Neurobiology of Aging 36 (2015) 2005.e15–2005.e22

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Genetic variability in *SQSTM1* and risk of early-onset Alzheimer dementia: a European early-onset dementia consortium study

Elise Cuyvers [a,b,c], Julie van der Zee [a,b,c], Karolien Bettens [a,b,c], Sebastiaan Engelborghs [b,c,d], Mathieu Vandenbulcke [e], Caroline Robberecht [a,b,c], Lubina Dillen [a,b,c], Céline Merlin [a,b,c], Nathalie Geerts [a,b,c], Caroline Graff [f,g], Håkan Thonberg [f,g], Huei-Hsin Chiang [f], Pau Pastor [h,i,j], Sara Ortega-Cubero [h,j], Maria A. Pastor [j,k,l], Janine Diehl-Schmid [m], Panagiotis Alexopoulos [m], Luisa Benussi [n], Roberta Ghidoni [n], Giuliano Binetti [n], Benedetta Nacmias [o], Sandro Sorbi [o], Raquel Sanchez-Valle [p], Albert Lladó [p], Ellen Gelpi [q], Maria Rosário Almeida [r], Isabel Santana [r], Jordi Clarimon [j,s], Alberto Lleó [j,s], Juan Fortea [j,s], Alexandre de Mendonça [t], Madalena Martins [t], Barbara Borroni [u], Alessandro Padovani [u], Radoslav Matěj [v,w], Zdenek Rohan [v,w,x], Agustín Ruiz [y], Giovanni B. Frisoni [z,aa], Gian Maria Fabrizi [bb], Rik Vandenberghe [cc], Peter P. De Deyn [b,c,d,dd], Christine Van Broeckhoven [a,b,c,*], Kristel Sleegers [a,b,c,**], on behalf of the BELNEU Consortium and of the EU EOD Consortium

[a] *Department of Molecular Genetics, VIB, Antwerp, Belgium*
[b] *Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium*
[c] *Laboratory of Neurochemistry and Behavior, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium*
[d] *Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken, Antwerp, Belgium*
[e] *Department of Old Age Psychiatry and Memory Clinic, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium*
[f] *Department of Neurobiology, Care Sciences and Society (NVS), Center for Alzheimer Research, Division of Neurogeriatrics, Karolinska Institutet, Huddinge, Sweden*
[g] *Department of Geriatric Medicine, Genetics Unit, Karolinska University Hospital, Stockholm, Sweden*
[h] *Neurogenetics Laboratory, Division of Neurosciences, Center for Applied Medical Research, Universidad de Navarra, Pamplona, Spain*
[i] *Department of Neurology, Hospital Universitari Mutua de Terrassa, Terrassa, Barcelona, Spain*
[j] *Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain*
[k] *Neuroimaging Laboratory, Division of Neurosciences, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain*
[l] *Department of Neurology, Clínica Universidad de Navarra, University of Navarra School of Medicine, Pamplona, Spain*
[m] *Department of Psychiatry and Psychotherapy, Technische Universität München, München, Germany*
[n] *Molecular Markers Laboratory—Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Centro San Giovanni di Dio-Fatebenefratelli, Brescia, Italy*
[o] *Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), University of Florence, Florence, Italy*
[p] *Alzheimer's Disease and Other Cognitive Disorders Unit, Neurology Department, Hospital Clínic, Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[q] *Neurological Tissue Bank of the Biobanc, Hospital Clinic, Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[r] *Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal*
[s] *Department of Neurology, IIB Sant Pau, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain*
[t] *Faculty of Medicine and Institute of Molecular Medicine, University of Lisbon, Lisbon, Portugal*
[u] *Neurology Unit, University of Brescia, Brescia, Italy*
[v] *Center of Clinical Neurosciences, Department of Neurology, First Medical Faculty, Charles University in Prague, Czech Republic*
[w] *Department of Pathology and Molecular Medicine, Thomayer Hospital, Prague, Czech Republic*
[x] *Institute of Pathology, Third Medical Faculty of Charles University in Prague, Prague, Czech Republic*
[y] *Memory Clinic of Fundaciò ACE, Institut Català de Neurociències Aplicades, Barcelona, Spain*
[z] *Départements de Médicine Interne Réhabilitation & Gériatrie et de Sante Mentale & Psychiatrie, Hôpitaux Universitaires de Genève et Université de Genève, Geneva, Switzerland*
[aa] *Alzheimer's Neuroimaging and Epidemiology Laboratory, IRCCS Fatebenefratelli, Brescia, Italy*
[bb] *Department of Neurological and Movements Sciences, Section of Neurology, University Hospital G.B. Rossi, University of Verona, Verona, Italy*
[cc] *Laboratory for Cognitive Neurology, Department of Neurology, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium*
[dd] *Department of Neurology and Alzheimer Research Center, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands*

