The Molecular Landscape of ALS in Turkey:

A Multifaceted Approach to the Complex Genetics of ALS

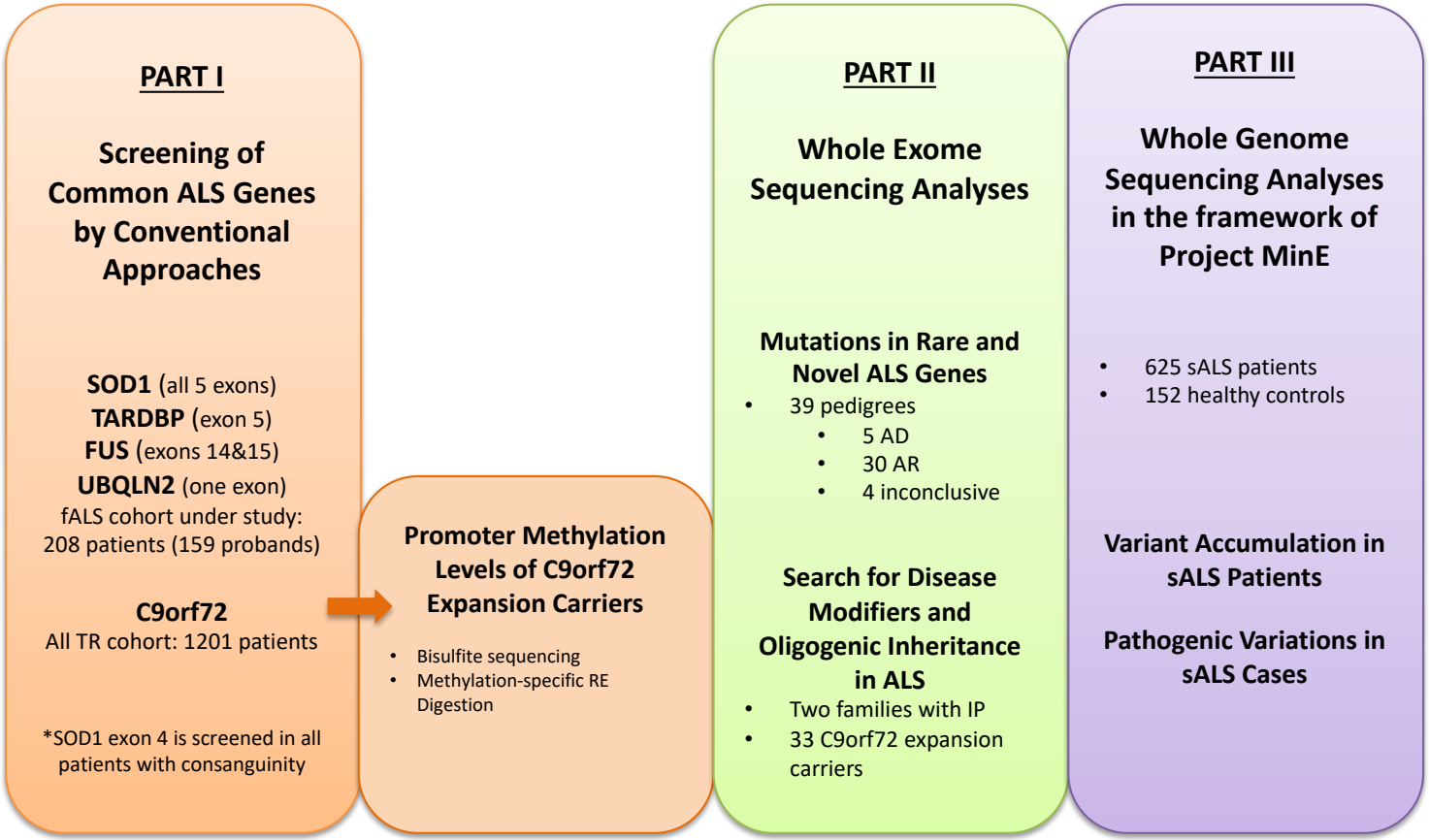
PhD Thesis Defense

Ceren İskender Tunca, MSc

October 10th, 2018

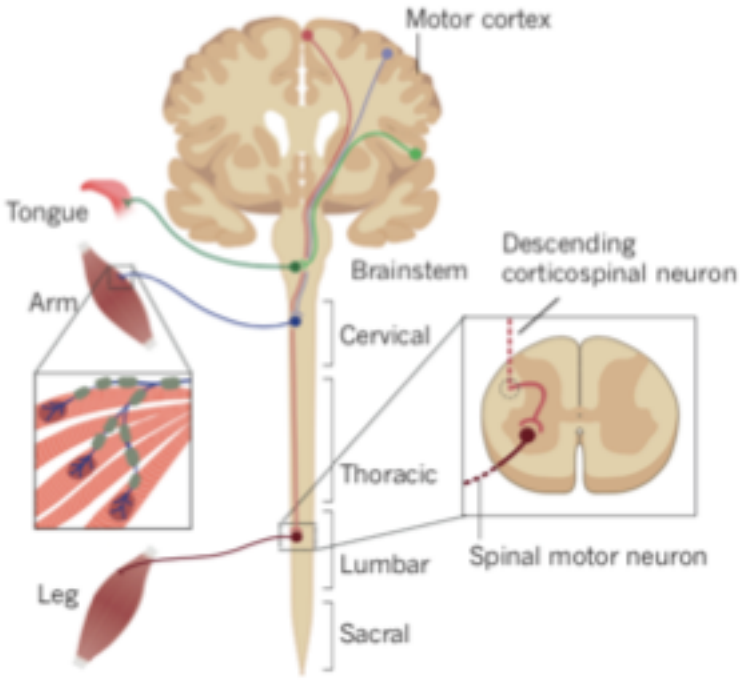
Outline

- ALS as a Complex Disease and Aim of the Thesis
- Strategy of the Thesis



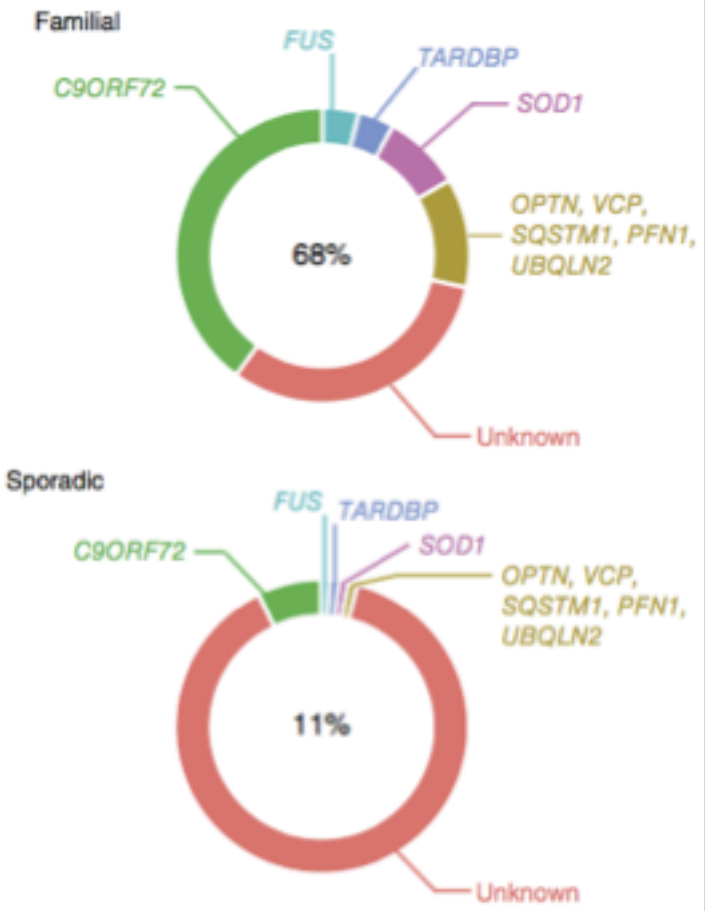
ALS: the hard facts

- 3rd most common NDD
- Incidence 2/100.000/ year
- Prevalance 4/100.000/ year
- Average age of onset 55-60
  - Juvenile cases with atypical symptoms
- Paralysis of motor function
  - Muscle wasting
  - Respiratory failure
- 2/3 spinal-onset, 1/3 bulbar-onset
- 2-5 years of survival
- fALS and sALS are clinically indistinguishable

