage the normal ganglioside metabolism. Abnormal ganglioside processing has long been observed in ALS (Moll, Shaw, and Cooper-Knock 2020; Desport et al. 2006).

It has been proposed that *GLT8D1* variants and CAMP Regulated Phosphoprotein 21 *(ARPP21)* variants act synergistically (Cooper-Knock et al. 2019). Unfortunately, we cannot confirm their synergistic effect in our ALS cohort as our identified variants (the *ARPP21* c.1724T>C, p.V521A and the *GLT8D1* c.13A>G, p.K5E variant) are carried by two different patients. Recent studies from Australia and mainland China reported *ARPP21* variants as occasional findings in ALS patients (Li et al. 2020; Chan Moi Fat et al. 2021). Our data confirms the same conclusion.

A novel angiogenin variant impairs both the ribonucleolytic activity and the nuclear translocation of the encoded protein

Relevant angiogenin (*ANG*) gene variants were detected in 2.19% (4/183) of our patients, which is higher than reported by other groups of sporadic ALS patients (Greenway et al. 2006). Literature data has shown the decreased stability of the protein and the decreased enzymatic activity which are proportional to the disease duration and to inversely proportional to the disease onset (Aluri et al. 2020). Although our cohort is too small to draw a far-fetched conclusion from it, our results support the above hypotheses.

The *ANG* c.3G>T, p.M1I may have a start loss effect on the protein and thus interfere with the physiological function of it. This variant was detected in a 79-year-old female patient who noticed dysphagia and dysarthria 6 months prior to the examination. These were signs of a pseudobulbar palsy. She presented with an atrophic tongue with restrained tongue movements and generalized brisk reflexes. She got an ALSFRS-R score of 33/48 upon initial examinations.

We identified the *ANG* c.379G>A, p.V127I in our cohort. This variant was first reported in 2011 (Zou et al. 2012). *In silico* studies suggested that the replacement of the wild type valine to isoleucine may precipitate the forming of a new hydrogen bond connecting the 127th and the 138th amino acid position. This new hydrogen bond could disrupt the physiological protein structure (Padhi et al. 2012). A ribonucleolytic assay similar to our experiment performed with the p.V127I variant showed comparable results to our assay of the p.R57W variant. The p.V127I exhibits approximately half of the ribonucleolytic activity of the wild type enzyme, just like we determined in case of the p.R57W variant (Bradshaw et al. 2017).

Our index patient carrying the c.379G>A, p.V127I variant was a 63-year-old man who had 1.5-year complaint of muscle twitching, muscle wasting and weakness of the right upper limb. Thorough neurological examination discovered bulbar- and pseudobulbar paresis as well. The patient scored 41/48 on the ALSFRS-R scale.

The *ANG* c.169C>T, p.R57W variant was identified in a 55-year-old male patient who presented at the neurologist's office one year after the onset of his symptoms. He showed severe atrophy and weakness of the distal muscles of the upper limbs with concomitant tongue atrophy, fasciculations and dysarthria. Electromyographic examination showed fibrillations in all myotomes. Deep tendon reflexes were brisk on the lower limbs, while no deep tendon reflexes could be elicited on the upper limbs. Upon initial examination the patient scored 35/48 on the ALSFRS-R scale.

Our functional assays have shown that the c.169C>T, p.R57W variant possesses a bit more than half of the wild type protein's ribonucleolytic activity. This piece of information corresponds to data published before: almost all deleterious *ANG* variants exhibit a decreased catalytic activity compared to the wild type protein (Wu et al. 2009; Bradshaw et al. 2017).

It has been shown that the c.169C>T, p.R57W variant also impairs the function of the nuclear localization signal built by the 53IMRRRGL59 amino acids of the encoded protein. The three arginines (amino acid positions 55-57) are known to govern the process. The nuclear localization sequence of the mutant protein forms a more closed off structure compared to the wild type protein's open structure of the 55RRR57 amino acids. This could be a consequence of the replacement of a hydrophilic arginine to a hydrophobic tryptophane. We suppose that the nuclear translocation activity of the protein shrinks due to the decreased solvent accessible surface area of the variant protein. Based on the results of our functional studies carried out we conclude that the arginine tryptophane change in the 57th amino acid position significantly impairs the protein's ability to translocate into the nucleus.

A different variant (c.169_170delCGinsGC) affecting the same amino acid, p.R57A has been shown to lack angiogenic activity and also the variant protein did not get an admission into the nucleus (Moroianu and Riordan 1994). Many of the ALS associated *ANG* variants act by inducing a folding change of the structure and thus limiting the solvent accessible surface area of the nuclear localization signal (Thiyagarajan et al. 2012; Padhi et al. 2014). Our studies have characterized the p.R57W variant from a clinical, a genetic and from molecular point of view and based on our results we consider the variant pathogenic.

Intermediate length repeat expansion of the *ATXN1* and *ATXN2* genes are common ALS genetic risk factors in the Hungarian ALS population

Alongside of identifying causative ALS genes, ALS risk factor genes have been also described in literature recently. The variations of these genes do not directly cause ALS but rather increas the risk of developing the disease. These genes include *ATXN1, AXTN2, SMN1* and *SMN2* (Lee et al. 2011; Elden et al. 2010; Blauw et al. 2012).

Almost 9% (8.79%, 16/182) of the investigated ALS patients were found to carry the intermediate length CAG repeat expansion in the *ATXN1* gene, while only 1.12% (2 out of the 178) control individuals carried an intermediate length allele.

Studies conducted on ethnically homogenous populations have reported lower rates of the intermediate length *ATXN1* allele among ALS patients. In Italy 7.07% of the 418 tested

ALS patients showed an elongated allele (Conforti et al. 2012). The fact that neither *ATXN1* nor *ATXN2* intermediate repeat expansions show an association with ALS among ALS patients of African origin highlights the need to conduct population specific studies to detect the diverse genetic architecture of ALS (Nel et al. 2021).

ATXN1 intermediate length repeat expansions are thought to act as contributing factors to TDP-43 protein aggregation. The abundance of ataxin-1 is believed to disturb the transport of TDP-43 protein from the cytoplasm to the nucleus. Thus in the presence of the *ATXN1* intermediate length repeat expansion, TDP-43 protein may become enriched in the cytoplasm and tends to form aggregates (Tazelaar et al. 2020; Woerner et al. 2016).

According to previous studies, intermediate length alleles of the *ATXN1* gene do not alter the age at onset, the survival, or the phenotype of the patients (Tazelaar et al. 2020). The age at onset of our *ATXN1* intermediate repeat expansion carrying patients was 60.18 years and on average they have had an 18-month-long history of complaints. Ataxia could not be observed in any of the patients. All of them had signs of upper- and lower motor neuron damage and most of them presented bulbar signs as well.

Two patients carried an additional variant in a major ALS gene beside the *ATXN1* intermediate repeat expansion. One patient carried the p.R84H variant of the *UBQLN2* gene and another patient also harbored the hexanucleotide repeat expansion in the *C9orf72* gene. Repeat expansion in the *ATXN1* and in the *C9orf72* gene have been found to coexist frequently (Lattante et al. 2018). The patient carrying both the *C9orf72* and the *ATXN1* repeat expansion was diagnosed with ALS at the age of 70 based on a three-month history of dysphagia and weakness. At initial examination she scored 37/48 on the ALS Functional Rating Scale and 29/30 on the Mini Mental State Examination. Further studies are needed to confirm the link between *C9orf72* and *ATXN1* repeat expansions in ALS, as the small size of our cohort does not allow us to establish an association.

ATXN2 intermediate length repeat expansions were first associated with an increased risk of ALS in 2010 (Elden et al. 2010). In the literature there has been much debate on the cut-off value of intermediate alleles: some authors suggested a cutoff repeat number as high as 32 CAG units (Corrado et al. 2011). In our study we used the values (≥ 24 repeats) which were proposed by Elden et al 2010.

The detected 18.3% (26/153) frequency of the intermediate length alleles of the ALS patients and 9.23% (18/195) of the control individuals is much higher than reported by comparable studies conducted on European population (Daoud et al. 2011). None of the *ATXN2*

intermediate length allele carrying patients showed clinical signs of type 2 spinocerebellar ataxia, which is consistent with previous reports (Corrado et al. 2011).

Table 13 shows data from larger populations regarding the carriership frequency of *ATXN1* and *ATXN2* intermediate length alleles among ALS patients. It is noteworthy that no other population shows as high carriership percentage as the examined Hungarian ALS patients.

Gene	Variant	Number of patients/ controls in this study	Frequency in ALS patients/ controls described by other groups (population size)	References	First report	Published functional studies
ATXN1	intermediate length CAG trinucleotide expansion	16/182 (8.79%)/ 2/178 (1.12%)	7.07%/ 2.38% (418 ALS patients and 296 healthy individuals); 5.84%/ 2.75% (411 ALS patients and 436 healthy control individuals)	Conforti *et al.* , 2012; Gonçalves *et al.* , 2020	Conforti *et al.* , 2012	Tazelaar *et al* , 2020.
ATXN2	intermediate length CAG trinucleotide expansion	28/153 (18.3%)/ 18/195 (9.23%)	3%/ 0% (232 ALS patients and 395 healthy control individuals) 7.2%/ 5.1% (471 ALS cases and 556 healthy controls)	Corrado *et al* ., 2011; Daoud *et al* ., 2011	Elden *et al.* , 2010	Elden *et al* , 2010.

Table 13. Literature data on the *ATXN1* and *ATXN2* intermediate repeat expansion carriership frequency among different populations.