* Corresponding author at: Neurodegenerative Brain Diseases Group, VIB Department of Molecular Genetics, University of Antwerp—CDE, Universiteitsplein 1, Antwerp B-2610, Belgium. Tel.: +32 3 265 1102; fax: +32 3 265 1112.

** Alternate corresponding author at: Neurodegenerative Brain Diseases Group, VIB Department of Molecular Genetics, University of Antwerp—CDE, Universiteitsplein 1, Antwerp B-2610, Belgium. Tel.: +32 3 265 1032; fax: +32 3 265 1112.

E-mail addresses: christine.vanbroeckhoven@molgen.vib-ua.be (C. Van Broeckhoven), kristel.sleegers@molgen.vib-ua.be (K. Sleegers).

http://dx.doi.org/10.1016/j.neurobiolaging.2015.02.014

ARTICLE INFO

Article history:
Received 5 February 2015
Accepted 12 February 2015
Available online 19 February 2015

Keywords:
SQSTM1/p62
Alzheimer's disease
Rare variants
Meta-analysis
European early-onset dementia consortium

ABSTRACT

Meta-analysis of existing genome-wide association studies on Alzheimer's disease (AD) showed subgenome-wide association of an intronic variant in the sequestosome 1 (*SQSTM1*) gene with AD. We performed targeted resequencing of *SQSTM1* in Flanders-Belgian AD patients selected to be enriched for a genetic background ($n = 435$) and geographically matched nonaffected individuals ($n = 872$) to investigate the role of both common and rare *SQSTM1* variants. Results were extended to the European early-onset dementia cohorts (926 early-onset Alzheimer's disease [EOAD] patients and 1476 nonaffected individuals). Of the 61 detected exonic variants in *SQSTM1*, the majority were rare ($n = 57$). Rare variant (minor allele frequency <0.01) burden analysis did not reveal an increased frequency of rare variants in EOAD patients in any of the separate study populations nor when meta-analyzing all cohorts. Common variants p.D292= and p.R312= showed nominal association with AD (odds ratio$_{p.D292=}$ = 1.11 [95% confidence interval = 1–1.22], $p = 0.04$), only when including the Flanders-Belgian cohort in the meta-analysis. We cannot exclude a role of *SQSTM1* genetic variability in late-onset AD, but our data indicate that *SQSTM1* does not play a major role in the etiology of EOAD.

