Supplementary Methods

Subjects

The study population consisted of incident patients identified through the Piemonte and Valle d'Aosta Register for ALS (PARALS), diagnosed with definite, probable, and probable laboratory-supported ALS according to the El Escorial revised diagnostic criteria[1] between January 1st 2007 and December 31st 2015. The PARALS is a prospective epidemiologic registry established in 1995 in Piemonte and Valle d'Aosta regions in Northern Italy.[2] Patients were followed at the ALS Centers in Turin and Novara. Patients with a history of other neurologic disorders affecting cognition (neurodegenerative diseases other than FTD, major stroke, severe head injuries, mental retardation), alcohol and drug dependence, severe mental illness, and use of high-dose psychoactive medications were excluded from the analysis.[3] Non-Italian speaking incident patients were also excluded. None of the study participants showed oxygen saturation <92% based on pulse oximetry at the time of their neuropsychological assessment. Six hundred seventy-seven healthy controls, matched by age, sex, and ancestry (based on the origin of both parents), were recruited from the patients' general practitioners. Out of 1313 incident cases in the period 2007-2015, 751 ALS patients from the participant ALS Centers (57.2%) underwent whole-genome sequencing and full neuropsychological assessment.

Neuropsychological evaluation

An extensive list of neuropsychological tests was performed on each patient.[4] These included: Mini-Mental State Examination (MMSE); Letter and category fluency tests; Frontal Assessment

Battery (FAB); Digit Span Forward and Backward; Trail-Making Test (TMT) A and B; Rey Auditory Verbal Learning Test (RAVLT), immediate and delayed recall; Babcock Story Recall Test (BSRT), immediate and delayed recall; Rey-Osterrieth Complex Figure (ROCF), copy and delayed recall; and Raven's Colored Progressive Matrices (CPM47). Neurobehavioral dysfunction was determined by patient history, direct observation by the neuropsychologist, and the family form of the Frontal Systems Behavior Scale completed by a close relative or caregiver. The Edinburgh Cognitive and Behavioral ALS Screen (ECAS) was not included in the battery since the Italian validation was published in 2018.[5] Anxiety and depression were assessed using the Hospital Anxiety and Depression Scale (HADS); the item "I feel slowed down" was discussed with patients in order to have them not to refer to physical disability.[3] The raw data of the neuropsychological tests were adjusted for age and years of education according to the Italian normative. The battery was administered following the same sequence to avoid differential interference. All study participants were tested at diagnosis or during the first follow-up visit (2 months later).

Cognitive categorization

Patients' cognitive status was classified according to the revised ALS-FTD Consensus Criteria[6] into five categories:

1. ALS with normal cognition (ALS-CN);
2. ALS with behavioral impairment (ALSbi);
3. ALS with cognitive impairment (ALSci);
4. ALS with cognitive and behavioral impairment (ALScbi)

5. ALS with FTD (ALS-FTD).

Patients were retrospectively classified blindly by two neuropsychologists with expertise in ALS (BI, LP). The concordance rate was over 90% for all diagnoses. When there was disagreement, the case was discussed until a consensus diagnosis was established.[4] In the analyses the ALSbi, ALSci and ALScbi group were collapsed into a single group, i.e. intermediate cognitive phenotype.

Whole-genome sequencing

All eligible patients and healthy subjects were screened for *GBA* variants through whole-genome sequencing (WGS). Blood samples and genomic DNA have been processed according to standard protocols: library preparation and read sequencing have been performed as per manufacturer protocol; read alignment, variant calling and quality control have been performed according to standard protocols. WGS methods have been fully described elsewhere[7]. Annotation was performed with KGGseq v1.0 (pgmlab.top/kggseq). A repeat-primed PCR determined the presence of the *C9orf72* hexanucleotide expansion. A threshold of ≥30 repeats with the typical sawtooth pattern was considered pathological.[8]

Statistical Analysis

Descriptive statistics were calculated for baseline demographics and cognitive testing. Survival was calculated from onset to death or censoring date (December 31st, 2019) using the Kaplan-Meier method. Patients with tracheostomy were coded as deceased on the date of the procedure.