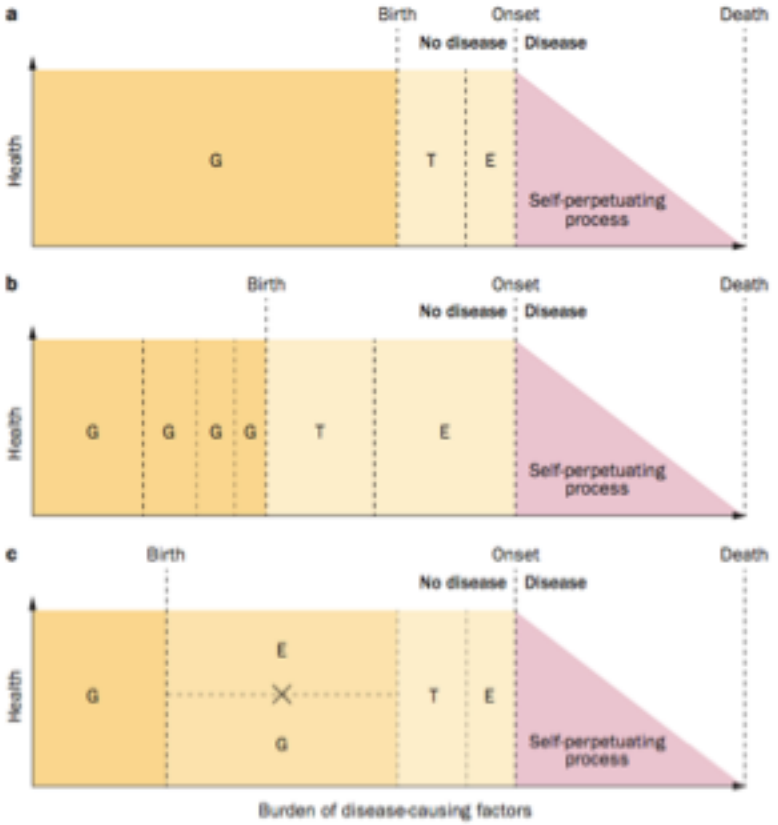


Taylor, Brown & Cleveland, *Nature Reviews*, 2016

Genetics of fALS and sALS



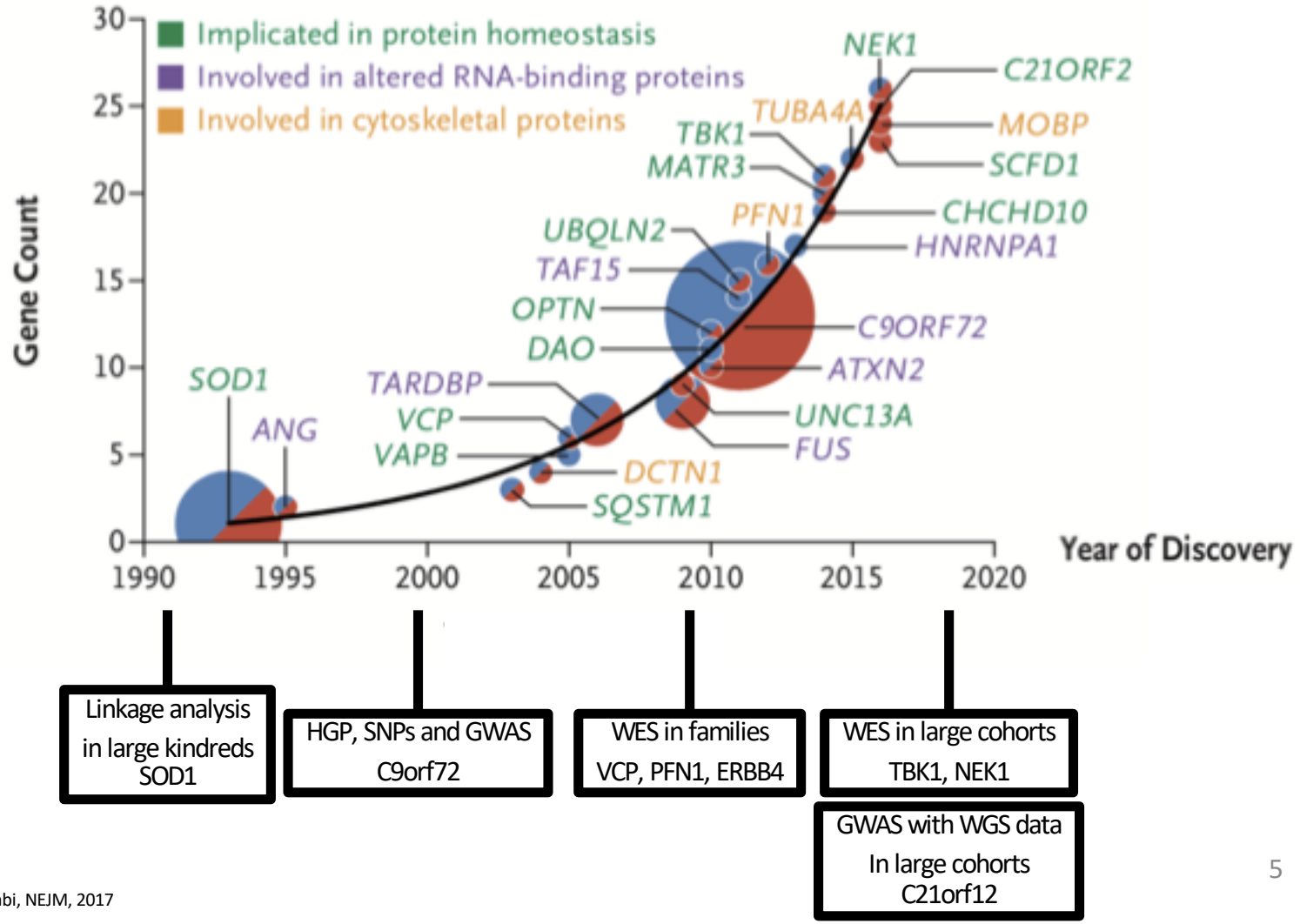
Renton, Chio & Traynor, *Nature Neuroscience*, 2013



Al-Chalabi & Hardiman, *Nature Reviews*, 2013

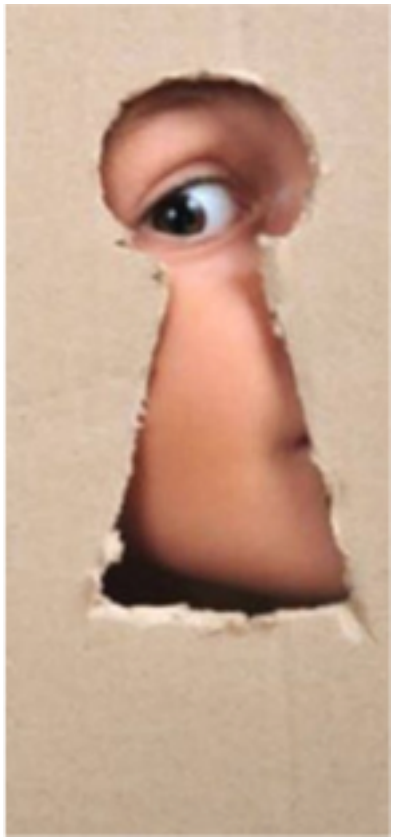
- Atxn-2 as inherited
- Epigenetics might be environmental

Gene Discovery in ALS and Impact of Next Generation Sequencing



adapted from Brown & Al-Chalabi, NEJM, 2017

Aim



Looking through the keyhole of genetics, this thesis aims to unravel the genetic factors that are either **disease-causing** or **disease-modifying** using conventional and next generation sequencing approaches, in familial ALS as well as in **sporadic disease**.

Clinical and Demographic Characteristics of the Cohort Under Study

		Total ALS	fALS	sALS
#	probands	722	159	563
	family members	49	49	-
	male/female	1.5	1.1	1.5
	consanguinity	139	40	99
	dementia	26	11	15
Site of onset	limb	515	121	394
	bulbar	131	29	102
	limb+bulbar	51	6	45
	unknown	25	3	22
Age of onset	juvenile (<25yrs)	61	20	41
	middle (25-45 yrs)	165	44	121
	late (>45 yrs)	480	94	386
	unkonwn	16	1	15
Mean age of onset	total ±SD	51±14,9	48±16,2	52±14,5

High percentage of fALS: 22%

- large kindreds
- close relationships

High consanguinity rates:

- 25% in fALS
- 17% in sALS

The younger AO of fALS expected

Gender bias: decreases when calculated in age-matched manner

- 34% AO<50, 44%, AO≥50
- male:female ratio decreases to 1.2 in older patients

Strategy Overview

PART I

Screening of Common ALS Genes by Conventional Approaches

SOD1 (all 5 exons)
TARDBP (exon 5)
FUS (exons 14&15)
UBQLN2 (one exon)
fALS cohort under study: 208 patients (159 probands)

C9orf72
All TR cohort: 1201 patients

*SOD1 exon 4 is screened in all patients with consanguinity

Promoter Methylation Levels of C9orf72 Expansion Carriers

- Bisulfite sequencing
- Methylation-specific RE Digestion

PART II

Whole Exome Sequencing Analyses

Mutations in Rare and Novel ALS Genes

- 39 pedigrees
  - 5 AD
  - 30 AR
  - 4 inconclusive

Search for Disease Modifiers and Oligogenic Inheritance in ALS

- Two families with IP
- 33 C9orf72 expansion carriers

PART III

Whole Genome Sequencing Analyses in the framework of Project MinE

- 625 sALS patients
- 152 healthy controls

Variant Accumulation in sALS Patients

Pathogenic Variations in sALS Cases

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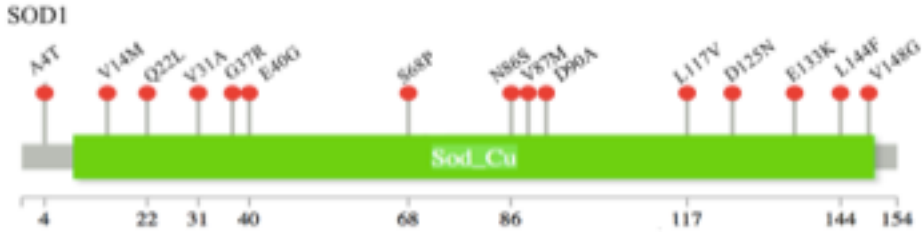
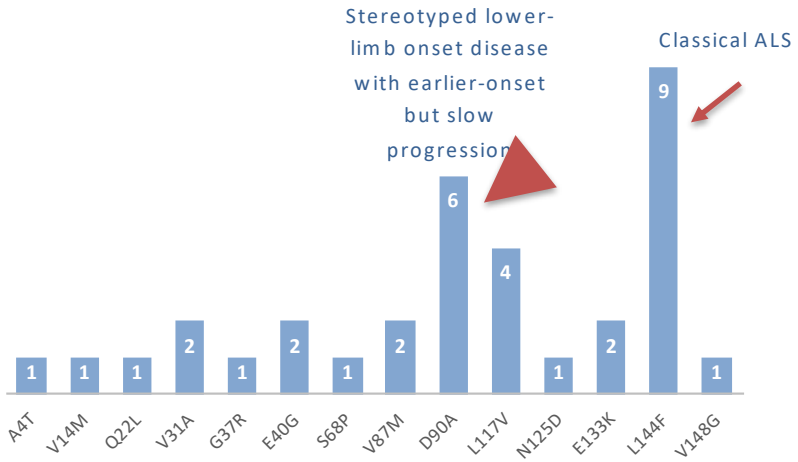
PART I