1. Introduction

Mega meta-analysis of existing genome-wide association studies (GWAS) on Alzheimer's disease (AD) performed by the International Genomics of Alzheimer's Project identified an intronic variant in the sequestosome 1 (*SQSTM1*) gene, which showed subgenome-wide association with AD (rs72807343, odds ratio [OR] = 1.35 [95% confidence interval {CI} = 1.20–1.52], $p = 7 \times 10^{-7}$) (Lambert et al., 2013). *SQSTM1* encodes the p62 protein that is a stress-responsive ubiquitin-binding protein commonly found in neuronal cytoplasmic inclusions in protein aggregation diseases like AD, Parkinson disease, Pick disease, etc. (Kuusisto et al., 2001; Zatloukal et al., 2002). P62 is involved in protein degradation via the proteasome, in protein aggregation, and in autophagy (Bjorkoy et al., 2006; Seibenhener et al., 2004). Mutations in this gene, especially affecting the ubiquitin-associated (UBA) domain of the p62 protein, have been found to be the most common cause of Paget disease of the bone (PDB), a disease that is characterized by malformed bones (Johnson-Pais et al., 2003). Using a hypothesis-driven candidate gene approach, a direct genetic role for *SQSTM1* in both familial and sporadic amyotrophic lateral sclerosis was identified in a European-American population (Fecto et al., 2011). Screening of additional amyotrophic lateral sclerosis (ALS) populations led to the identification of novel variations in the gene (Hirano et al., 2013; Teyssou et al., 2013). These results suggested that presumably ALS and PDB share a common molecular pathomechanism (Hirano et al., 2013), reminiscent of PDB, and frontotemporal lobar degeneration (FTLD) in *VCP* mutation carriers (Kimonis et al., 2008; Watts et al., 2007; van der Zee et al., 2009). Adding to the firmly established clinicopathologic relationship between ALS and FTLD, studies were conducted to investigate the frequency of *SQSTM1* variants in FTLD patients (Rubino et al., 2012; van der Zee et al., 2014). Rare mutations clustering in the UBA domain of p62 were found to be associated with a 2-fold increased risk to develop FTLD (Rubino et al., 2012; van der Zee et al., 2014).

In this study, we investigated the contribution of both rare and common variations in the *SQSTM1* exonic region to the occurrence of AD in a cohort of Flanders-Belgian AD patients selected to be enriched for a genetic background (early disease onset and/or familial AD, $n = 435$) and geographically matched nonaffected individuals ($n = 872$). Our results were extended to a European early-onset dementia (EU EOD) cohort comprising 926 early-onset Alzheimer's disease (EOAD) patients and 1476 nonaffected individuals.

2. Materials and methods

2.1. Study population

2.1.1. Flanders-Belgian cohort

We selected 435 AD patients with early-onset age (age of onset [AAO] <65 years) and/or familial disease (at least one first-degree relative with the disease) (mean AAO = 67.7 ± 8.2 years, women = 62.2%) from a large prospective cohort of Belgian AD patients ascertained at the Memory Clinic of the ZNA Middelheim and Hoge Beuken, Antwerp, Belgium (PPDD and SE) (Engelborghs et al., 2003, 2006) and the Memory Clinic of the University Hospitals of Leuven, Leuven, Belgium (MV and RV) (Table 1). Consensus diagnosis of possible and probable AD was given by at least 2 neurologists based on the National Institute of Neurological and Communication Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria (McKhann et al., 1984, 2011). Each patient underwent a neuropsychological examination and structural and/or functional neuroimaging (Bettens et al., 2009).

The Flanders-Belgian control cohort ($n = 872$, mean age at inclusion = 66 ± 12.7 years, women = 55.6%) consisted primarily of community-dwelling volunteers, for whom subjective memory complaints, neurologic or psychiatric antecedents, and a familial history of neurodegeneration were ruled out by means of an interview. Cognitive screening was performed using the Mini-Mental State Examination (cutoff score ≥26) (Folstein et al., 1975). The control cohort additionally included spouses of patients, examined at the Memory Clinic of ZNA Middelheim and Hoge Beuken, Antwerp, Belgium and the Memory Clinic at the University Hospitals of Leuven, Gasthuisberg, Leuven, Belgium.

All participants and/or their legal guardian gave written informed consent for participation in clinical and genetic studies. Clinical study protocol and the informed consent forms for patient ascertainment were approved by the ethics committee of the respective hospitals at the cohort sampling sites in Belgium. The genetic study protocols and informed consent forms were approved by the Ethics Committees of the University of Antwerp and the University Hospital of Antwerp, Belgium.