Comparisons were performed using the log-rank test. In the first step of the analyses, only variants known to be a risk factor for cognitive decline in PD were considered. A single-variant association test was used to compare the frequency of *GBA* variants between ALS cases and healthy subjects.[9] A binomial test was used to assess the prevalence of *GBA* mutations across cognitive groups. Given the strong influence of *C9orf72* on cognitive status, we performed the binomial test with and without *C9orf72* expansion carriers to rule out its impact on the results. Linear mixed-effects models were used to test for associations between *GBA* genotype and cognitive functioning. The covariates included in this model were age at onset, sex, site of onset (classified as bulbar or spinal), the presence of bulbar signs at diagnosis, the rate of ALS Functional Rating Scale-Revised (ALS-FRS-R) decline at the time of diagnosis, and *C9orf72* status. ALS-FRS-R decline was calculated as follows: (48 - ALS-FRS-R at diagnosis) / months from disease onset to diagnosis. All statistical analyses were performed in R v.3.6.0 (http://www.r-project. org). R scripts are available on GitHub (http://github.com). To assess whether pathogenic rare variants in *GBA* contribute to cognitive decline risk in ALS, a gene-based rare variants association test was also performed. This analysis provided an independent approach from the assumption that only *GBA* variants associated with PD may influence cognitive status. Only rare variants were included, and minor allele frequency threshold was set at < 5%. The analysis was performed confronting ALS patients with cognitive decline (i.e. ALS-FTD and ALSCi/Cbi/Bi) vs ALS patients with normal cognitive function. The rare variant burden was assessed using the Sequence Kernel Association Test (SKAT) and the Sequence Kernel Association Test - Optimized (SKAT-O) as implemented in RVtests (http://zhanxw.github.io/rvtests/).

Supplementary Tables

Supplementary Table 1. Genes and Risk Factors associated with Parkinson Disease (PD), Dementia with Lewy Bodies (DLB), frontotemporal dementia (FTD) and Amyotrophic Lateral Sclerosis (ALS) that have been implicated in Lysosomal function.

Gene	Disease	Functions involving lysosomes
SNCA	Early-onset PD, DLB	Recruitment of proteins to lysosomal damage sites
PARK2	Early-onset PD	Mitophagy
PINK1	Early-onset PD	Mitophagy
LRRK2	Late-onset PD	Regulattion of lysosomal pH and homesostasis, and lysosome-Golgi trafficking
GBA	Late-onset PD, DLB	Accumulation of cholesterol in lysosomes
VPS35	Late-onset PD	Endosome–Golgi trafficking; autophagy
C9ORF72	ALS/FTD	Autophagy induction and stress granule autophagy
GRN	FTD	Regulation of lysosomal pH
TBK1	ALS/FTD	Clearance of damaged mitochondria, recruitment to lysosomes by α-synuclein

OPTN	ALS/FTD	Clearance of damaged mitochondria, recruitment to lysosomes by α-synuclein
CHMP2B	ALS/FTD	Regulation of lysosomal trafficking
CHCHD2	PD	Mitochondrial quality control
CHCHD10	ALS/FTD	Mitochondrial quality control
VCP	ALS/FTD	Autophagosome maturation and clearance of damaged lysosomes
SQSTM1	ALS/FTD	It targets protein aggregates for lysosomal degradation

Supplementary Table 2. Results from the gene-based rare variant association test

Burden test	**FTD**	**ALS-Bi / ALS-Ci / ALS-Cbi**
SKAT	0.196	0.0125
SKAT-O	0.184	0.00005

Supplementary Table 3. List of *GBA* variants detected in our ALS series. We identified three common *GBA* polymorphisms (p.E365K, p.T408M, p.N409S), which are known risk factors for DLB and cognitive impairment in PD[10–13]. Six of the other variants have not been reported to be associated with PD.

Nucleotide	AA	Exon	SNP id	gnomAD NFE	CADD score	ClinVar	Disease
c.C1319T	p.P440L	10	rs74598136	.	26.2	Pathogenic	Gaucher's disease
c.G1279A	p.E427K	10	rs149171124	0.0003	23.2	.	.
c.A1226G	p.N409S	10	rs76763715	0.002	22.7	Pathogenic	Gaucher's disease, PD, DLB
c.C1223T	p.T408M	9	rs75548401	0.0091	22.2	Likely benign	PD
c.G1093A	p.E365K	9	rs2230288	0.0121	17.33	Pathogenic	PD
c.A928G	p.S310G	8	rs1057942	0	14.81	.	.
c.C740G	p.T247S	7	.	.	22	.	.
c.T634A	p.S212T	7	rs398123533	.	1.937	VUS	
c.G626A	p.R209H	7	rs749416070	3.92E-05	17.53	.[§]	Reported in PD[14]
c.A38G	p.K13R	3	rs150466109	0.0002	0.003	Benign	

AA: amino acid; SNP: Single Nucleotide Polymorphism; gnomAD NFE: exome frequency in Non-Finnish European.