SOD1 in Our Cohort

15% of fALS, 14 different mutations in 35 cases and 9 affected family members

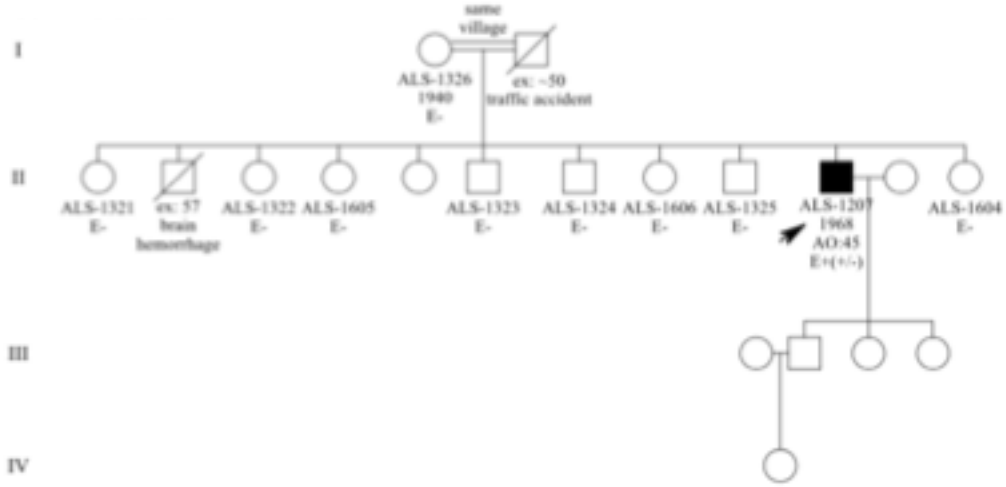




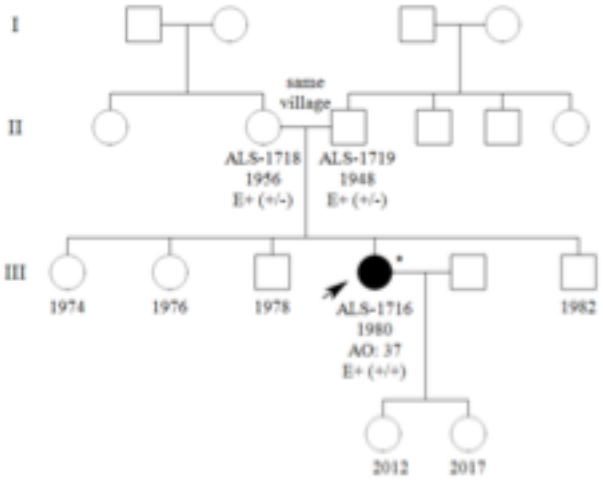
PART I

Three Rare SOD1 Mutations with Distinct Inheritance Patterns

SOD1-Asn86Ser (het)

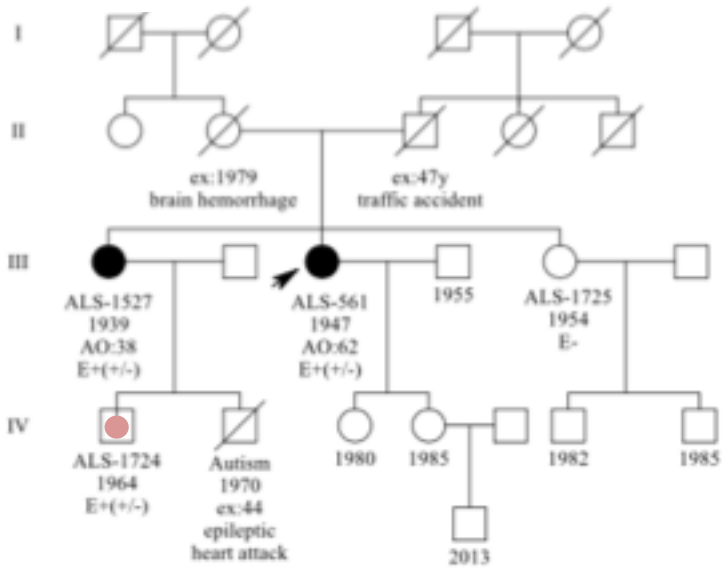


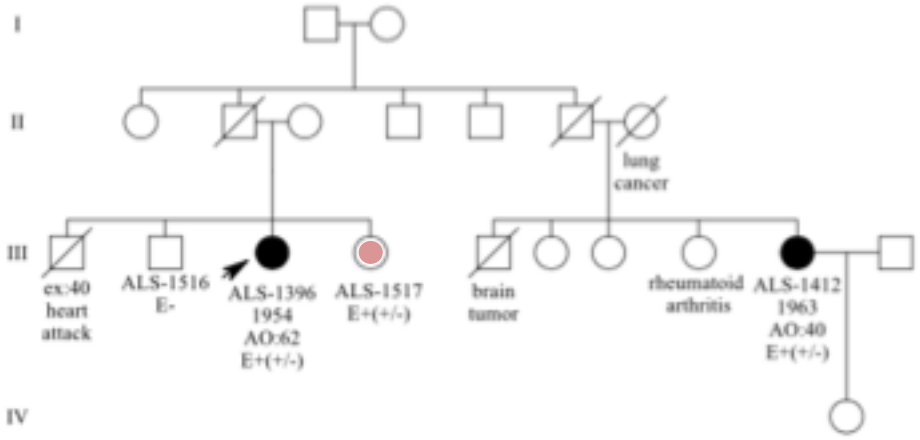
SOD1-Glu133Lys (hom)

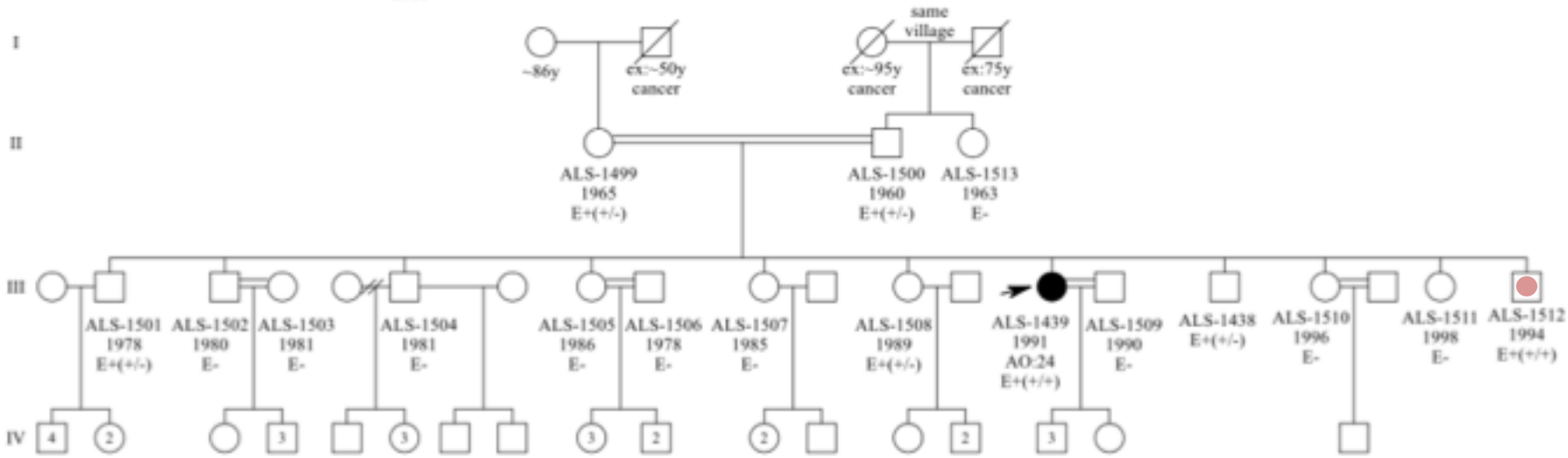


PART I

SOD1-L117V



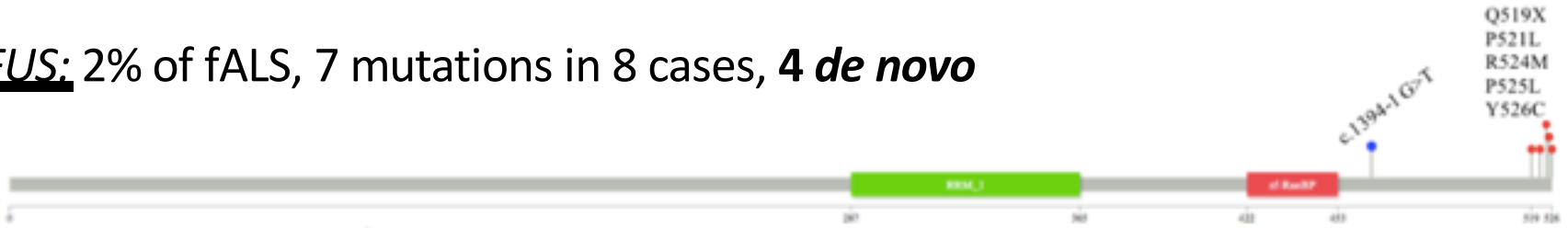




TARDBP and *FUS:* RNA-processing in ALS (2008)