With our results we confirm that both *ATXN1* and *ATXN2* intermediate length repeat expansions are risk factors of ALS and both repeat expansions are more common in the Hungarian ALS population compared to other European ethnicities.

Gene	Variant	Number of patients/ controls in this study	Frequency in ALS patients/ controls described by other groups (population size)	References	First report
SMN1	duplication	6/148 (4.05%)/ 7/148 (4.73%)	7.8%/ 1.8% (167 ALS patients and 167 healthy controls); 5.1%/2.6% (433 ALS patients and 454 healthy controls)	Corcia *et al.* , 2002; Corcia *et al.* , 2006	Corcia *et al.* , 2002
SMN2	homozygous deletion	7/148 (4.73%)/ 10/148 (6.76%)	20%/ 2% (25 ALS patients and 25 healthy controls); 5.8%/7.4% (502 ALS patients and 502 healthy controls)	Lee *et al.* , 2012; Corcia *et al.* , 2012	Lee *et al.* , 2012

Table 14. Literature data on *SMN1* duplications and *SMN2* homozygous deletion in ALS patients.

Table 14 reports the frequency of copy number variations of the *SMN1* and *SMN2* genes in other populations. *SMN1* duplications and *SMN2* homozygous deletions were present in a similar fraction of ALS patients of other European ethnicities as we have reported in case of the Hungarian ALS patients.

Our results suggest that neither *SMN1* duplications nor *SMN2* homozygous deletions are a potent genetic risk factors of ALS, since the frequency of these genetic variations are similar in the ALS and the control group. Studies have shown that carrying three copies of the *SMN1* gene is as common among ALS patients as 7%, and the duplication of the gene is associated with a more than twofold increased risk of ALS (Corcia et al. 2002).

Conflicting reports have emerged whether homozygous deletions of *SMN2* are risk factors for developing ALS or the complete opposite: homozygous *SMN2* deletions may have a protecting role against ALS (Lee et al. 2012; Corcia et al. 2012). Our data supports neither of these strong statements. Up to date no effect of *SMN1* or *SMN2* copy number variation has been described in the literature regarding the age at onset or disease duration (Blauw et al. 2012).

Conclusion

In this PhD dissertation we report the first detailed genetic investigations in the Hungarian amyotrophic lateral sclerosis population that resulted in the identification of novel putative ALS-associated genetic factors. With the accurate and detailed phenotypic data our sample collection represents a great value to the Hungarian and to the international scientific community as well.

Within the frameworks of our project, a novel angiogenin mutation was detected and functionally characterized to prove its disease-causing role. We also performed the whole exome sequencing of 21 ALS patients and carried out a thorough custom design panel sequencing of additional 86 ALS patients. These panels were reanalyzed 5 years later. Furthermore, we investigated the role of established genetic risk factors of ALS in the Hungarian ALS patient's cohort.

Genetic factors contributing to the development of ALS vary from one ethnic group to the other (Zou et al. 2017). Characterizing a different population adds crucial data to the growing amount of available information about the population-specific aspects of ALS. By deciphering the genetic background of ALS, we might better understand the diverse pathological processes behind the disease. Well understood pathology of the disease paves the way to the development of effective and personalized therapeutic options (van Es et al. 2017).

Our project identified the genetic background of ALS in almost 41% of our patients and our work also revealed novel genotype-phenotype correlations. To stratify the patients, genetic risk factors were also investigated which helped to understand the different phenotypic characteristics of patients with the same main or major ALS gene variant.

Particularly interesting is the oligogenic basis of ALS, for which more and more evidence has stockpiled in recent years (Blitterswijk et al. 2012; Kuuluvainen et al. 2019; Ross et al. 2020; McCann et al. 2021). It is believed that a more potent, penetrant variant or several less penetrant variants contribute to the development of the disease. The subgroup of patients carrying multiple major ALS gene variants identified in our cohort support this hypothesis.

Acknowledgements

I am immensely grateful for my wonderful supervisors, Professor Márta Széll and Dr. Margit Pál. I thank Professor Széll for believing in me and for the opportunity to join the research group. I thank Dr. Margit Pál for her great enthusiasm and for her excellent comments and suggestions regarding my work.

I would like to thank Dr. Kornélia Tripolszki for introducing me to this project and for helping me in the first years of my work. Thank you for familiarizing me with the wet lab techniques and for showing me for exciting research can be.

I would like to express my gratitude towards Dr. András Salamon who has provided invaluable clinical characteristics about the patients and helped with the genotype-phenotype correlations.

Special thanks to Professor József I Engelhardt, Professor Péter Klivényi from the Department of Neurology for the enrollment of patients and their detailed neurological examination.

I am grateful for the support of all my colleagues at the Department of Medical Genetics, especially Dr. Gloria Kafui Esi Zodanu and Dalma Füstös for their help in performing some of the analyses. I owe a thank you to Dr. Dóra Nagy, a former colleague of ours, for her contribution to the establishment of genotype-phenotype correlations.

I thank Dr. Piyush Gampawar and Dr. Helena Schmidt from the Medical University of Graz, Austria for providing control samples for our studies. Thank you for all our collaborating partners in Delhi, India from the Kusuma School of Biological Sciences, Indian Institute of Technology, from the Department of Dermatology and Allergology, University of Szeged and from the MTA-SZTE Dermatological Research Group.

Lastly, without the support and encouragement of my family this work would not have been possible, I owe it all to you.

References

Aguila, M. A. Del, W. T. Longstreth, V. McGuire, T. D. Koepsell, and G. Van Belle. 2003. “Prognosis in Amyotrophic Lateral Sclerosis: A Population-Based Study.” *Neurology* 60 (5): 813–19. https://doi.org/10.1212/01.WNL.0000049472.47709.3B.

Al-Chalabi, Ammar, and Orla Hardiman. 2013. “The Epidemiology of ALS: A Conspiracy of Genes, Environment and Time.” *Nature Reviews Neurology* 9 (11): 617–28. https://doi.org/10.1038/nrneurol.2013.203.

Alcaz, S, M Jarebinski, T Pekmezovic, S Pavlovic, and S Apostolski. 1996. “Epidemiological and Clinical Characteristics of ALS in Belgrade, Yugoslavia.” *Acta Neurol Scand* 94 (4): 264–68. https://doi.org/10.1111/j.1600-0404.1996.tb07063.x.

Aluri, Krishna C., Joseph P. Salisbury, Jochen H.M. Prehn, and Jeffrey N. Agar. 2020. “Loss of Angiogenin Function Is Related to Earlier ALS Onset and a Paradoxical Increase in ALS Duration.” *Scientific Reports* 10 (1): 1–13. https://doi.org/10.1038/s41598-020-60431-6.

Andersen, PM. 2006. “Amyotrophic Lateral Sclerosis Associated with Mutations in the CuZn Superoxide Dismutase Gene.” *Current Neurology and Neuroscience Reports* 6 (1): 37–46.

Arai, Tetsuaki, Masato Hasegawa, Haruhiko Akiyama, Kenji Ikeda, Takashi Nonaka, Hiroshi Mori, David Mann, et al. 2006. “TDP-43 Is a Component of Ubiquitin-Positive Tau-Negative Inclusions in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis.” *Biochemical and Biophysical Research Communications* 351 (3): 602–11. https://doi.org/10.1016/j.bbrc.2006.10.093.

Benatar, Michael, Joanne Wuu, Peter M. Andersen, Robert C. Bucelli, Jinsy A. Andrews, Markus Otto, Nita A. Farahany, et al. 2022. “Design of a Randomized, Placebo-Controlled, Phase 3 Trial of Tofersen Initiated in Clinically Presymptomatic SOD1 Variant Carriers: The ATLAS Study.” *Neurotherapeutics* 19 (4): 1248–58. https://doi.org/10.1007/s13311-022-01237-4.

Bensimon, G, L Lacomblez, and V Meininger. 1999. “A Controlled Trial of Riluzole in Amyotrophic Lateral Sclerosis. ALS/Riluzole Study Group.” *New England Journal of Medicine* 3 (330): 585–91. https://doi.org/10.1056/NEJM199403033300901.

Bhardwaj, Amit, Michael P. Myers, Emanuele Buratti, and Francisco E. Baralle. 2013. “Characterizing TDP-43 Interaction with Its RNA Targets.” *Nucleic Acids Research* 41 (9): 5062–74. https://doi.org/10.1093/nar/gkt189.

Blauw, H. M., C. P. Barnes, P. W.J. Van Vught, W. Van Rheenen, M. Verheul, E. Cuppen, J. H. Veldink, and L. H. Van Den Berg. 2012. “SMN1 Gene Duplications Are Associated with Sporadic ALS.” *Neurology* 78 (11): 776–80. https://doi.org/10.1212/WNL.0b013e318249f697.

Blitterswijk, Marka Van, Michael A Van Es, Eric A M Hennekam, Dennis Dooijes, Wouter Van Rheenen, Jelena Medic, Pierre R Bourque, et al. 2012. “Evidence for an Oligogenic Basis of Amyotrophic Lateral Sclerosis” 21 (17): 3776–84. https://doi.org/10.1093/hmg/dds199.

Boer, Eva Maria Johanna De, Viyanti K. Orie, Timothy Williams, Mark R. Baker, Hugo M. De Oliveira, Tuomo Polvikoski, Matthew Silsby, et al. 2021. “TDP-43 Proteinopathies: A New Wave of Neurodegenerative Diseases.” *Journal of Neurology, Neurosurgery and Psychiatry* 92 (1): 86–95. https://doi.org/10.1136/jnnp-2020-322983.