§ Classified as likely pathogenic according to the following American College of Medical Genetic (ACMG) criteria[15]: PM1 moderate (Hot-spot of length 17 amino-acids has 7 non-VUS missense/in-frame variants, 6 pathogenic and 1 benign), PM2 moderate (GnomAD exomes homozygous allele count = 0 is less than 3 for recessive gene GBA), PM5 moderate (Alternative variants Arg209Pro and Arg209Cys are classified as Pathogenic), PP2 supporting (269 out of 282 non-VUS missense variants in *GBA* gene are pathogenic).

Supplementary Table 4. Comparison of *GBA* risk variants frequency between ALS patients and healthy controls. These variants were found in 18 ALS patients (2.40% of our cohort) and 15 healthy controls (2.22%). The variant frequencies observed in our Italian population are consistent with those reported in gnomAD. The single-variant analysis confirmed that *GBA* variants are not a risk factor for developing ALS. We did not observe homozygote cases or compound heterozygous cases for *GBA* variants. Furthermore, we did not observe mutations in ALS-related genes (*SOD1*, *TARDBP* and *FUS*) in *GBA* risk variants carriers. We identified one patient who carried both a *GBA* risk variant (p.N409S) and the *C9orf72* repeat expansion.

GBA variant	n. Heterozygous ALS cases (n=751)	n. Heterozygous HC (n=677)	p-value	gnomAD NFE
c.1093G>A (p.E365K)	3	5	0.49	0.0121
c.1223C>T (p.T408M)	8	6	0.61	0.0091
c.1226A>G (p.N409S)	7	4	0.76	0.002

Supplementary Table 5. Clinical and demographic characteristics of ALS patients grouped by *GBA* genotype. The *GBA* risk variants did not influence survival or the motor phenotype. Only two of the ALS patients carrying a *GBA* risk variant exhibited extrapyramidal signs. Both patients had slowly progressive disease, and they developed extrapyramidal signs years after the initial ALS diagnosis. None of the *GBA* risk variants carriers had a family history of Parkinson's disease or other neurodegenerative diseases. The effect of *GBA* mutations on cognitive but not motor phenotype could be explained by a lower susceptibility of motor neurons: it should be noted that while extrapyramidal involvement is established in *GBA* homozygous carriers, motor neuron involvement has only been recently reported in few cases with Gaucher's Disease and one subject with a heterozygous *GBA* mutation. [16,17]

	***GBA* carriers (n=18)**	***GBA* non carriers (n=733)**	**P**
N (% male)	5 (27.8%)	404 (55.1%)	0.04
Age onset (yrs, SD)	65.2 (10.5)	65.7 (9.9)	0.84
Bulbar onset, n (%)	4 (22.2%)	244 (33.3%)	0.46
Bulbar signs at diagnosis, n (%)	9 (50.0%)	369 (49.2%)	1
C9orf72 expansion carriers (%)	1 (5.6%)	66 (9.0%)	0.93
Median survival (years, SD)	2.65 (1.68-5.26)	3.26 (1.68-5.89)	0.43*
Mean ALSFRS-R slope	1.31 (1.21)	0.99 (1.74)	0.33

* Survival was calculated with Kaplan-Meier curves and significance with Log-rank test

Supplementary Table 6. Cognitive phenotypes of *GBA* risk variant carriers. Due to the relatively small number of *GBA* variant carriers, we could not properly draw any conclusion on whether *GBA* variants were predominantly associated with cognitive or behavioural impairment or with a distinct pattern of cognitive deficits. However, it should be noted that the only variant causing Gaucher's Disease of this group (p.N409S) was associated with cognitive impairment in 7 out of the 8 carriers (87.5%): it could be postulated that more disruptive variants have a larger influence on cognitive function. Longitudinal neuropsychological studies and the application of neuroimaging techniques might allow further clarification on this issue. As recently reported in cross-sectional study using ^{18}F-2-fluoro-2-deoxy-D-glucose-PET,[18] the extent of metabolic brain changes in ALS patients reflects the degree of cognitive impairment, paralleling brain metabolic alterations observed in FTD over time.[19]

GBA variant	FTD	ALSBi	ALSCi	ALSCbi	CN
c.626G>A (p.R209H)	0	0	1	0	0
c.1093G>A (p.E365K)	0	2	0	0	1
c.1223C>T (p.T408M)	3	0	2	0	3
c.1226A>G (p.N409S)	2 §	1	2	1	1

§ One subject carrying the p.N409S variant and presenting FTD also carried the *C9orf72* repeat expansion

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