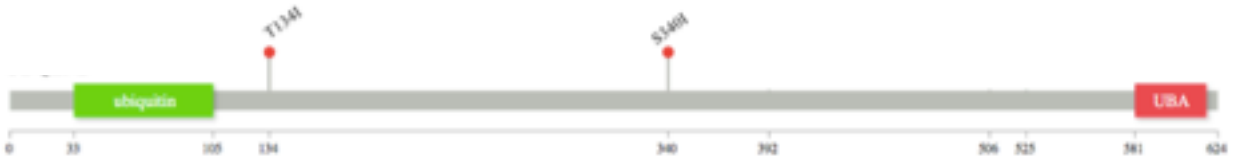
TARDBP: 3% of fALS, 5 mutations in 7 cases

FUS: 2% of fALS, 7 mutations in 8 cases, **4 *de novo***



Juvenile disease in 41 isolated/sporadic cases
17 had consanguineous parents
Aggressive disease in *de novo* mutation carriers

UBQLN2 *(protein homeostasis): 2* VUS



PART I

C9orf72 Hexanucleotide Repeat Expansion

- The non-coding GGGGCC repeat expansion is located in the promoter region of the gene
  - Pathogenic over 30
  - Expansion is usually more than 100 or even 1000
  - Anticipation is not shown

- Chr 9p21 Locus was determined via linkage studies but the repeat is detected with RP-PCR
- The most common cause of ALS and Frontotemporal dementia (FTD)
  - Different neuronal populations
  - First repeat expansion mutation for both diseases
- Identified in several other NDDs, but very rare

PART I

C9orf72 in Turkish ALS Cases

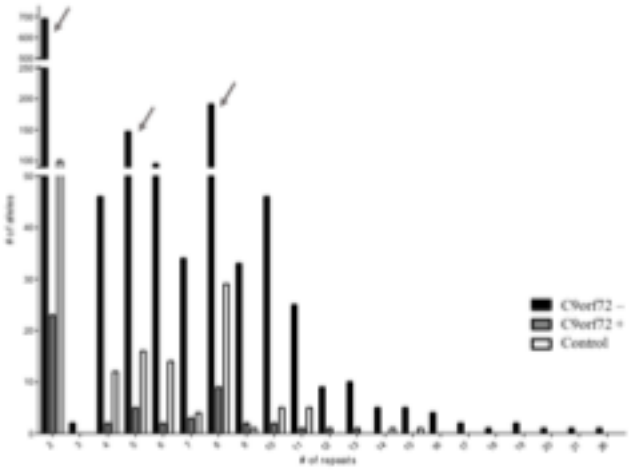
33 families with 39 affected individuals, 5 yet asymptomatic gene carriers
32 sporadic cases

16% of all fALS and 3% of all sALS (much lower than European populations)

		Total ALS	fALS	sALS	FTD
#	probands	65	33	32	-
	family members	6+ 5 assymptomatic	6	-	2
	male:female		1,3	1,7	
SO	limb	39	22	21	-
	bulbar	18	13	5	-
	limb+bulbar	5	1	3	-
	unknown	6	3	3	-
dementia		14	6	6	2
AO	juvenile (<25yrs)	1	1	-	-
	middle (25-45 yrs)	9	5	4	-
	late (>45 yrs)	61	33	28	2
	range (years)	32-80	32-80	40-71	57-59
MAO	total ±SD	54±10,9	55±9,6	53±8,4	-

#: numbers; AO: age of onset; MAO: mean age of onset; SO: site of onset.

Mean AO represents sALS
27% bulbar compared to 18% in the cohort



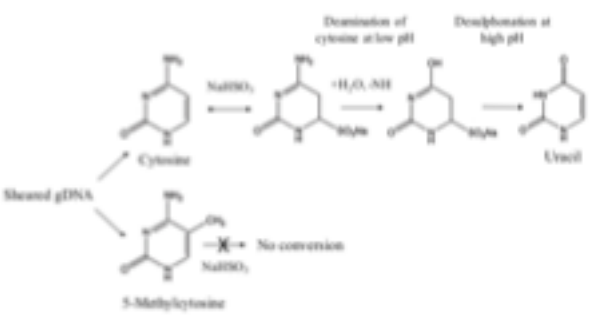
2, 5 and 8 repeats are common for the non-expanded allele of the *C9orf72* gene in ALS cases with and without the expansion and in controls.

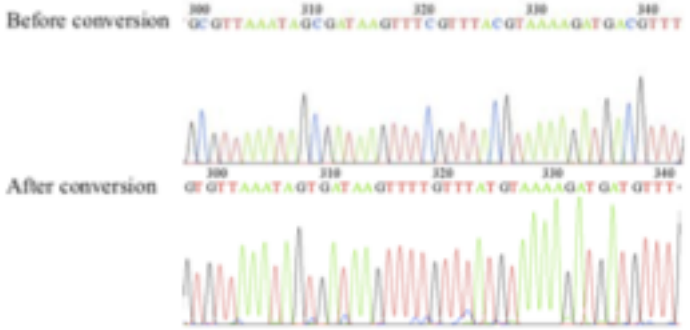
PART I

C9orf72 Promoter Hypermethylation

- DNA hypermethylation is common in repeat expansion disorders
  - FRDA, Fragile X, DM
- Promoter hypermethylation and histone trimethylation (H3K9, H3K27, H3K79, H4K20) is associated with C9orf72-based ALS and FTD, resulting in decreased mRNA levels
- Effect of hypermethylation on C9-mechanism
  - Reduction in 50% of the C9orf72 expression can explain LOF
  - Protective: decrease in mRNA transcript leads to less toxicity based on GOF

BST-PCR





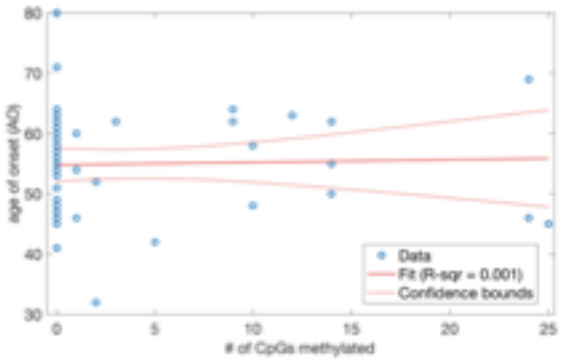
PART I

Promoter Hypermethylation is Evident in TR C9-Cases

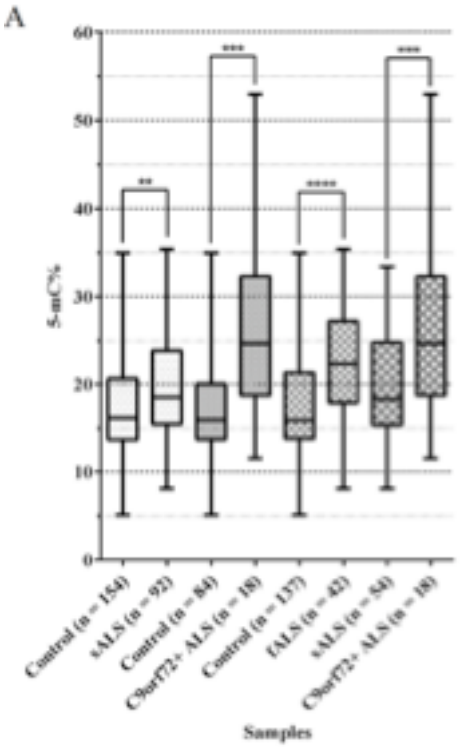
Bisulfite sequencing assay revealed a significant increase in promoter hypermethylation for the expansion carriers.

	ALS expanded		Controls	
Methylation level	# of Individuals	Frequency	# of Individuals	Frequency
No Methylation (0)	34	0,65	29	0,94
Low Methylation (1-2)	5	0,10	2	0,06
High Methylation (3-26)	13	0,25	0	0
Total	52	-	31	-
p-value (high methylation)	0.0023			

No correlation between age of onset and the number of methylated CpG sites



Higher global methylation levels detected in fALS/sALS cases (Elisa)



Hamzeiy, Tunca et al., *Neurodegenerative Disease*, 2018

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Whole Exome Sequencing (WES) Pipeline of NDAL

Alignment of raw reads
- BWA (Burrows Wheeler Algorithm)
- Reference: Human genome b37 plus the decoy

Variant calling
- GATK (Genome Analysis Toolkit)
- Low quality variants are filtered