Bonne, G., E. Mercuri, A. Muchir, A. Urtizberea, H. M. Bécane, D. Recan, L. Merlini, et al. 2000. “Clinical and Molecular Genetic Spectrum of Autosomal Dominant Emery-Dreifuss Muscular Dystrophy Due to Mutations of the Lamin A/C Gene.” *Annals of Neurology* 48 (2): 170–80. https://doi.org/10.1002/1531-8249(200008)48:2<170::AID-ANA6>3.0.CO;2-J.

Bourke, Stephen C., Mark Tomlinson, Tim L. Williams, Robert E. Bullock, Pamela J. Shaw, and G. John Gibson. 2006. “Effects of Non-Invasive Ventilation on Survival and Quality of Life in Patients with Amyotrophic Lateral Sclerosis: A Randomised Controlled Trial.” *Lancet Neurology* 5 (2): 140–47. https://doi.org/10.1016/S1474-4422(05)70326-4.

Bradshaw, William J., Saima Rehman, Tram T.K. Pham, Nethaji Thiyagarajan, Rebecca L. Lee, Vasanta Subramanian, and K. Ravi Acharya. 2017. “Structural Insights into Human Angiogenin Variants Implicated in Parkinson’s Disease and Amyotrophic Lateral Sclerosis.” *Scientific Reports* 7 (February): 1–10. https://doi.org/10.1038/srep41996.

Brenner, David, Thomas Wieland, Patrick Weydt, Sarah Bo, Kathrin Mu, Annemarie Hu, Christoph Neuwirth, et al. 2016. “NEK1 Mutations in Familial Amyotrophic Lateral Sclerosis.” *Brain* 139: 1–4. https://doi.org/10.1093/brain/aww033.

Carvalho, Mamede D E, and Michael Swash. 2009. “Awaji Diagnostic Algorithm Increases Sensitivity of El Escorial Criteria for ALS Diagnosis,” no. August 2008: 53–57. https://doi.org/10.1080/17482960802521126.

Carvalho, Mamede de, Adriano Chio, Reinhard Dengler, Martin Hecht, Markus Weber, and Michael Swash. 2005. “Neurophysiological Measures in Amyotrophic Lateral Sclerosis: Markers of Progression in Clinical Trials.” *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders* 6 (1): 17–28. https://doi.org/10.1080/14660820410020600.

Chan Moi Fat, Sandrine, Emily P. McCann, Kelly L. Williams, Lyndal Henden, Natalie A. Twine, Denis C. Bauer, Roger Pamphlett, et al. 2021. “Genetic Analysis of GLT8D1 and ARPP21 in Australian Familial and Sporadic Amyotrophic Lateral Sclerosis.” *Neurobiology of Aging* 101: 297.e9-297.e11. https://doi.org/10.1016/j.neurobiolaging.2021.01.005.

Chernyatina, Anastasia A., Stefan Nicolet, Ueli Aebi, Harald Herrmann, and Sergei V. Strelkov. 2012. “Atomic Structure of the Vimentin Central α-Helical Domain and Its Implications for Intermediate Filament Assembly.” *Proceedings of the National Academy of Sciences of the United States of America* 109 (34): 13620–25. https://doi.org/10.1073/pnas.1206836109.

Chiò, A., G. Logroscino, B. J. Traynor, J. Collins, J. C. Simeone, L. A. Goldstein, and L. A. White. 2013. “Global Epidemiology of Amyotrophic Lateral Sclerosis: A Systematic Review of the Published Literature.” *Neuroepidemiology* 41 (2): 118–30. https://doi.org/10.1159/000351153.

Chio, A., C. Magnani, and D. Schiffer. 1995. “Gompertzian Analysis of Amyotrophic Lateral Sclerosis Mortality in Italy, 1957 1987; Application to Birth Cohorts.” *Neuroepidemiology* 14 (6): 269–77. https://doi.org/10.1159/000109802.

Chiò, Adriano, Giuseppe Borghero, Gabriella Restagno, Gabriele Mora, Carsten Drepper, Bryan J. Traynor, Michael Sendtner, et al. 2012. “Clinical Characteristics of Patients with Familial Amyotrophic Lateral Sclerosis Carrying the Pathogenic GGGGCC Hexanucleotide Repeat Expansion of C9ORF72.” *Brain* 135 (3): 784–93. https://doi.org/10.1093/brain/awr366.

Chiò, Adriano, Letizia Mazzini, and Gabriele Mora. 2020. “Disease-Modifying Therapies in Amyotrophic Lateral Sclerosis.” *Neuropharmacology* 167 (November 2019): 107986. https://doi.org/10.1016/j.neuropharm.2020.107986.

Chou, Ching Chieh, Yi Zhang, Mfon E. Umoh, Spencer W. Vaughan, Ileana Lorenzini, Feilin Liu, Melissa Sayegh, et al. 2018. “TDP-43 Pathology Disrupts Nuclear Pore Complexes and Nucleocytoplasmic Transport in ALS/FTD.” *Nature Neuroscience* 21 (2): 228–39. https://doi.org/10.1038/s41593-017-0047-3.

Cirulli, Elizabeth T, Meg Watson, Dawn M Holman, Kathleen A Fox, Gery P Guy Jr, Andrew B Seidenberg, Blake P Sampson, and Craig Sinclair. 2015. “Exome Sequencing in Amyotrophic Lateral Sclerosis Identifies Risk Genes and Pathways.” *Sciencek* 44 (6): 682–89. https://doi.org/10.1016/j.amepre.2013.02.015.Preventing.

Conforti, Francesca L., Rossella Spataro, William Sproviero, Rosalucia Mazzei, Francesca Cavalcanti, Francesca Condino, Isabella L. Simone, et al. 2012. “Ataxin-1 and Ataxin-2 Intermediate-Length PolyQ Expansions in Amyotrophic Lateral Sclerosis.” *Neurology* 79 (24): 2315–20. https://doi.org/10.1212/WNL.0b013e318278b618.

Cooper-Knock, Johnathan, Tobias Moll, Tennore Ramesh, Lydia Castelli, Alexander Beer, Henry Robins, Ian Fox, et al. 2019. “Mutations in the Glycosyltransferase Domain of GLT8D1 Are Associated with Familial Amyotrophic Lateral Sclerosis.” *Cell Reports* 26 (9): 2298-2306.e5. https://doi.org/10.1016/j.celrep.2019.02.006.

Cooper-Knock, Johnathan, Pamela J. Shaw, and Janine Kirby. 2014. “The Widening Spectrum of C9ORF72-Related Disease; Genotype/Phenotype Correlations and Potential Modifiers of Clinical Phenotype.” *Acta Neuropathologica* 127 (3): 333–45. https://doi.org/10.1007/s00401-014-1251-9.

Cooper-Knock, Johnathan, Matthew J. Walsh, Adrian Higginbottom, J. Robin Highley, Mark J. Dickman, Dieter Edbauer,

Paul G. Ince, et al. 2014. “Sequestration of Multiple RNA Recognition Motif-Containing Proteins by C9orf72 Repeat Expansions.” *Brain* 137 (7): 2040–51. https://doi.org/10.1093/brain/awu120.

Corcia, P., P. Petiot, Z. Stevic, P. Vourc’h, R. Morales, P. H. Gordon, N. Pageot, C. Andres, and W. Camu. 2011. “Respiratory Onset in an ALS Family with L144F SOD1 Mutation.” *Journal of Neurology, Neurosurgery and Psychiatry* 82 (7): 747–49. https://doi.org/10.1136/jnnp.2009.197558.

Corcia, Philippe, Caroline Ingre, Helene Blasco, Rayomand Press, Julien Praline, Catherine Antar, Charlotte Veyrat-Durebex, et al. 2012. “Homozygous SMN2 Deletion Is a Protective Factor in the Swedish ALS Population.” *European Journal of Human Genetics* 20 (5): 588–91. https://doi.org/10.1038/ejhg.2011.255.

Corcia, Philippe, Véronique Mayeux-Portas, Jawad Khoris, Bertrand De Toffol, Alain Autret, Jean Pierre Müh, William Camu, and Christian Andres. 2002. “Abnormal SMN1 Gene Copy Number Is a Susceptibility Factor for Amyotrophic Lateral Sclerosis.” *Annals of Neurology* 51 (2): 243–46. https://doi.org/10.1002/ana.10104.

Corrado, Lucia, Letizia Mazzini, Gaia Donata Oggioni, Bernadetta Luciano, Michela Godi, Alfredo Brusco, and Sandra D. Alfonso. 2011. “ATXN-2 CAG Repeat Expansions Are Interrupted in ALS Patients.” *Human Genetics* 130 (4): 575–80. https://doi.org/10.1007/s00439-011-1000-2.

Dal Bello-Haas, Vanina. 2018. “Physical Therapy for Individuals with Amyotrophic Lateral Sclerosis: Current Insights.” *Degenerative Neurological and Neuromuscular Disease* Volume 8: 45–54. https://doi.org/10.2147/dnnd.s146949.

Damme, Philip Van, Wim Robberecht, and Ludo Van Den Bosch. 2017. “Modelling Amyotrophic Lateral Sclerosis: Progress and Possibilities.” *DMM Disease Models and Mechanisms* 10 (5): 537–49. https://doi.org/10.1242/dmm.029058.

Daoud, Hussein PhD;, Véronique MSc; Belzil, Sandra PhD; Martins, Mike MD; Sabbagh, Pierre MSc; MD; Provencher, Lacomblez Lucette, Vincent MD; Meininger, et al. 2011. “Association of Long ATXN2 CAG Repeat Sizes With Increased Risk of Amyotrophic Lateral Sclerosis.” *Arch Neurol* 68 (6): 739–42. https://doi.org/10.1001/archneurol.2011.111.