2.1.2. EU EOD cohort

Patients and control individuals ascertained through the EU EOD consortium were included as replication cohort (van der Zee et al., 2013, 2014). For this study, DNA and medical/demographic

Table 1
All exonic variants in *SQSTM1* that were identified in the Flanders-Belgian cohort

Exonic variants	Protein domain	Genomic position	Protein position	dbSNP137	Minor allele count AD	Freq AD	OR (95% CI), *p* value	Minor allele count control	Freq control	Freq EVS
Exon 1	PB1	g.179248023C>G	p.P29=		0	0.000		2	0.001	—
	PB1	g.179248034C>T	p.A33V	rs200396166	1	0.001		0	0.000	0.0008
	PB1	g.179248119C>T	p.G61=		1	0.001		2	0.001	—
Exon 3	PB1<>ZZ	g.179250885G>A	p.R110H		0	0.000		1	0.001	—
	PB1<>ZZ	g.179250906C>T	p.A117V	rs147810437	0	0.000		2	0.001	0.0012
	PB1<>ZZ	g.179250930A>G	p.N125S		0	0.000		1	0.001	—
	ZZ	g.179251013G>A	p.V153I	rs145056421	1	0.001		3	0.002	0.001
Exon 4	ZZ<>TRAF6	g.179251313G>A	p.T221=		0	0.000		1	0.001	—
Exon 5	TRAF6	g.179252168G>A	p.P232=	rs145688323	1	0.001		0	0.000	0
	TRAF6	g.179252184A>G	p.K238E	rs11548633	8	0.009		7	0.004	0.0035
Exon 6	PEST1	g.179260072A>C	p.R265S		0	0.000		1	0.001	—
	PEST1	g.179260073A>C	p.S266R		0	0.000		1	0.001	—
	PEST1	g.179260099G>C	p.E274D	rs55793208	21	0.025	1.67 (0.91–3.05), 0.096	31	0.018	0.0253
	PEST1	g.179260110C>T	p.T278I	rs200445838	0	0.000		1	0.001	—
	PEST1	**g.179260153C>T**	**p.D292=**	**rs4935**	**489**	**0.575**	**1.22 (1.01–1.47), 0.035**	**933**	**0.535**	**0.5294**
	PEST1<>LIR	g.179260165G>A	p.P296=	rs148984239	0	0.000		1	0.001	0.0001
	PEST1<>LIR	g.179260183C>T	p.G302=	rs11548642	1	0.001		1	0.001	0.0002
	PEST1<>LIR	g.179260202G>A	p.A308=		0	0.000		1	0.001	—
	PEST1<>LIR	**g.179260213G>A**	**p.R312=**	**rs4797**	**479**	**0.564**	**1.23 (1.02–1.48), 0.03**	**920**	**0.528**	**0.5227**
	PEST1<>LIR	g.179260231C>T	p.S318=	rs56092424	20	0.024	1.56 (0.85–2.89), 0.151	31	0.018	0.0212
	PEST1<>LIR	g.179260232G>A	p.E319K	rs61748794	0	0.000		1	0.001	0.0002
Exon 7	LIR	g.179260601G>A	p.S328=	rs146164139	5	0.006		11	0.006	0.0045
	LIR<>PEST2	g.179260649A>G	p.K344=	—	0	0.000		3	0.002	—
	PEST2	g.179260661G>A	p.P348=	rs10058037	0	0.000		1	0.001	0.0002
	PEST2	g.179260700C>T	p.S361=	rs201591177	1	0.001		0	0.000	0.0001
Exon 8	UBA	g.179263445C>T	p.P392L	rs104893941	2	0.002		4	0.002	0.0021
	UBA	g.179263543G>A	p.G425R		0	0.000		1	0.001	—
	UBA	g.179263544G>A	p.A426=		0	0.000		2	0.001	—
	UBA	g.179263583C>T	p.P438L		2	0.002		0	0.000	—
	UBA	g.179263586C>T	p.P439L		0	0.000		1	0.001	—

SQSTM1 (NM_003900.4). Genomic position in base pairs according to hg19 (GRCh37), and "—" denotes not applicable. Total allele count for the Belgian population is 870 AD and 1744 control alleles. A comparison of frequencies with those in EVS in European samples (at least 7388 alleles) is provided. The variations with MAF >1% and significant *p* values are indicated in bold. Chi^2 *p* values are corrected for AAO, gender, and APOE genotype. Protein domains are based on UniProt information; transcript level on NM_003900.4, and protein level on the GenPept Accession Number NP_003891.1. Protein domains were assigned as described previously (van der Zee et al., 2014). "<>" denotes between protein domains. Rare nonsynonymous variants p.P438L and p.A33V were present in the same AD patient (female, AAO = 75 years). In addition, variants p.R265S and p.S266R were identified in the same control individual (male, AAI = 70 years).
Key: AAO, age at onset; AD, Alzheimer's disease; EVS, Exome Variant Server; freq, frequency; MAF, multiple allele frequency; OR, odds ratio; *SQSTM1*, sequestosome 1; TRAF6, tumor necrosis factor receptor–associated factor 6; UBA, ubiquitin associated.