Annotation
- ANNOVAR

Primary Analysis

Variant visualization & Filtering

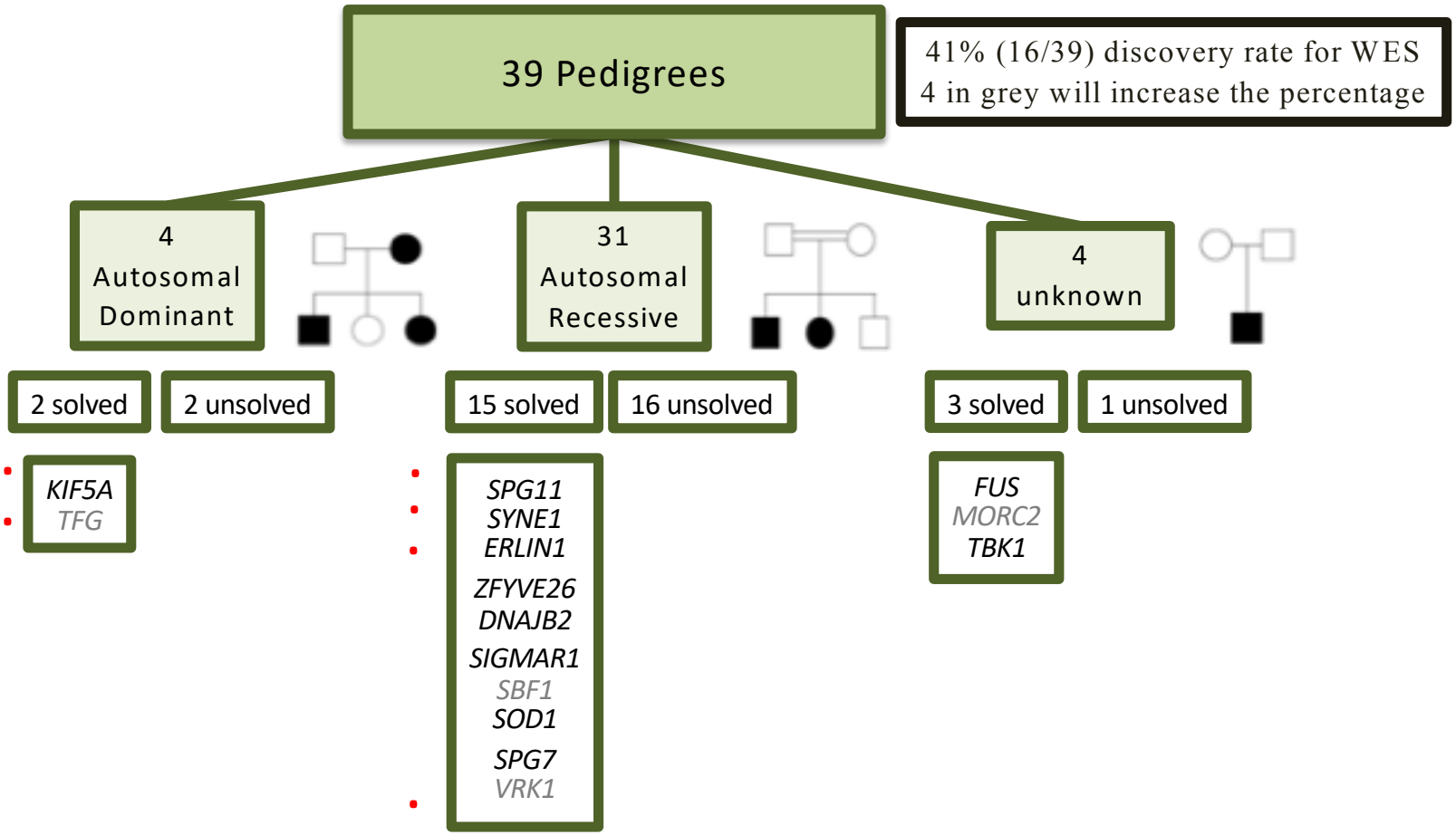
VarSifter Filtering

- Exonic nonsynonymous, splicing variants
- Mode of inheritance
  - Recessive variants (homozygous in cases, heterozygous in controls)
  - Heterozygous variants in dominant cases
  - de novo variants
- 1000G& ESP6500 MAF
  - (Multiple Allele Frequency > 0,01)
- Our in-house exome database of 420 samples

Variant & Gene Prioritization

PART II

WES in ALS and non-ALS MNDs



PART II

Variants Identified in Cases Subjected to WES

Family No	Age of Onset	Consanguinity	Family History	Inheritance	Clinical Diagnosis	Gene	Zygosity	OMIM
Family 1	50	no	yes	AD	ALS	KIF5A	Het	ALS, SPG10
Family 2	20	no	no	inconclusive	ALS	TBK1	Het	ALS
Family 3	17	no	no	inconclusive	ALS	FUS	*de novo*	ALS
Family 4	23	yes	yes	AR	ALS	SPG11	Hom	ARJALS, ARHSP, CMT2X
Family 5	14	yes	yes	AR	ALS	SPG11	Hom	
Family 6	13	yes	no	AR	HSP	SPG11	Hom	
Family 7	23	yes	no	AR	HSP	SPG11	Hom	
Family 8	21	yes	yes	AR	ALS	SYNE1	Hom	ARCA1, ALS
Family 9	17	yes	yes	AR	ALS	SYNE1	Hom	ARCA1, ALS
Family 10	15	yes	yes	AR	ALS	ERLIN1	Hom	SPG62, ALS
Family 11	17	yes	no	AR	ALS	ZFYVE26	Hom	SPG15
Family 12	22	yes	no	AR	ALS	DNAJB2	Hom	Distal SMA
Family 13	17	yes	no	AR	ALS	SIGMAR1	Hom	ALS, SMA
Family 14	childhood	yes	no	AR	ALS/CMT	SBF1	Hom	CMT4B3
Family 15	37	yes	no	AR	ALS	SOD1	Hom	ALS
Family 16	36	yes	no	AR	HSP	SPG7	Hom	SPG7
Family 17	37	yes	no	AR	ALS/HSP	SPG7	Hom	SPG7
Family 18	22	yes	yes	AR	ALS	VRK1	Hom	PCH1A
Family 19	47	no	yes	AD	MND/Sensory Neuropathy	TFG	Het	HMSN Okinawa type
Family 20	21	no	no	inconclusive	MND	MORC2	Het	CMT2Z

M:male, F:female, AD:autosomal dominant, AR:autosomal recessive

PART II

n novel

Family 1, *KIF5A*, c.3005A>G, p.(Asp1002Gly)[n]

Index (ALS-1539):

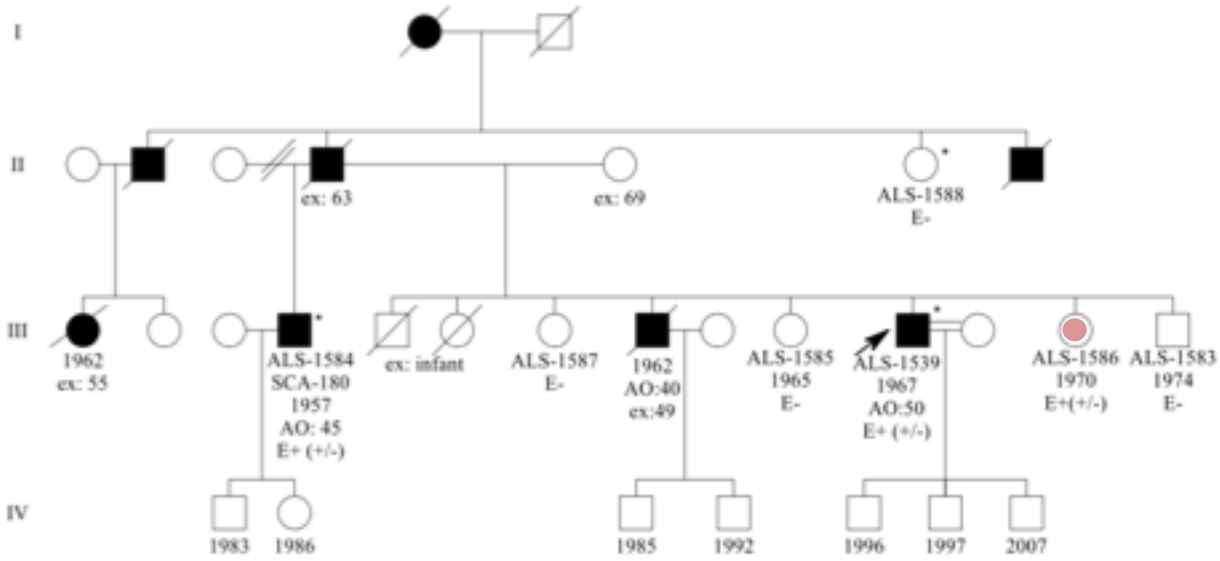
- weakness in his upper limbs at the age of 50, followed by bulbar involvement.
- amyotrophy, muscle atrophy, fasciculations and spasticity.
- motor deficit, hyperactive reflexes in the lower limbs, positive Hoffman's sign and plantar reflex.

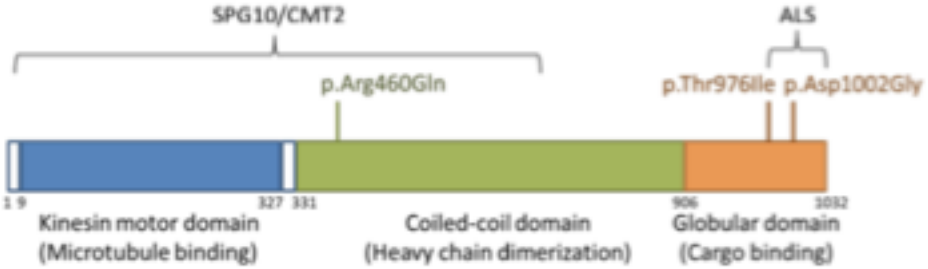
Family history: several affected members

Suspected Inheritance: AD

WES: 3 individuals (*)

Result: novel heterozygous change in the KIF5A





PART II

Family 19, *TFG*, c.854C>T, p.(Pro285Leu)

Index (ALS-731):

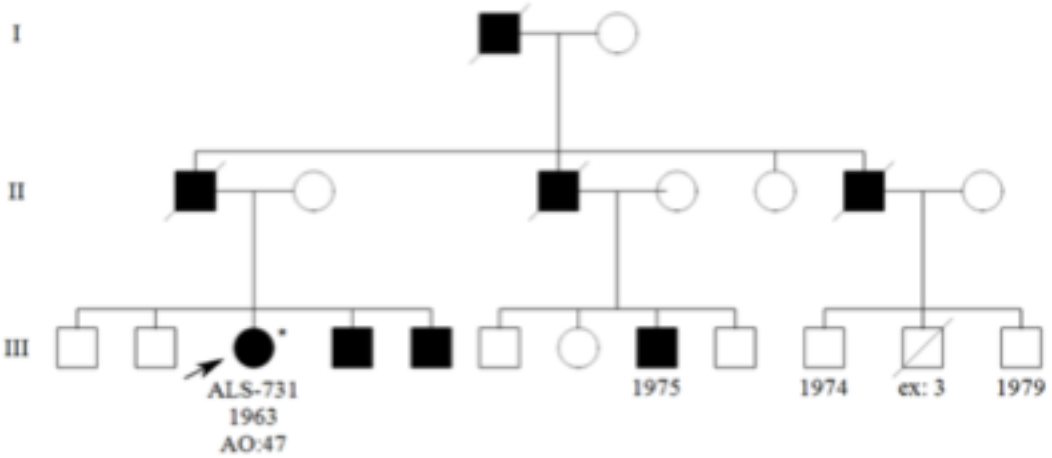
- Diagnosed with ALS with accompanying sensory neuropathy symptoms