DeJesus-Hernandez, Mariely, Ian R Mackenzie, Bradley F Boeve, Adam L Boxer, Matt Baker, Nicola J Rutherford, Alexandra M Nicholson, et al. 2012. “Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS.” *Neuron* 72 (2): 245–56. https://doi.org/10.1016/j.neuron.2011.09.011.Expanded.

Desport, Jean Claude, Frédéric Torny, Mathieu Lacoste, Pierre Marie Preux, and Philippe Couratier. 2006. “Hypermetabolism in ALS: Correlations with Clinical and Paraclinical Parameters.” *Neurodegenerative Diseases* 2 (3–4): 202–7. https://doi.org/10.1159/000089626.

Dorst, Johannes, Albert C. Ludolph, and Annemarie Huebers. 2018. “Disease-Modifying and Symptomatic Treatment of Amyotrophic Lateral Sclerosis.” *Therapeutic Advances in Neurological Disorders* 11: 1–16. https://doi.org/10.1177/1756285617734734.

Drory, Vivian E., Miriam Birnbaum, Leah Peleg, Boleslaw Goldman, and Amos D. Korczyn. 2003. “Hexosaminidase a Deficiency Is an Uncommon Cause of a Syndrome Mimicking Amyotrophic Lateral Sclerosis.” *Muscle and Nerve* 28 (1): 109–12. https://doi.org/10.1002/mus.10371.

Duff, Rachael M., Valerie Tay, Peter Hackman, Gianina Ravenscroft, Catriona McLean, Paul Kennedy, Alina Steinbach, et al. 2011. “Mutations in the N-Terminal Actin-Binding Domain of FilamiInvestigating the Role of Filamin C in Belgian Patients with Frontotemporal Dementia Linked to GRN Deficiency in FTLD-TDP Brains n C Cause a Distal Myopathy.” *American Journal of Human Genetics* 88 (6): 729–40. https://doi.org/10.1016/j.ajhg.2011.04.021.

Dupuis, Luc, Pierre François Pradat, Albert C. Ludolph, and Jean Philippe Loeffler. 2011. “Energy Metabolism in Amyotrophic Lateral Sclerosis.” *The Lancet Neurology* 10 (1): 75–82. https://doi.org/10.1016/S1474-4422(10)70224-6.

Elden, Andrew C., Hyung Jun Kim, Michael P. Hart, Alice S. Chen-Plotkin, Brian S. Johnson, Xiaodong Fang, Maria Armakola, et al. 2010. “Ataxin-2 Intermediate-Length Polyglutamine Expansions Are Associated with Increased Risk for ALS.” *Nature* 466 (7310): 1069–75. https://doi.org/10.1038/nature09320.

Eleutherio, Elis Cristina Araujo, Rayne Stfhany Silva Magalhães, Aline de Araújo Brasil, José Raphael Monteiro Neto, and Luan de Holanda Paranhos. 2021. "SOD1, More than Just an Antioxidant." *Archives of Biochemistry and Biophysics* 697 (September 2020). https://doi.org/10.1016/j.abb.2020.108701.

Es, Michael A. van, Orla Hardiman, Adriano Chio, Ammar Al-Chalabi, R. Jeroen Pasterkamp, Jan H. Veldink, and Leonard H. van den Berg. 2017. "Amyotrophic Lateral Sclerosis." *The Lancet* 390 (10107): 2084–98. https://doi.org/10.1016/S0140-6736(17)31287-4.

Farg, Manal A., Vinod Sundaramoorthy, Jessica M. Sultana, Shu Yang, Rachel A.K. Atkinson, Vita Levina, Mark A. Halloran, et al. 2014. "C9ORF72, Implicated in Amytrophic Lateral Sclerosis and Frontotemporal Dementia, Regulates Endosomal Trafficking." *Human Molecular Genetics* 23 (13): 3579–95. https://doi.org/10.1093/hmg/ddu068.

Fecto, Faisal, Jianhua Yan, S Pavan Vemula, Erdong Liu, Yi Yang, Wenjie Chen, Jian Guo Zheng, et al. 2011. "SQSTM1 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis." *Arch Neurol* 68 (11): 1440–46. https://doi.org/10.1001/archneurol.2011.250.

Ferrera, Loretta, Claudia Caponnetto, Valeria Marini, Domenica Rizzi, Domenico Bordo, Silvana Penco, Antonio Amoroso, Paola Origone, and Cecilia Garrè. 2003. "An Italian Dominant FALS Leu144Phe SOD1 Mutation: Genotype-Phenotype Correlation." *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders* 4 (3): 167–70. https://doi.org/10.1080/aml.4.3.167.170.

Figlewicz, Denise a, Aldis Krizus, Maria G Martinoli, Vincent Meininger, Michel Dib, Guy a Rouleau, and Jean-pierre Julien. 1994. "Variants of the Heavy Neurofilament Subunit Are Associated with the Development of Amyotrophic Lateral Sclerosis." *Human Molecular Genetics* 3 (10): 1757–61.

Forgrave, Lauren M., Matthew Ma, John R. Best, and Mari L. DeMarco. 2019. "The Diagnostic Performance of Neurofilament Light Chain in CSF and Blood for Alzheimer's Disease, Frontotemporal Dementia, and Amyotrophic Lateral Sclerosis: A Systematic Review and Meta-Analysis." *Alzheimer's and Dementia: Diagnosis, Assessment and Disease Monitoring* 11: 730–43. https://doi.org/10.1016/j.dadm.2019.08.009.

Freischmidt, Axel, Thomas Wieland, Benjamin Richter, Wolfgang Ruf, Veronique Schaeffer, Kathrin Müller, Nicolai Marroquin, et al. 2015. "Haploinsufficiency of TBK1 Causes Familial ALS and Fronto-Temporal Dementia," no. March. https://doi.org/10.1038/nn.4000.

Gaiottino, Johanna, Niklas Norgren, Ruth Dobson, Joanne Topping, Ahuva Nissim, Andrea Malaspina, Jonathan P. Bestwick, et al. 2013. "Increased Neurofilament Light Chain Blood Levels in Neurodegenerative Neurological Diseases." *PLoS ONE* 8 (9): 1–9. https://doi.org/10.1371/journal.pone.0075091.

Gal, Jozsef, Lisha Kuang, Kelly R. Barnett, Brian Z. Zhu, Susannah C. Shissler, Konstantin V. Korotkov, Lawrence J. Hayward, Edward J. Kasarskis, and Haining Zhu. 2016. "ALS Mutant SOD1 Interacts with G3BP1 and Affects Stress Granule Dynamics." *Acta Neuropathologica* 132 (4): 563–76. https://doi.org/10.1007/s00401-016-1601-x.

Geier, Ethan G., Mathieu Bourdenx, Nadia J. Storm, J. Nicholas Cochran, Daniel W. Sirkis, Ji Hye Hwang, Luke W. Bonham, et al. 2019. "Rare Variants in the Neuronal Ceroid Lipofuscinosis Gene MFSD8 Are Candidate Risk Factors for Frontotemporal Dementia." *Acta Neuropathologica* 137 (1): 71–88. https://doi.org/10.1007/s00401-018-1925-9.

Goldstein, Orly, Merav Kedmi, Mali Gana-Weisz, Beatrice Nefussy, Batel Vainer, Yaara Fainmesser, Vivian E. Drory, and Avi Orr-Urtreger. 2020. "A Novel Mutation in TARDBP Segregates with Amyotrophic Lateral Sclerosis in a Large Family with Early Onset and Fast Progression." *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration* 21 (3–4): 280–85. https://doi.org/10.1080/21678421.2020.1747496.

Greenway, Matthew J., Peter M. Andersen, Garsten Russ, Sean Ennis, Susan Cashman, Colette Donaghy, Victor Patterson, et al. 2006. "ANG Mutations Segregate with Familial and 'sporadic' Amyotrophic Lateral Sclerosis." *Nature Genetics* 38 (4): 411–13. https://doi.org/10.1038/ng1742.

Gregory, Jenna M., Delphine Fagegaltier, Hemali Phatnani, and Matthew B. Harms. 2020. "Genetics of Amyotrophic Lateral Sclerosis." *Current Genetic Medicine Reports* 8 (1): 121–31. https://doi.org/10.1024/1661-8157/a003153.

Hadano, Shinji, Ryota Kunita, Asako Otomo, Kyoko Suzuki-utsunomiya, and Joh-e Ikeda. 2007. “Molecular and Cellular Function of ALS2 / Alsin : Implication of Membrane Dynamics in Neuronal Development and Degeneration” 51: 74–84. https://doi.org/10.1016/j.neuint.2007.04.010.

Hall, P, and M Harcup. 1969. “A Trial of Lipotropic Enzymes in Atheromatous (‘arteriosclerotic’) Dementia.” *Angiology* 20 (5): 287–300. https://doi.org/10.1177/000331976902000509.

Hasegawa, Masato, Tetsuaki Arai, Takashi Nonaka, Fuyuki Kametani, Mari Yoshida, Yoshio Hashizume, Thomas G. Beach, et al. 2008. “Phosphorylated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis.” *Annals of Neurology* 64 (1): 60–70. https://doi.org/10.1002/ana.21425.