information on 926 EOAD patients (disease onset = <65 years), originating from Spain (n = 329), Portugal (n = 107), Italy (n = 210), Sweden (n = 175), Germany (n = 98), and Czech Republic (n = 7), was contributed by members of the consortium (Supplementary Table 1). Patients were diagnosed following the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association International Working Group criteria (McKhann et al., 1984, 2011). Diagnosis of pathology confirmed patients was based on currently accepted diagnostic criteria (Montine et al., 2012). Genetic profiling of AD-associated genes was previously generated for a subset of patients *APP* (n = 227), presenilin-1 (*PSEN1*) (n = 248), *PSEN2* (n = 225), *GRN* (n = 11), and *MAPT* (n = 11) (Supplementary Materials and Methods). This revealed 3 *APP*, 14 *PSEN1*, and 5 *PSEN2* missense mutations and 1 *GRN* frameshift mutation. Genotyping of *APOE* was performed in the total patient population.

As control group, we sequenced 1476 age- and origin-matched European individuals (Spain [n = 484], Portugal [n = 127], Italy [n = 518], Sweden [n = 340], and Czech Republic [n = 7]) tested for normal cognition for age and education and Mini-Mental State Examination cutoff score >26. For all EU EOD participants, informed consent for participation, approved by the ethics committee of the respective hospitals or sampling sites, was obtained. A more detailed description of the EU EOD consortium cohort can be found in the Supplementary Table 1.

2.2. SQSTM1 *sequencing*

For the Flanders-Belgian cohort, genomic DNA was extracted from peripheral blood lymphocytes using MagDEA DNA Whole-Blood (8Lx) kit (Precision System Science, Pleasanton, CA, USA). Resequencing of the full *SQSTM1* exonic DNA sequence (coding sequence [CDS]) of the Flanders-Belgian sample (n = 1307) was performed by polymerase chain reaction (PCR)–based amplification of DNA followed by Sanger sequencing of the 8 exons and intron-exon boundaries (NM_003900.4). Primers were designed using the PCR primer design tool Primer3 (primer sequences are available on request, http://primer3.sourceforge.net/). All sequences were analyzed with Seqman (DNASTAR, Madison, WI, USA) and NovoSNP software packages (Reumers et al., 2011; Weckx et al., 2005).

For the EU EOD cohort, DNA samples were subjected to quality control procedures as previously described (van der Zee et al., 2014). Resequencing of *SQSTM1* was performed by massive parallel resequencing after multiplex amplicon enrichment. To this end, we designed a target enrichment assay based on MASTR technology (Multiplicom, Niel, Belgium) covering *SQSTM1* coding exons 2–8, flanking intron-exon boundaries and untranslated regions. *SQSTM1* exon 1 was screened by Sanger sequencing as described previously. Primers for multiplex PCR were designed using mPCR (Multiplicom). Multiplex PCR was performed for amplification of the target region, followed by purification of the equimolar pooled

amplicon libraries using Agencourt AMPureXP beads (Beckman Coulter, CA, USA). Patient-specific barcodes (Illumina Nextera XT) were incorporated in a universal PCR step. Barcoded samples were pooled before bridge amplification and sequencing on an Illumina MiSeq platform, using the Illumina reagent kit v2, generating 250-bp paired-end reads. A subset of the control cohort ($n = 707$) was screened using both MASTR massive parallel resequencing and Sanger sequencing. This dual analysis showed a high concordance of 99.4% between both used technologies.