Family history: several affected members with 10-15 years of survival

Suspected Inheritance: AD

WES: only index

Result: well-characterized heterozygous variation associated with HSMN-Okinawa type



TFG is involved in ER trafficking

Common Japanese variant: fasciculations, proximal muscle weakness, atrophy and sensory involvement

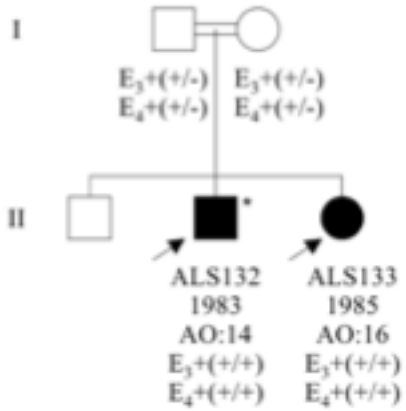
Same variant in Korean patients: more severe and progressive disease

TR haplotype should be investigated

PART II

Families with Truncating *SPG11* Mutations

Family 5, *SPG11,* c.2250delT, p.(Phe750Leufs*3)[n]



Index (ALS-132):

- Limb-onset disease with walking difficulties, twitching, spasticity, and fasciculations in legs
- UMN and LMN involvement and dysarthria

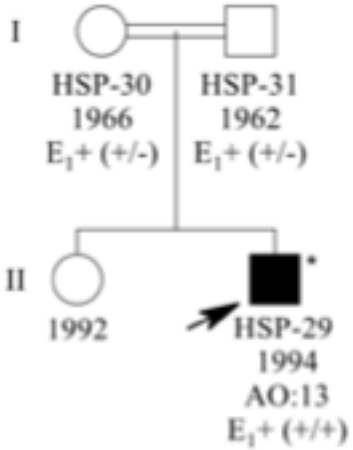
Family history: none/cons.

Suspected Inheritance: AR

WES: only ALS-132

Result: SPG11-ARJALS

Family 6, *SPG11,* c.7076delG, p.(Gly2359Glufs*14)[n]



Index (HSP-29):

- difficulty in walking and imbalance
- bilateral nystagmus, spastic paraparesis, Babinski positive
- thin corpus callosum (TCC)

Family history: none/cons.

Suspected Inheritance: AR

WES: only index

Result: SPG11-ARHSP

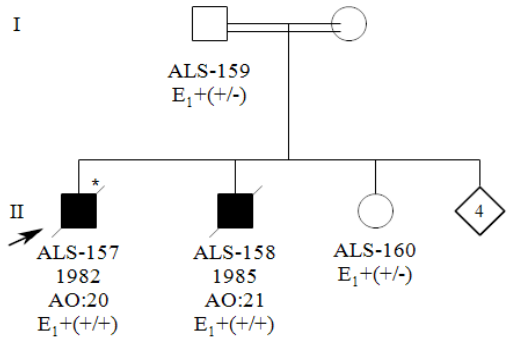
Iskender et al., *Neurology: Genetics,* 2015

PART II

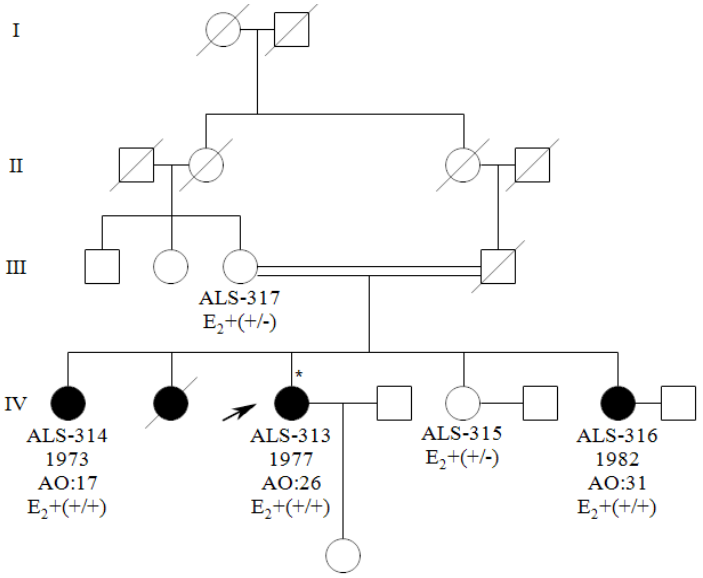
SYNE1: a Multisystemic Disorder

- Pleiotropy in genes has been shown to bring Ataxia and ALS together
  - recessive mutations in the *SETX* cause AOA2, dominant mutations cause autosomal dominant juvenile ALS
  - recessive SYNE1 mutations cause either a pure ataxia called autosomal recessive cerebellar ataxia type 1 (ARCA1) or ataxia accompanying motor neuron disease

Family 8, *SYNE1*, c.22930C>T, p.(Gln7644Ter)[n]



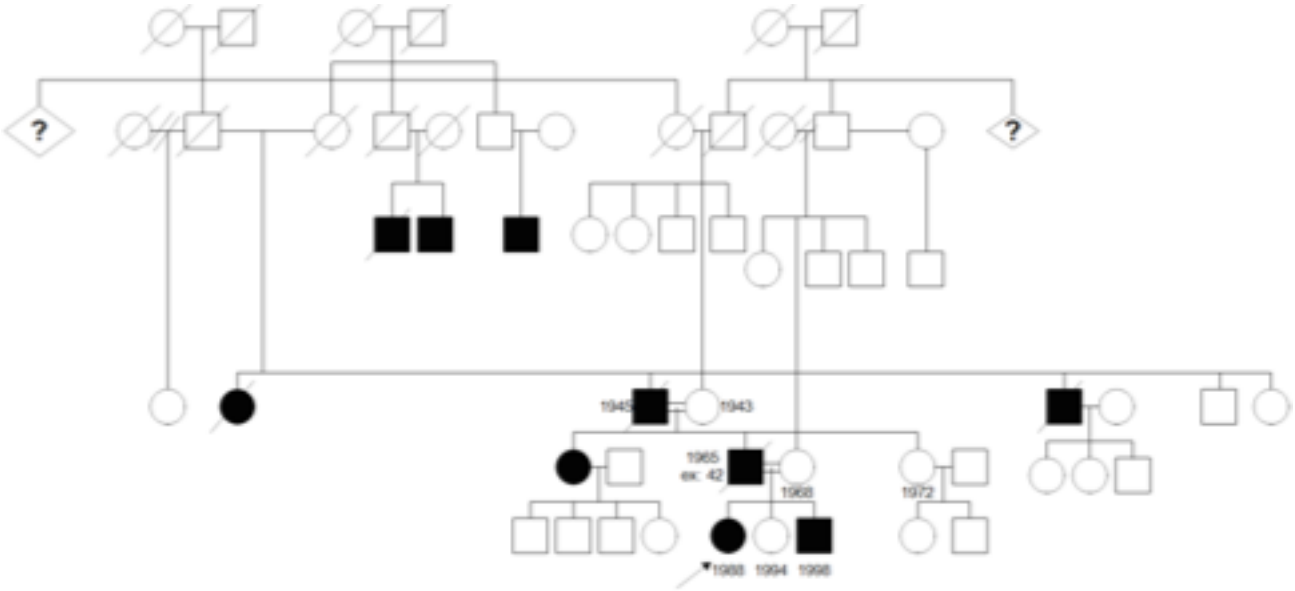
Family 9, *SYNE1*, c.23524C>T, p.(Arg7842Ter)



UMN & LMN signs
pyramidal cerebellar syndrome
spastic ataxic gait, dysarthria and dysmetria

PART II

Family 10, An ALS Family with Pseudo-dominant Inheritance



- Teenage onset patient with mild walking difficulties
- Father diagnosed with definitive ALS and succumbed to disease
- Several affected deceased family members
- Did not respond to common ALS genes
- WES did not identify variation in a rare ALS gene