Hu, William T., Matthew Shelnutt, Ashley Wilson, Nicole Yarab, Crystal Kelly, Murray Grossman, David J. Libon, et al. 2013. “Behavior Matters-Cognitive Predictors of Survival in Amyotrophic Lateral Sclerosis.” *PLoS ONE* 8 (2). https://doi.org/10.1371/journal.pone.0057584.

Huang, Ling, Zhen Liu, Yanchun Yuan, Lu Shen, Hong Jiang, Beisha Tang, Junling Wang, and Lifang Lei. 2021. “Mutation Analysis of MFSD8 in an Amyotrophic Lateral Sclerosis Cohort from Mainland China.” *European Journal of Neuroscience* 53 (4): 1197–1206. https://doi.org/10.1111/ejn.15058.

Huang, Xiao, Shen Shen, and Dongsheng Fan. 2017. “No Evidence for Pathogenic Role of UBQLN2 Mutations in Sporadic Amyotrophic Lateral Sclerosis in the Mainland Chinese Population,” no. December 2014: 1–5. https://doi.org/10.1371/journal.pone.0170943.

Humphrey, William, Andrew Dalke, and Klaus Schulten. 1996. “VMD: Visual Molecular Dynamics.” *Journal of Molecular Graphics* 14 (October 1995): 33–38. https://www.tapbiosystems.com/tap/products/index.htm.

Janssens, Jonathan, Stéphanie Philtjens, Gernot Kleinberger, Sara Van Mossevelde, Julie van der Zee, Rita Cacace, Sebastiaan Engelborghs, et al. 2015. “Investigating the Role of Filamin C in Belgian Patients with Frontotemporal Dementia Linked to GRN Deficiency in FTLD-TDP Brains.” *Acta Neuropathologica Communications* 3: 68. https://doi.org/10.1186/s40478-015-0246-7.

Joensen, P. 2012. “Incidence of Amyotrophic Lateral Sclerosis in the Faroe Islands.” *Acta Neurologica Scandinavica* 126 (1): 62–66. https://doi.org/10.1111/j.1600-0404.2011.01611.x.

Karam, Chafic Y., Sabrina Paganoni, Nanette Joyce, Gregory T. Carter, and Richard Bedlack. 2016. “Palliative Care Issues in Amyotrophic Lateral Sclerosis: An Evidenced-Based Review.” *American Journal of Hospice and Palliative Medicine* 33 (1): 84–92. https://doi.org/10.1177/1049909114548719.

Kaufmann, P., G. Levy, J. L.P. Thompson, M. L. DelBene, V. Battista, P. H. Gordon, L. P. Rowland, B. Levin, and H. Mitsumoto. 2005. “The ALSFRSr Predicts Survival Time in an ALS Clinic Population.” *Neurology* 64 (1): 38–43. https://doi.org/10.1212/01.WNL.0000148648.38313.64.

Kenna, Kevin P., Perry T.C. Van Doormaal, Annelot M. Dekker, Nicola Ticozzi, Brendan J. Kenna, Frank P. Diekstra, Wouter Van Rheenen, et al. 2016. “NEK1 Variants Confer Susceptibility to Amyotrophic Lateral Sclerosis.” *Nature Genetics* 48 (9): 1037–42. https://doi.org/10.1038/ng.3626.

Kimura, F., C. Fujimura, S. Ishida, H. Nakajima, D. Furutama, H. Uehara, K. Shinoda, M. Sugino, and T. Hanafusa. 2006. “Progression Rate of ALSFRS-R at Time of Diagnosis Predicts Survival Time in ALS.” *Neurology* 66 (2): 265–67. https://doi.org/10.1212/01.wnl.0000194316.91908.8a.

Kochin, Vitaly, Takeshi Shimi, Elin Torvaldson, Stephen A. Adam, Anne Goldman, Chan Gi Pack, Johanna Melo-Cardenas, Susumu Y. Imanishi, Robert D. Goldman, and John E. Eriksson. 2014. “Interphase Phosphorylation of Lamin A.” *Journal of Cell Science* 127 (12): 2683–96. https://doi.org/10.1242/jcs.141820.

Kopanos, Christos, Vasilis Tsiolkas, Alexandros Kouris, Charles E. Chapple, Monica Albarca Aguilera, Richard Meyer, and Andreas Massouras. 2019. “VarSome: The Human Genomic Variant Search Engine.” *Bioinformatics* 35 (11): 1978–80. https://doi.org/10.1093/bioinformatics/bty897.

Kotan, Dilcan, Zeynep Özözen Ayas, Ceren Tunca, Belma Dogan Gungen, Fulya Akçimen, and A. Nazlı Başak. 2020. “Phenotypic and Genotypic Features of Patients Diagnosed with ALS in the City of Sakarya, Turkey.” *Acta*

Neurologica Belgica 120 (6): 1411–18. https://doi.org/10.1007/s13760-020-01441-z.

Krüger, Stefanie, Thomas Gasser, Johannes Prudlo, Marita Munz, and Ludger Schöls. 2016. "Rare Variants in Neurodegeneration Associated Genes Revealed by Targeted Panel Sequencing in a German ALS Cohort." *Frontiers in Molecular Neuroscience* 9 (October). https://doi.org/10.3389/fnmol.2016.00092.

Kuuluvainen, Liina, Karri Kaivola, Saana Mönkäre, Hannu Laaksovirta, Manu Jokela, Bjarne Udd, Miko Valori, et al. 2019. "Oligogenic Basis of Sporadic ALS: The Example of SOD1 p.Ala90Val Mutation." *Neurology: Genetics* 5 (3): 1–6. https://doi.org/10.1212/NXG.0000000000000335.

Lamb, Ruth, Jonathan D. Rohrer, Raquel Real, Steven J. Lubbe, Adrian J. Waite, Derek J. Blake, R. Jon Walters, et al. 2019. "A Novel TBK1 Mutation in a Family with Diverse Frontotemporal Dementia Spectrum Disorders." *Cold Spring Harbor Molecular Case Studies* 5 (3): 1–10. https://doi.org/10.1101/mcs.a003913.

Lamsoul, Isabelle, Loïc Dupré, and Pierre G. Lutz. 2020. "Molecular Tuning of Filamin A Activities in the Context of Adhesion and Migration." *Frontiers in Cell and Developmental Biology* 8 (November): 1–10. https://doi.org/10.3389/fcell.2020.591323.

Laššuthová, P., R. Mazanec, P. Vondráček, D. Šišková, J. Haberlová, J. Sabová, and P. Seeman. 2011. "High Frequency of SH3TC2 Mutations in Czech HMSN I Patients." *Clinical Genetics* 80 (4): 334–45. https://doi.org/10.1111/j.1399-0004.2011.01640.x.

Lattante, Serena, Maria Grazia Pomponi, Amelia Conte, Giuseppe Marangi, Giulia Bisogni, Agata Katia Patanella, Emiliana Meleo, et al. 2018. "ATXN1 Intermediate-Length Polyglutamine Expansions Are Associated with Amyotrophic Lateral Sclerosis." *Neurobiology of Aging* 64: 157.e1-157.e5. https://doi.org/10.1016/j.neurobiolaging.2017.11.011.

Laurin, Nancy, Jacques P. Brown, Arnaud Lemainque, Annie Duchesne, Denys Huot, Yves Lacourcière, Gervais Drapeau, Jean Verreault, Vincent Raymond, and Jean Morissette. 2001. "Paget Disease of Bone: Mapping of Two Loci at 5q35-Qter and 5q31." *American Journal of Human Genetics* 69 (3): 528–43. https://doi.org/10.1086/322975.

Lee, Jun Beom, Kyung A. Lee, Ji Man Hong, Gyoung Im Suh, and Young Chul Choi. 2012. "Homozygous SMN2 Deletion Is a Major Risk Factor among Twenty-Five Korean Sporadic Amyotrophic Lateral Sclerosis Patients." *Yonsei Medical Journal* 53 (1): 53–57. https://doi.org/10.3349/ymj.2012.53.1.53.

Lee, T., Y. R. Li, A. Chesi, M. P. Hart, D. Ramos, N. Jethava, D. Hosangadi, et al. 2011. "Evaluating the Prevalence of Polyglutamine Repeat Expansions in Amyotrophic Lateral Sclerosis." *Neurology* 76 (24): 2062–65. https://doi.org/10.1212/WNL.0b013e31821f4447.

Leonidas, Demetres D., Robert Shapiro, Simon C. Allen, Gowtham V. Subbarao, Kasinadar Veluraja, and K. Ravi Acharya. 1999. "Refined Crystal Structures of Native Human Angiogenin and Two Active Site Variants: Implications for the Unique Functional Properties of an Enzyme Involved in Neovascularisation during Tumour Growth." *Journal of Molecular Biology* 285 (3): 1209–33. https://doi.org/10.1006/jmbi.1998.2378.

Li, Wanzhen, Zhen Liu, Weining Sun, Yanchun Yuan, Yiting Hu, Jie Ni, Bin Jiao, et al. 2020. "Mutation Analysis of GLT8D1 and ARPP21 Genes in Amyotrophic Lateral Sclerosis Patients from Mainland China." *Neurobiology of Aging* 85: 156.e1-156.e4. https://doi.org/10.1016/j.neurobiolaging.2019.09.013.

López-Gómez, Juan J., María D. Ballesteros-Pomar, Beatriz Torres-Torres, Begoña Pintor-De la Maza, María A. Penacho-Lázaro, José M. Palacio-Mures, Cristina Abreu-Padín, Irene Sanz-Gallego, and Daniel A. De Luis-Román. 2021. "Impact of Percutaneous Endoscopic Gastrostomy (Peg) on the Evolution of Disease in Patients with Amyotrophic Lateral Sclerosis (Als)." *Nutrients* 13 (8). https://doi.org/10.3390/nu13082765.