Fastq-mcf was used to trim the MiSeq (Illumina) adapters of the paired-end reads. Alignment and mapping of the reads against the whole genome (hg19) was performed with Burrows-Wheeler Aligner (Li and Durbin, 2009). Variant calling and annotation was performed using GATK, version 2.2 (McKenna et al., 2010) in combination with GenomeComb software (Reumers et al., 2011). Raw reads of rare variants were manually checked using the integrative genomics viewer (IGV; Broad Institute, Cambridge, MA, USA). Rare variants were validated on genomic DNA using Sanger sequencing. Numbering of variations at genomic DNA level was based on the GenBank Accession Number NC_000005.9, transcript level on NM_003900.4, and protein level on the GenPept Accession Number NP_003891.1.

2.3. In silico prediction

The effects of coding *SQSTM1* variations were predicted using Polymorphism Phenotyping (PolyPhen-2, version 2, http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/www/SIFT_enst_submit.html), and SNPs&Go (http://snps.uib.es/snps-and-go//snps-and-go.html). PolyPhen-2 predicts a possible impact of amino acid substitutions on the structure and function of human proteins. The Polyphen-2 score ranges from 0 to 1 and indicates the probability of a damaging effect. SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and physical proportions of amino acids. A SIFT score <0.05 suggests pathogenicity. SNPs&Go predicts human disease–related mutations in functionally annotated proteins. The reliability index reports the reliability of the prediction, scoring from 0 (unreliable) to 10 (reliable). If the disease probability is >0.5, the variation is predicted as disease associated. MutationTaster was used to predict the effect of synonymous variants (Schwarz et al., 2014). If the probability value is close to 1, this indicates a high certainty of the prediction.

2.4. Statistical analyses

For common *SQSTM1* variants with minor allele frequency (MAF) >1%, deviations from Hardy-Weinberg equilibrium were assessed using an exact Hardy-Weinberg equilibrium test (www.pharmgat.org/IIPGA2/Bioinformatics/exacthweform), and allele frequencies were compared between AD patients and healthy control individuals using χ^2 statistics. ORs (calculated relative to the common genotype) and 95% CIs were calculated using a logistic regression model, using SPSS, version 20.0, for Windows (IBM SPSS Inc, Chicago, IL, USA), corrected for onset age (AAO), gender, and *APOE* ε4. A 2-sided *p*-value of 0.05 was considered statistically significant. Fixed-effects (Mantel-Haenszel) meta-analysis of the common variants was performed based on raw allele data of the different EU EOD cohorts. The Czech (7 patients and 7 control individuals) and German (patients only) cohorts were not included in the association analysis. Mantel-Haenszel summary OR and Woolf test for heterogeneity were computed in R using the library rmeta, version 2.16. We performed rare variant burden analysis on the cumulative frequency of nonsynonymous variant alleles with MAF <1% either spanning the full exonic region of *SQSTM1* or affecting different protein domains using χ^2 statistics. As for the common variants, meta-analysis (Mantel-Haenszel) of rare variant alleles was performed, following the same procedures as described earlier. Protein domains were assigned as described previously (van der Zee et al., 2014).

3. Results

3.1. SQSTM1 mutation screening in the Flanders-Belgian cohort

Sequencing of the *SQSTM1* CDS in the Flanders-Belgian cohort resulted in the identification of 26 rare variants (MAF <0.01), of which 14 variations were nonsynonymous (Table 1). Two of these variants (p.A33V and p.P438L) were absent from 872 Belgian control individuals. The amino acid substitution p.P438L, located in the C-terminal region of the UBA domain of the protein and predicted to be damaging for protein structure and/or function, was previously described in a patient with ALS (Rubino et al., 2012). The mutation was found in 2 AD patients with onset ages of 67 and 75 years. The 2 AD patients shared a second nonsynonymous variation, p.E274D, which is a low-frequency variant (MAF 0.025). The AD patient with AAO of 75 years also carried a third rare nonsynonymous variant, which is the other variant that was absent from control individuals, that is, p.A33V. This variant is located in the first exon of *SQSTM1*, encoding the Phox and Bem1p (PB1) domain. This variation was absent from our Flanders-Belgian control cohort, but has been reported before at low frequency in public databases, and is predicted benign based on impact on protein structure and function (Supplementary Table 3). Review of clinical records of both patients did not show evidence of ALS or PDB, although on X-ray of the skull of the patient with AAO of 75 years, a diploic skull was noted. Further, 2 synonymous variants (p.P232= and p.S361=) were found in patients only located in tumor necrosis factor receptor–associated factor 6 (TRAF6) and proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST2) domains. Sixteen variants were observed in control individuals only, of which 9 were nonsynonymous.