Tunca et al., *EJHG,* 2018

PART II

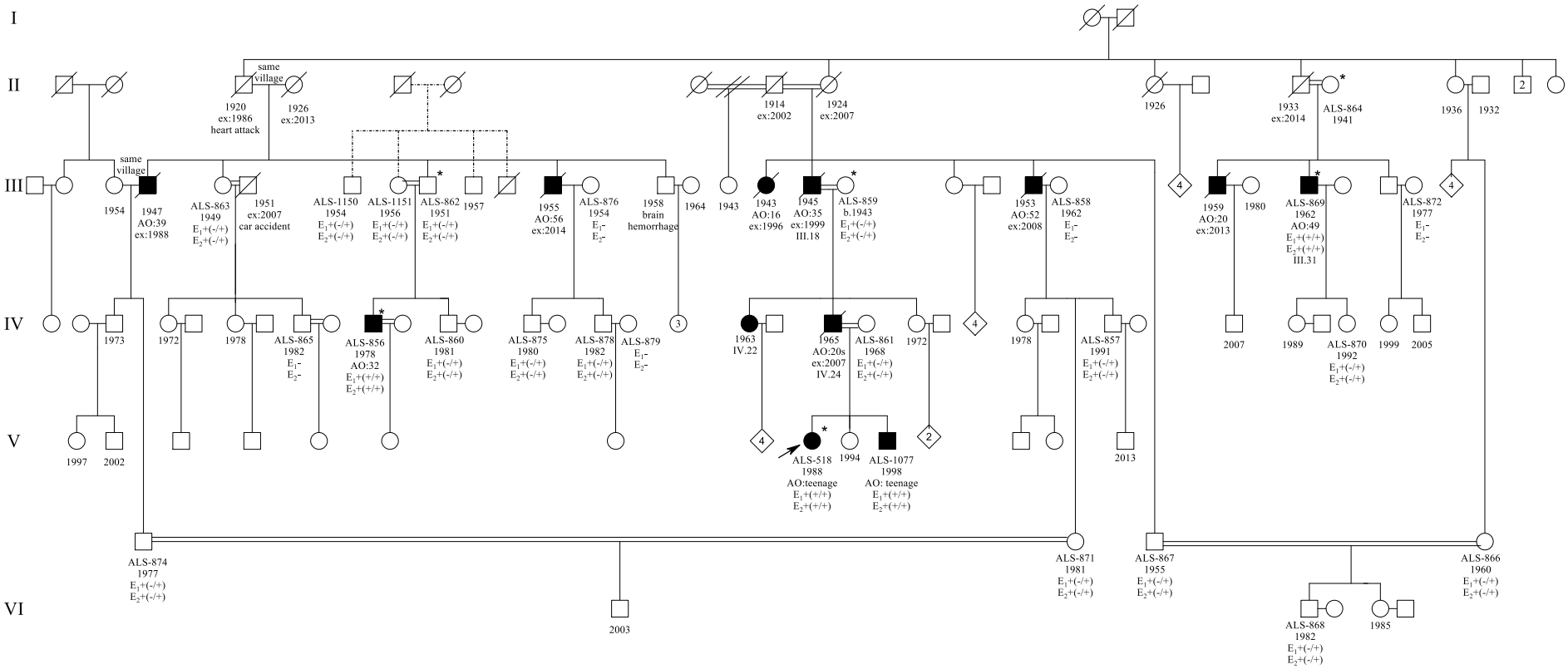
Family 10, Clinical Findings

Grandfather (III.18) walking difficulties at 35 swallowing proplems and death at 54
Father (IV.24) limped mildly after returning from military service at 20, spinal stenosis surgery EMG: fibrillation and fasciculation in genioglossus, right first dorsal interossei, biceps, quadriceps, gastrocnemius and tibialis anterior muscles died at 42
Index (V.12) abnormal gait at late-teens increased DTR in both lower extremities and atrophy in left interosseous muscles EMG: fibrillation and fasciculation in genioglossus, right first interossei, biceps and both tibialis anterior muscles
Brother (V.14) quadriparesis with mild atrophy in both intrinsic hand muscles and spasticity in both legs slowed motor evoked potential in asymmetrically bilateral lower limbs EMG: LMN involvement
Male relative 1 (III.31) mild walking problem (muscle contraction) hyperreflexia in the legs, positive Babinski sign on the right and unresponsive plantar reflex on the left foot with bilateral clonus
Male relative 2 (IV.11) difficulty in step climbing

Tunca et al., *EJHG,* 2018

PART II

Family 10, ERLIN1 c.281T>C p.(Val94Ala)[n]



- *Endoplasmic Reticulum Lipid Raft-Associated Protein 1* linked to pure HSP in three families (SPG62)
  - Gly50Val, Arg255Ter, Tyr288-Gln289del
- Homozygosity Mapping showed the ROH in patients but not controls
- Segregation analyses in 26 family members
- Clinical Re-evaluation

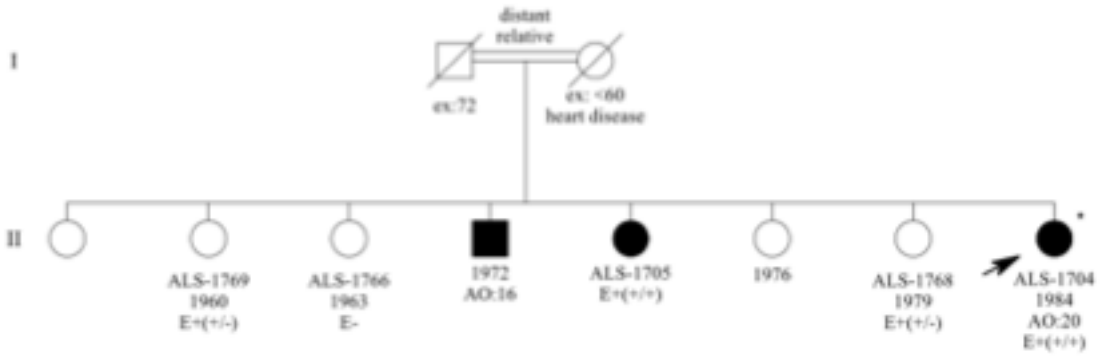
Tunca et al., *EJHG,* 2018

PART II

Family 18, *VRK1*, c.961C>T, p.(Arg321Cys)

Index (ALS-1704):

- walking disability and muscle weakness
- cannot walk long distances
- ataxic walk
- cannot walk on her toes
- UMN&LMN involvement
- sensory ataxia



Family history: two older sibs are affected

Suspected Inheritance: AR

WES: only index

Result: A pathogenic missense variant in *VRK1* gene associated with PCH1A

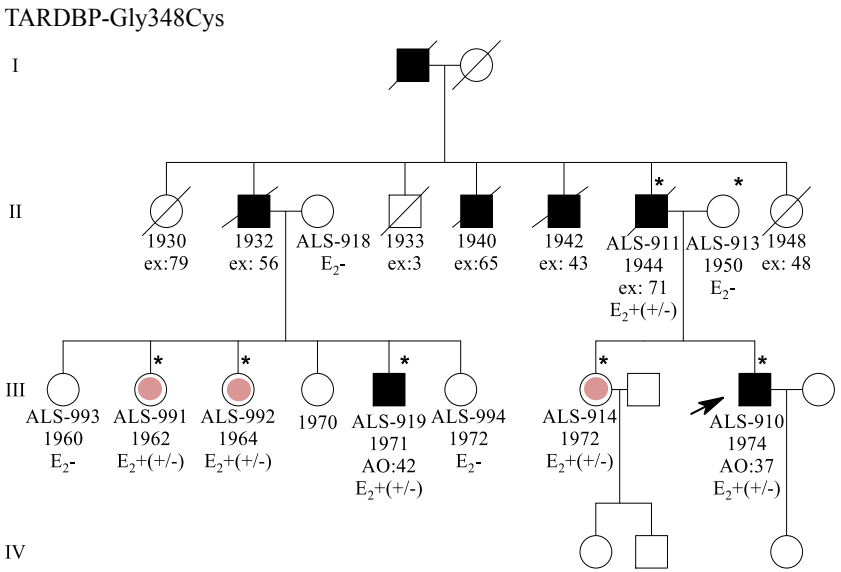
Homozygous and compound heterozygous variants in the *VRK1* > complex motor and sensory neuropathy

Two recent reports present patients with suspected ALS, SBMA, CMT or distal SMA diagnoses without PCH

- ages of onset between 15-20

PART II

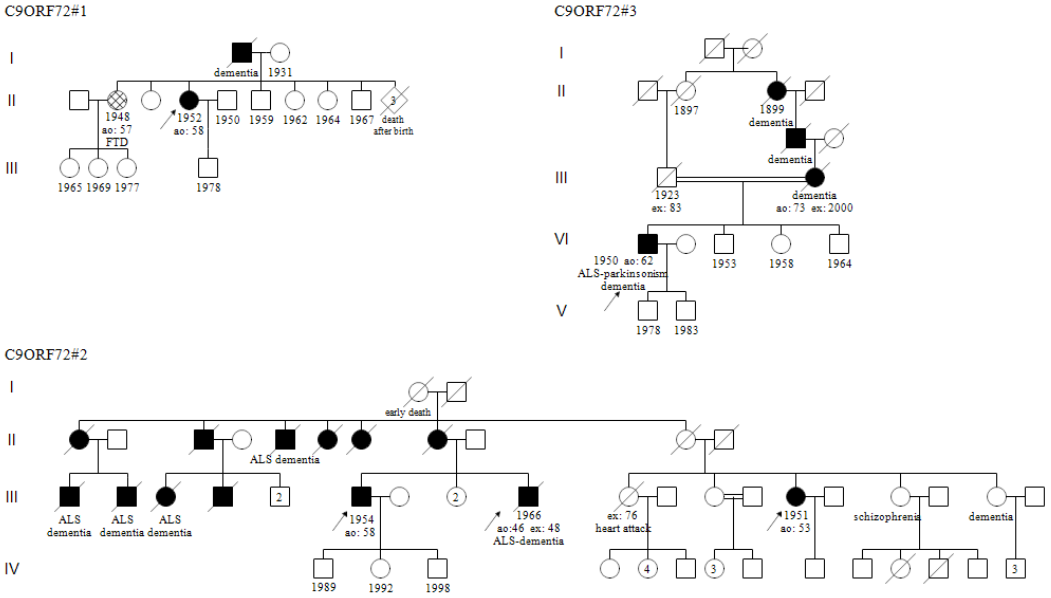
Disease-modifiers and Oligogenic Inheritance

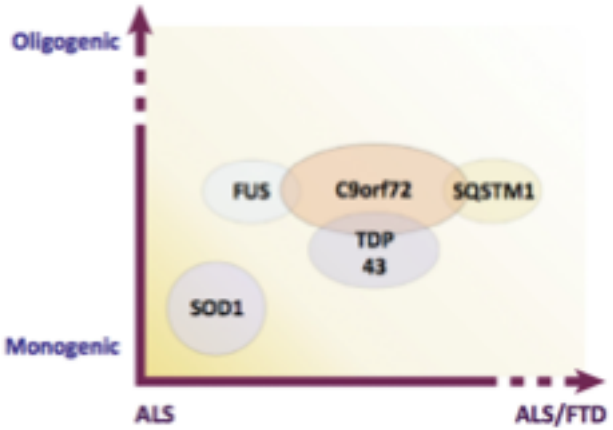


Filtering Purpose	Filter Criteria	Family	# of variants
Heterozygous protective factor in asymptomatic carriers	ALS914 ALS991 ALS992 het ALS910 ALS911 ALS919 reference	TARDBP-Gly348Cys	38
X-linked protective variant in asymptomatic females	ALS914 ALS991 ALS992 het or homvar ALS910 ALS911 ALS919 reference	TARDBP-Gly348Cys	2
het: heterozygous, homref: homozygous reference, homvar: homozygous variant, nothom: not homozygous			

PART II

Oligogenic Inheritance of Common ALS Genes is not Prominent in our *C9orf72* cohort





Lattante et al., 2015, Trends in Genetics

WES in 33 C9orf72 expansion carriers;

- rs143144050 in the VABP gene
- rs75087725 in the C21orf2 gene (risk factor for ALS)
- rs72824736 in GRN (ExAC MAF: 0.04)
- rs75795663 in UNC13A genes in two affected brothers, which was absent in two other affected family members.