Ludolph, Albert, Vivian Drory, Orla Hardiman, Imaharu Nakano, John Ravits, Wim Robberecht, and Jeremy Shefner. 2015. "A Revision of the El Escorial Criteria - 2015." *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, 2015. https://doi.org/10.3109/21678421.2015.1049183.

Majounie, Elisa, Alan E. Renton, Kin Mok, Elise G.P. Dopper, Adrian Waite, Sara Rollinson, Adriano Chiò, et al. 2012. "Frequency of the C9orf72 Hexanucleotide Repeat Expansion in Patients with Amyotrophic Lateral Sclerosis and Frontotemporal Dementia: A Cross-Sectional Study." *The Lancet Neurology* 11 (4): 323–30.

https://doi.org/10.1016/S1474-4422(12)70043-1.

Masè, G., S. Ros, A. Gemma, L. Bonfigli, N. Carraro, G. Cazzato, M. Rolfo, et al. 2001. “ALS with Variable Phenotypes in a Six-Generation Family Caused by Leu144phe Mutation in the SOD1 Gene.” *Journal of the Neurological Sciences* 191 (1–2): 11–18. https://doi.org/10.1016/S0022-510X(01)00625-6.

Masrori, P., and P. Van Damme. 2020. “Amyotrophic Lateral Sclerosis: A Clinical Review.” *European Journal of Neurology* 27 (10): 1918–29. https://doi.org/10.1111/ene.14393.

Maystadt, Isabelle, René Rezsöhazy, Martine Barkats, Sandra Duque, Pascal Vannuffel, Sophie Remacle, Barbara Lambert, et al. 2007. “The Nuclear Factor KB-Activator Gene PLEKHG5 Is Mutated in a Form of Autosomal Recessive Lower Motor Neuron Disease with Childhood Onset.” *American Journal of Human Genetics* 81 (1): 67–76. https://doi.org/10.1086/518900.

Mazón, Miguel, Juan Francisco Vázquez Costa, Amadeo Ten-Esteve, and Luis Martí-Bonmatí. 2018. “Imaging Biomarkers for the Diagnosis and Prognosis of Neurodegenerative Diseases. The Example of Amyotrophic Lateral Sclerosis.” *Frontiers in Neuroscience* 12 (October): 1–19. https://doi.org/10.3389/fnins.2018.00784.

McCann, Emily P., Lyndal Henden, Jennifer A. Fifita, Katharine Y. Zhang, Natalie Grima, Denis C. Bauer, Sandrine Chan Moi Fat, et al. 2021. “Evidence for Polygenic and Oligogenic Basis of Australian Sporadic Amyotrophic Lateral Sclerosis.” *Journal of Medical Genetics* 58 (2): 87–95. https://doi.org/10.1136/jmedgenet-2020-106866.

Miller, Timothy M., Merit E. Cudkowicz, Angela Genge, Pamela J. Shaw, Gen Sobue, Robert C. Bucelli, Adriano Chiò, et al. 2022. “ Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS .” *New England Journal of Medicine* 387 (12): 1099–1110. https://doi.org/10.1056/nejmoa2204705.

Millul, A., E. Beghi, G. Logroscino, A. Micheli, E. Vitelli, and A. Zardi. 2005. “Survival of Patients with Amyotrophic Lateral Sclerosis in a Population-Based Registry.” *Neuroepidemiology* 25 (3): 114–19. https://doi.org/10.1159/000086353.

Mitchell, Jacqueline C., Philip McGoldrick, Caroline Vance, Tibor Hortobagyi, Jemeen Sreedharan, Boris Rogelj, Elizabeth L. Tudor, et al. 2013. “Overexpression of Human Wild-Type FUS Causes Progressive Motor Neuron Degeneration in an Age- and Dose-Dependent Fashion.” *Acta Neuropathologica* 125 (2): 273–88. https://doi.org/10.1007/s00401-012-1043-z.

Mitsumoto, Hiroshi, Robert J. Sliman, Irwin A. Schafer, Cary S. Sternick, Benjamin Kaufman, Asa Wilbourn, and Samuel J. Horwitz. 1985. “Motor Neuron Disease and Adult Hexosaminidase a Deficiency in Two Families: Evidence for Multisystem Degeneration.” *Annals of Neurology* 17 (4): 378–85. https://doi.org/10.1002/ana.410170413.

Mizielinska, Sarah, Sebastian Grönke, Teresa Niccoli, Charlotte E. Ridler, Emma L. Clayton, Anny Devoy, Thomas Moens, et al. 2014. “C9orf72 Repeat Expansions Cause Neurodegeneration in Drosophila through Arginine-Rich Proteins.” *Science* 345 (6201): 1192–94. https://doi.org/10.1126/science.1256800.

Moll, Tobias, Pamela J. Shaw, and Johnathan Cooper-Knock. 2020. “Disrupted Glycosylation of Lipids and Proteins Is a Cause of Neurodegeneration.” *Brain* 143 (5): 1332–40. https://doi.org/10.1093/brain/awz358.

Morelot-Panzini, Capucine, Gaëlle Bruneteau, and Jesus Gonzalez-Bermejo. 2019. “NIV in Amyotrophic Lateral Sclerosis: The ‘When’ and ‘How’ of the Matter.” *Respirology* 24 (6): 521–30. https://doi.org/10.1111/resp.13525.

Moroianu, Junona, and James F. Riordan. 1994. “Nuclear Translocation of Angiogenin in Proliferating Endothelial Cells Is Essential to Its Angiogenic Activity.” *Proceedings of the National Academy of Sciences of the United States of America* 91 (5): 1677–81. https://doi.org/10.1073/pnas.91.5.1677.

Nahm, Minyeop, Su Min Lim, Young Eun Kim, Jinseok Park, Min Young Noh, Sanggon Lee, Ju Eun Roh, et al. 2020. “ANXA11 Mutations in ALS Cause Dysregulation of Calcium Homeostasis and Stress Granule Dynamics.” *Science Translational Medicine* 12 (566): 1–16. https://doi.org/10.1126/scitranslmed.aax3993.

Nel, Melissa, Thandeka Mavundla, Kayleigh Gultig, Gerrit Botha, Nicola Mulder, Michael Benatar, Joanne Wu, et al. 2021. “Repeats Expansions in ATXN2, NOP56, NIPA1 and ATXN1 Are Not Associated with ALS in Africans.” *IBRO Neuroscience Reports* 10 (February): 130–35. https://doi.org/10.1016/j.ibneur.2021.02.002.

Nguyen, Dao K.H., Ravi Thombre, and Jiou Wang. 2019. “Autophagy as a Common Pathway in Amyotrophic Lateral Sclerosis.” *Neuroscience Letters* 697: 34–48. https://doi.org/10.1016/j.neulet.2018.04.006.

Nguyen, Hung Phuoc, Sara Van Mossevelde, Lubina Dillen, Jan L. De Bleecker, Matthieu Moisse, Philip Van Damme, Christine Van Broeckhoven, et al. 2018. “NEK1 Genetic Variability in a Belgian Cohort of ALS and ALS-FTD Patients.” *Neurobiology of Aging* 61: 255.e1-255.e7. https://doi.org/10.1016/j.neurobiolaging.2017.08.021.

Okada, S., M. L. Veath, J. Leroy, and J. S. O’Brien. 1971. “Ganglioside GM2 Storage Diseases: Hexosaminidase Deficiencies in Cultured Fibroblasts.” *American Journal of Human Genetics* 23 (1): 55–61.

Orlacchio, Antonio, Toshitaka Kawarai, Anna Maria Massaro, Peter H. St George-Hyslop, and Sandro Sorbi. 2000. “Absence of Linkage between Familial Amyotrophic Lateral Sclerosis and Copper Chaperone for the Superoxide Dismutase Gene Locus in Two Italian Pedigrees.” *Neuroscience Letters* 285 (2): 83–86. https://doi.org/10.1016/S0304-3940(00)01032-6.

Osmanovic, Alma, Maylin Widjaja, Alisa Förster, Julia Weder, Mike P. Wattjes, Inken Lange, Anastasia Sarikidi, et al. 2020. “SPG7 Mutations in Amyotrophic Lateral Sclerosis: A Genetic Link to Hereditary Spastic Paraplegia.” *Journal of Neurology* 267 (9): 2732–43. https://doi.org/10.1007/s00415-020-09861-w.

Özoğuz, Aslihan, Özgün Uyan, Güneş Birdal, Ceren Iskender, Ece Kartal, Suna Lahut, Özgür Ömür, et al. 2015. “The Distinct Genetic Pattern of ALS in Turkey and Novel Mutations.” *Neurobiology of Aging* 36 (4): 1764.e9-1764.e18. https://doi.org/10.1016/j.neurobiolaging.2014.12.032.

Padhi, Aditya K., Kamalika Banerjee, James Gomes, and Manidipa Banerjee. 2014. “Computational and Functional Characterization of Angiogenin Mutations, and Correlation with Amyotrophic Lateral Sclerosis.” *PLoS ONE* 9 (11). https://doi.org/10.1371/journal.pone.0111963.

Padhi, Aditya K., Hirdesh Kumar, Suhas V. Vasaikar, Bhyravabhotla Jayaram, and James Gomes. 2012. “Mechanisms of Loss of Functions of Human Angiogenin Variants Implicated in Amyotrophic Lateral Sclerosis.” *PLoS ONE* 7 (2): 1–20. https://doi.org/10.1371/journal.pone.0032479.

Pang, Weilun, and Fenghua Hu. 2021. “Cellular and Physiological Functions of C9ORF72 and Implications for ALS/FTD.” *Journal of Neurochemistry* 157 (3): 334–50. https://doi.org/10.1111/jnc.15255.

Patai, Roland, Bernát Nógrádi, József I. Engelhardt, and László Siklós. 2017. “Calcium in the Pathomechanism of Amyotrophic Lateral Sclerosis – Taking Center Stage?” *Biochemical and Biophysical Research Communications* 483 (4): 1031–39. https://doi.org/10.1016/j.bbrc.2016.08.089.

Peters, J. M., R. Barnes, L. Bennett, W. M. Gitomer, A. M. Bowcock, and A. Garg. 1998. “Localization of the Gene for Familial Partial Lipodystrophy (Dunnigan Variety) to Chromosome 1q21-22.” *Nature Genetics* 18 (3): 292–98. https://doi.org/10.1038/ng0398-292.

Pozzi, Laura, Fabiola Valenza, Lorena Mosca, Andrea Dal Mas, Teuta Domi, Alessandro Romano, Claudia Tarlarini, et al. 2017. “TBK1 Mutations in Italian Patients with Amyotrophic Lateral Sclerosis: Genetic and Functional Characterization.” *Journal of Neurology, Neurosurgery and Psychiatry* 88 (10): 869–75. https://doi.org/10.1136/jnnp-2017-316174.

Preux, P M, P Couratier, F Boutros-Toni, Salle J Y, F Tabaraud, P Bernet-Bernady, J M Vallat, and M Dumas. 1996. “Survival Prediction in Sporadic Amyotrophic Lateral Sclerosis. Age and Clinical Form at Onset Are Independent Risk Factors” 15 (3): 153–60. https://doi.org/10.1159/000109902.

Rahman, Shamima, and William C. Copeland. 2019. “POLG-Related Disorders and Their Neurological Manifestations.” *Nature Reviews Neurology* 15 (1): 40–52. https://doi.org/10.1038/s41582-018-0101-0.

Ratti, Antonia, Lucia Corrado, Barbara Castellotti, Roberto Del Bo, Isabella Fogh, Cristina Cereda, Cinzia Tiloca, et al. 2012. “C9ORF72 Repeat Expansion in a Large Italian ALS Cohort: Evidence of a Founder Effect.” *Neurobiology of Aging* 33 (10): 2528.e7-2528.e14. https://doi.org/10.1016/j.neurobiolaging.2012.06.008.

Reichert, Cadiele Oliana, Debora Levy, and Sergio P. Bydlowski. 2021. “Paraoxonase Role in Human Neurodegenerative Diseases.” *Antioxidants* 10 (1): 1–26. https://doi.org/10.3390/antiox10010011.

Renton, Alan E, Adriano Chiò, and Bryan J Traynor. 2014a. “State of Play in Amyotrophic Lateral Sclerosis Genetics.” *Nature Publishing Group* 17 (1): 17–23. https://doi.org/10.1038/nn.3584.

———. 2014b. “State of Play in Amyotrophic Lateral Sclerosis Genetics.” *Nat Neuroscience* 17 (1): 17–23. https://doi.org/10.1038/nn.3584.State.

Renton, Alan E, Elisa Majounie, Adrian Waite, Javier Simón-sánchez, Sara Rollinson, J Raphael Gibbs, Jennifer C Schymick, et al. 2012. “A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD.” *Neuron* 72 (2): 257–68. https://doi.org/10.1016/j.neuron.2011.09.010.A.

Ribeiro, João V., Juan A.C. Tamames, Nuno M.F.S.A. Cerqueira, Pedro A. Fernandes, and Maria J. Ramos. 2013. “Volarea - A Bioinformatics Tool to Calculate the Surface Area and the Volume of Molecular Systems.” *Chemical Biology and Drug Design* 82 (6): 743–55. https://doi.org/10.1111/cbdd.12197.

Richards, Sue, Nazneen Aziz, Sherri Bale, David Bick, Soma Das, Julie Gastier-Foster, Wayne W. Grody, et al. 2015. “Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.” *Genetics in Medicine* 17 (5): 405–24. https://doi.org/10.1038/gim.2015.30.

Rosen, Daniel R., Teepu Siddique, David Patterson, Denise A. Figlewicz, Peter Sapp, Afif Hentati, Deirdre Donaldson, et al. 1993. “Mutations in Cu/Zn Superoxide Dismutase Gene Are Associated with Familial Amyotrophic Lateral Sclerosis.” *Nature* 362 (6415): 59–62. https://doi.org/10.1038/362059a0.

Ross, Jay P., Claire S. Leblond, Sandra B. Laurent, Dan Spiegelman, Alexandre Dionne-Laporte, William Camu, Nicolas Dupré, Patrick A. Dion, and Guy A. Rouleau. 2020. “Oligogenicity, C9orf72 Expansion, and Variant Severity in ALS.” *Neurogenetics* 21 (3): 227–42. https://doi.org/10.1007/s10048-020-00612-7.

Rothstein, Jeffrey D. 2017. “Edaravone: A New Drug Approved for ALS.” *Cell* 171 (4): 725. https://doi.org/10.1016/j.cell.2017.10.011.

Sajko, Sara, Irina Grishkovskaya, Julius Kostan, Melissa Graewert, Kim Setiawan, Linda Trübestein, Korbinian Niedermüller, et al. 2020. *Structures of Three MORN Repeat Proteins and a Re-Evaluation of the Proposed Lipid-Binding Properties of MORN Repeats*. *PLoS ONE*. Vol. 15. https://doi.org/10.1371/journal.pone.0242677.

Sareen, Dhruv, Jacqueline G. O’Rourke, Pratap Meera, A. K.M.G. Muhammad, Sharday Grant, Megan Simpkinson, Shaughn Bell, et al. 2013. “Targeting RNA Foci in IPSC-Derived Motor Neurons from ALS Patients with a C9ORF72 Repeat Expansion.” *Science Translational Medicine* 5 (208). https://doi.org/10.1126/scitranslmed.3007529.

Sau, Daniela, Silvia De Biasi, Laura Vitellaro-Zuccarello, Patrizia Riso, Serena Guarnieri, Marisa Porrini, Silvia Simeoni, et al. 2007. “Mutation of SOD1 in ALS: A Gain of a Loss of Function.” *Human Molecular Genetics* 16 (13): 1604–18. https://doi.org/10.1093/hmg/ddm110.

Seibenhener, M. Lamar, Jeganathan Ramesh Babu, Thangiah Geetha, Hing C. Wong, N. Rama Krishna, and Marie W. Wooten. 2004. “Sequestosome 1/P62 Is a Polyubiquitin Chain Binding Protein Involved in Ubiquitin Proteasome Degradation.” *Molecular and Cellular Biology* 24 (18): 8055–68. https://doi.org/10.1128/mcb.24.18.8055-8068.2004.

Shefner, Jeremy M., Ammar Al-Chalabi, Mark R. Baker, Li Ying Cui, Mamede de Carvalho, Andrew Eisen, Julian Grosskreutz, et al. 2020. “A Proposal for New Diagnostic Criteria for ALS.” *Clinical Neurophysiology* 131 (8): 1975–78. https://doi.org/10.1016/j.clinph.2020.04.005.

Siintola, Eija, Meral Topcu, Nina Aula, Hannes Lohi, Berge A. Minassian, Andrew D. Paterson, Xiao Qing Liu, et al. 2007. “The Novel Neuronal Ceroid Lipofuscinosis Gene MFSD8 Encodes a Putative Lysosomal Transporter.” *American Journal of Human Genetics* 81 (1): 136–46. https://doi.org/10.1086/518902.

Silahtaroglu, Asli N., Karen Brondum-Nielsen, Ole Gredal, Lene Werdelin, Marios Panas, Michael B. Petersen, Niels Tommerup, and Zeynep Tümer. 2002. “Human CCS Gene: Genomic Organization and Exclusion as a Candidate for Amyotrophic Lateral Sclerosis (ALS).” *BMC Genetics* 3: 1–5. https://doi.org/10.1186/1471-2156-3-5.

Smith, Bradley N., Simon D. Topp, Claudia Fallini, Hideki Shibata, Han Jou Chen, Claire Troakes, Andrew King, et al. 2017. “Mutations in the Vesicular Trafficking Protein Annexin A11 Are Associated with Amyotrophic Lateral

Sclerosis Bradley N. Smith." *Science Translational Medicine* 9 (388): 1–16. https://doi.org/10.1126/scitranslmed.aad9157.

Son, Marjatta, Krishna Puttaparthi, Hibiki Kawamata, Bhagya Rajendran, Philip J. Boyer, Giovanni Manfredi, and Jeffrey L. Elliott. 2007. "Overexpression of CCS in G93A-SOD1 Mice Leads to Accelerated Neurological Deficits with Severe Mitochondrial Pathology." *Proceedings of the National Academy of Sciences of the United States of America* 104 (14): 6072–77. https://doi.org/10.1073/pnas.0610923104.

Strydom, Daniel J., James W. Fett, Roy R. Lobb, Edward M. Alderman, J. Lemuel Bethune, James F. Riordan, and Bert L. Vallee. 1985. "Amino Acid Sequence of Human Tumor Derived Angiogenic." *Biochemistry* 24 (20): 5486–94. https://doi.org/10.1021/bi00341a031.