- the rs1800435 in the ALAD gene, previously associated with prolonged survival was found in three out of four affected relatives, all three represented different survival times.

Strategy Overview

PART I

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208 p

All TR

*SOD1 exon 4 is screened in all patients with consanguinity

Turkey
625 sALS patients
152 healthy controls

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PART II

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- Two families with IP
- 33 C9orf72 expansion carriers

PART III

Whole Genome Sequencing Analyses in the framework of Project MinE

Variant Accumulation in sALS Patients

Pathogenic Variations in sALS Cases

PART III

Project MinE: Variant Accumulation in Sporadic Disease

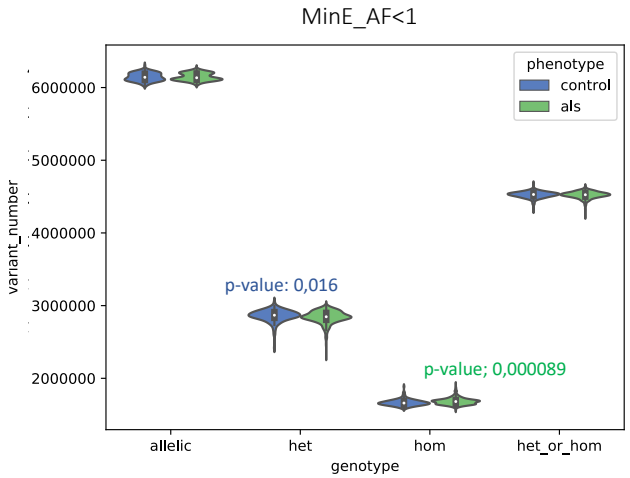
Rare/common Variant Accumulation in Patients (625sALS, 152 control)

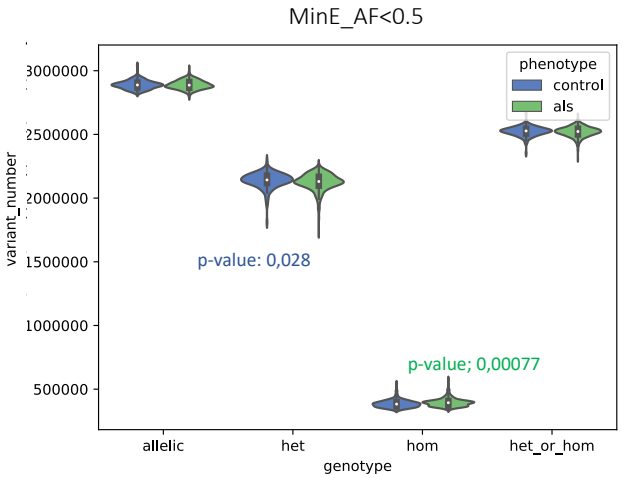
Hypothesis: Rare and/or common variants accumulated in patients, but not in controls might confer to increased disease risk.

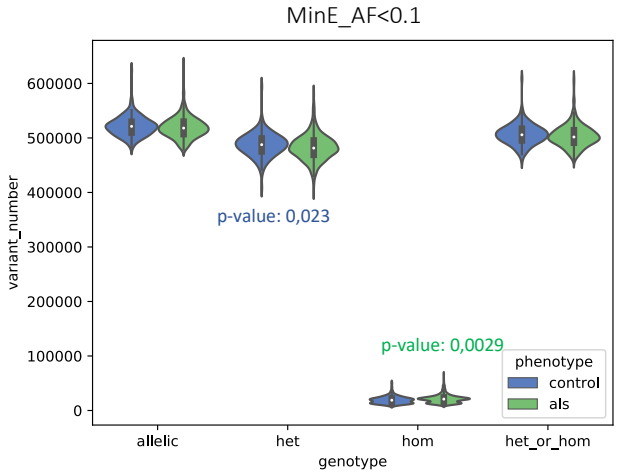
Method:
Number of protein coding and non-coding variants per individual was calculated and plotted for

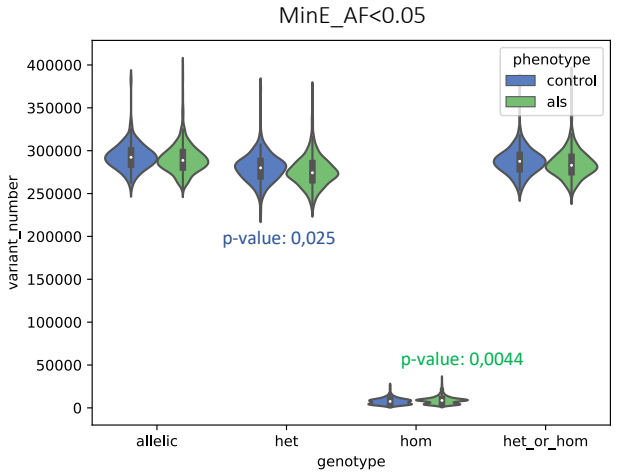
- five internal (among Turkish samples) frequency thresholds (1, 0.5, 0.1, 0.05, 0.01)
- four categories
  - allelic
  - heterozygous (het)
  - homozygous (hom)
  - heterozygous or homozygous (het_or_hom)

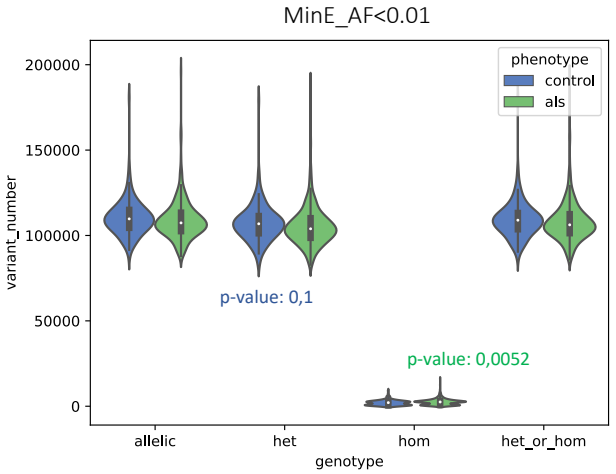
PART III











Result: No significant accumulation associated with disease pathogenesis. Population-specific properties result in significant increase in rare and common homozygous variants in patients compared to controls.

PART III

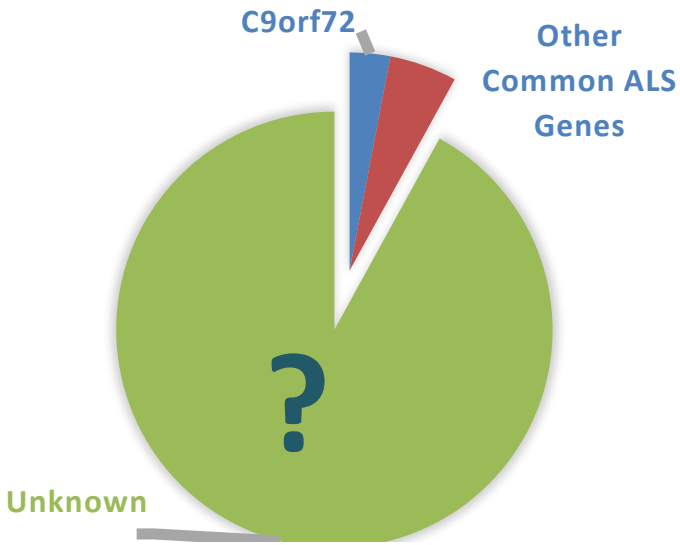
Project MinE: fALS Genes Contributing to sALS

Pathogenic Variants in 625 sALS Cases

~60 patients carry pathogenic variants in ALS-causing and ALS-associated genes

- 6 SOD1
- 1 TARDBP
- 2 FUS
- TBK1, OPTN, VCP, ERBB4, CHCHD10

- PON3 5 cases, 1 control



Concluding Remarks

- Common ALS Genes > 70% in European populations, 50% in Turkish cohort > heterogeneous genetic make-up although inbred

- ALS is a heterogeneous condition with several pathogenic mechanisms and different clinical manifestations > many ALS-linked genes are associated with a wide spectrum of diseases
  - WES results > gene-based disease concept and extended the phenotypes of known genes (genetic pleiotropy)

- sALS cases is explained by known ALS-causing genes > reduced penetrance also in genes from which we expect high penetrance.
  - ALS gene mutations in sALS explains only 10% > modifiers and oligogenicity, unidentified mutations