Suh, Eun Ran, Kaitlyn Grando, and Vivianna M. Van Deerlin. 2018. "Validation of a Long-Read PCR Assay for Sensitive Detection and Sizing of C9orf72 Hexanucleotide Repeat Expansions." *Journal of Molecular Diagnostics* 20 (6): 871–82. https://doi.org/10.1016/j.jmoldx.2018.07.001.

Sztriha, L, Z Vekerdy, S Túri, C Panzeri, N Bresolin, MT Bassi, E Endreffy, N Szabó, R Kálmánchey, and C Baschirotto. 2009. "First Case of Compound Heterozygosity in ALS2 Gene in Infantile-Onset Ascending Spastic Paralysis with Bulbar Involvement." *Clinical Genetics* 73 (6): 591–93. https://doi.org/10.1111/j.1399-0004.2008.00993.x.

Tazelaar, Gijs HP, Steven Boeynaems, Mathias De Decker, Joke JFA van Vugt, Lindy Kool, H Stephan Goedee, Russell L McLaughlin, et al. 2020. "ATXN1 Repeat Expansions Confer Risk for Amyotrophic Lateral Sclerosis and Contribute to TDP-43 Mislocalization Running Title: ATXN1 Repeat Expansions in ALS Authors and Affiliations." *Brain Communications* 11 (2020): 10. https://academic.oup.com/braincomms/advance-article-abstract/doi/10.1093/braincomms/fcaa064/5840473.

Thiyagarajan, Nethaji, Ross Ferguson, Vasanta Subramanian, and K. Ravi Acharya. 2012. "Structural and Molecular Insights into the Mechanism of Action of Human Angiogenin-ALS Variants in Neurons." *Nature Communications* 3: 1114–21. https://doi.org/10.1038/ncomms2126.

Traynor, B. J., M. B. Codd, B. Corr, C. Forde, E. Frost, and Orla Hardiman. 2000. "ALS Mimic Syndromes: A Population-Based Study." *Archives of Neurology* 57 (1): 109–13.

Tripolszki, Kornélia, Bernadett Csányi, Dóra Nagy, Antonia Ratti, Cinzia Tiloca, Vincenzo Silani, Éva Kereszty, et al. 2017. "Genetic Analysis of the SOD1 and C9ORF72 Genes in Hungarian Patients with Amyotrophic Lateral Sclerosis." *Neurobiology of Aging* 53: 195.e1-195.e5. https://doi.org/10.1016/j.neurobiolaging.2017.01.016.

Tripolszki, Kornélia, Dóra Török, David Goudenége, Katalin Farkas, Adrienn Sulák, Nóra Török, József I. Engelhardt, et al. 2017. "High-Throughput Sequencing Revealed a Novel SETX Mutation in a Hungarian Patient with Amyotrophic Lateral Sclerosis" 7 (January): 1–7. https://doi.org/10.1002/brb3.669.

Tsuiji, Hitomi, Yohei Iguchi, Asako Furuya, Ayane Kataoka, Hiroyuki Hatsuta, Naoki Atsuta, Fumiaki Tanaka, et al. 2013. "Spliceosome Integrity Is Defective in the Motor Neuron Diseases ALS and SMA." *EMBO Molecular Medicine* 5 (2): 221–34. https://doi.org/10.1002/emmm.201202303.

Turner, Martin R. 2022. "Diagnosing ALS: The Gold Coast Criteria and the Role of EMG." *Practical Neurology*, 176–78. https://doi.org/10.1136/practneurol-2021-003256.

Verde, Federico, Markus Otto, and Vincenzo Silani. 2021. "Neurofilament Light Chain as Biomarker for Amyotrophic Lateral Sclerosis and Frontotemporal Dementia." *Frontiers in Neuroscience* 15 (June). https://doi.org/10.3389/fnins.2021.679199.

Volkening, Kathryn, Sali M.K. Farhan, Jessica Kao, Cheryl Leystra-Lantz, Lee Cyn Ang, Adam McIntyre, Jian Wang, Robert A. Hegele, and Michael J. Strong. 2021. "Evidence of Synergism among Three Genetic Variants in a Patient with LMNA-Related Lipodystrophy and Amyotrophic Lateral Sclerosis Leading to a Remarkable Nuclear Phenotype." *Molecular and Cellular Biochemistry* 476 (7): 2633–50. https://doi.org/10.1007/s11010-021-04103-7.

Wahiduzzaman, Vijay Kumar, Farah Anjum, Alaa Shafie, Abdelbaset Mohamed Elasbali, Asimul Islam, Faizan Ahmad, and Md Imtaiyaz Hassan. 2021. "Delineating the Aggregation-Prone Hotspot Regions (Peptides) in the Human Cu/Zn

Superoxide Dismutase 1." *ACS Omega* 6 (49): 33985–94. https://doi.org/10.1021/acsomega.1c05321.

Walsh, Matthew J., Johnathan Cooper-Knock, Jennifer E. Dodd, Matthew J. Stopford, Simeon R. Mihaylov, Janine Kirby, Pamela J. Shaw, and Guillaume M. Hautbergue. 2015. "Invited Review: Decoding the Pathophysiological Mechanisms That Underlie RNA Dysregulation in Neurodegenerative Disorders: A Review of the Current State of the Art." *Neuropathology and Applied Neurobiology* 41 (2): 109–34. https://doi.org/10.1111/nan.12187.

Wang, Kai, Mingyao Li, and Hakon Hakonarson. 2010. "ANNOVAR: Functional Annotation of Genetic Variants from High-Throughput Sequencing Data." *Nucleic Acids Research* 38 (16): 1–7. https://doi.org/10.1093/nar/gkq603.

Woerner, Andreas C., Frédéric Frottin, Daniel Hornburg, Li R. Feng, Felix Meissner, Maria Patra, Jörg Tatzelt, et al. 2016. "Cytoplasmic Protein Aggregates Interfere with Nucleocytoplasmic Transport of Protein and RNA." *Science* 351 (6269): 173–76. https://doi.org/10.1126/science.aad2033.

Wu, David, Wenhao Yu, Hiroko Kishikawa, Rebecca D Folkerth, a John Iafrate, Yiping Shen, Winnie Xin, and Katherine Sims. 2009. "Angiogenin Loss-of-Function Mutations in Amyotrophic Lateral Sclerosis." *Annals of Neurology* 62 (6): 609–17. https://doi.org/10.1002/ana.21221.Angiogenin.

Yamaguchi, Atsushi, and Keisuke Takanashi. 2016. "FUS Interacts with Nuclear Matrix-Associated Protein SAFB1 as Well as Matrin3 to Regulate Splicing and Ligand-Mediated Transcription." *Scientific Reports* 6 (October): 1–14. https://doi.org/10.1038/srep35195.

Yilmaz, Rüstem, Torsten Grehl, Lukas Eckrich, Ines Marschalkowski, Kanchi Weishaupt, Ivan Valkadinov, Melita Simic, et al. 2023. "Frequency of C9orf72 and SOD1 Mutations in 302 Sporadic ALS Patients from Three German ALS Centers." *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration* 24 (5–6): 414–19. https://doi.org/10.1080/21678421.2023.2165946.

Yoshino, Hiide. 2019. "Edaravone for the Treatment of Amyotrophic Lateral Sclerosis." *Expert Review of Neurotherapeutics* 19 (3): 185–93. https://doi.org/10.1080/14737175.2019.1581610.

Zee, Julie van der, Ilse Gijselinck, Lubina Dillen, Tim Van Langenhove, Jessie Theuns, Sebastiaan Engelborghs, Stéphanie Philtjens, et al. 2013. "A Pan-European Study of the C9orf72 Repeat Associated with FTLD: Geographic Prevalence, Genomic Instability, and Intermediate Repeats." *Human Mutation* 34 (2): 363–73. https://doi.org/10.1002/humu.22244.

Zhan, Yiqiang, and Fang Fang. 2019. "Smoking and Amyotrophic Lateral Sclerosis: A Mendelian Randomization Study." *Annals of Neurology* 85 (4): 482–84. https://doi.org/10.1002/ana.25443.

Zhang, Katharine Y., Shu Yang, Sadaf T. Warraich, and Ian P. Blair. 2014. "Ubiquilin 2: A Component of the Ubiquitin-Proteasome System with an Emerging Role in Neurodegeneration." *International Journal of Biochemistry and Cell Biology* 50 (1): 123–26. https://doi.org/10.1016/j.biocel.2014.02.018.

Zou, Zhang Yu, Xin Ning Wang, Ming Sheng Liu, Qin Sun, Xiao Guang Li, Li Ying Cui, and Jiming Kong. 2012. "Identification of a Novel Missense Mutation in Angiogenin in a Chinese Amyotrophic Lateral Sclerosis Cohort." *Amyotrophic Lateral Sclerosis* 13 (3): 270–75. https://doi.org/10.3109/17482968.2011.643900.

Zou, Zhang Yu, Zhi Rui Zhou, Chun Hui Che, Chang Yun Liu, Rao Li He, and Hua Pin Huang. 2017. "Genetic Epidemiology of Amyotrophic Lateral Sclerosis: A Systematic Review and Meta-Analysis." *Journal of Neurology, Neurosurgery and Psychiatry* 88 (7): 540–49. https://doi.org/10.1136/jnnp-2016-315018.

Zuo, Xinxin, Jie Zhou, Yinming Li, Kai Wu, Zonggui Chen, Zhiwei Luo, Xiaorong Zhang, et al. 2021. "TDP-43 Aggregation Induced by Oxidative Stress Causes Global Mitochondrial Imbalance in ALS." *Nature Structural and Molecular Biology* 28 (2): 132–42. https://doi.org/10.1038/s41594-020-00537-7.