3.2. Rare variant association analysis in the Flanders-Belgian cohort

No significant difference in total number of rare variations (MAF <0.01) was identified between the Belgian AD (14/870 = 0.016) and control individuals (24/1744 = 0.014) (relative risk [RR] = 0.94 [95% CI = 0.57–1.55], allelic $p = 0.8$) (Table 2). When investigating rare variant burden in the different functional protein domains of SQSTM1, we did not observe a significant increase in rare variants in specific domains in AD patients versus control individuals. The low-frequent variant p.E274D (and p.S318=, in strong LD) was observed slightly more often in patients (MAF 0.025) than control individuals (MAF 0.018), but this did not reach statistical significance (OR = 1.67 [95% CI = 0.91–3.05], allelic $p = 0.096$). Inclusion of this variant in the whole gene burden analysis (RR = 1.16 [95% CI = 0.91–1.49], allelic $p = 0.23$) or analysis of the PEST1 domain (RR = 1.2 [95% CI = 0.72–2.16], allelic $p = 0.44$) in which it is located did not change the observations.

3.3. Association of common SQSTM1 variants in the Flanders-Belgian cohort

Two common polymorphisms with MAF >0.05 were observed in the CDS of *SQSTM1*, both synonymous (p.D292= and p.R312=). Allelic association with AD was observed for both variants p.D292= (OR = 1.22 [95% CI = 1.01–1.46], allelic $p = 0.037$) and p.R312= (OR = 1.23 [95% CI = 1.02–1.48], allelic $p = 0.03$) that are in strong pairwise linkage disequilibrium (HapMap $D' = 0.915$ in Centre d'Etude du

Table 2
Whole-gene rare variant burden analysis per country

Country	Rare alleles/total alleles AD patients	Rare alleles/total alleles control individuals	Fisher exact (p value)
Belgium	14/870 (1.6%)	24/1744 (1.4%)	0.61
Spain	15/658 (2.3%)	21/968 (2.2%)	0.87
Italy	10/420 (2.4%)	11/1036 (1.1%)	0.09
Portugal	5/214 (2.3%)	6/254 (2.4%)	1.00
Sweden	6/350 (1.7%)	6/680 (0.9%)	0.24
Meta-analysis	50/2512 (2%)	68/4682 (1.5%)	OR = 1.32 (95% CI = 0.91–1.91) p value = 0.14 Heterogeneity – p value = 0.6

All nonsynonymous rare alleles were taken into account to perform the burden analysis. Fisher exact 2-tailed p values are shown for the individual populations. Mantel-Haenszel summary OR and Woolf test for heterogeneity are shown for the meta-analysis of the 5 cohorts.
Key: AD, Alzheimer's disease; CI, confidence interval; OR, odds ratio.

Polymorphisme Humain [Utah residents with ancestry from northern and western Europe] [CEU] population). Conditional logistic regression was performed to investigate if the observed association between AD and these common variants was mediated by the borderline effect of the low-frequency variant p.E274D (OR = 1.22 [95% CI = 1.01–1.47], nominal allelic $p = 0.04$) or the presence of rare alleles (OR = 1.23 [95% CI = 1.02–1.49], nominal allelic $p = 0.034$). None of these conditions could affect the association with AD.

3.4. Replication analyses in the EU EOD cohort

To increase power to interpret the findings of the Flanders-Belgian AD cohort, we extended our analysis to the EU EOD cohort, including 926 patients and 1476 control individuals originating from Spain, Portugal, Italy, Sweden, Germany, and Czech Republic. In total, 48 variations, both synonymous and nonsynonymous, were identified in the exonic sequence of the *SQSTM1* gene. Of these, 44 variants were rare (MAF <1%) of which 23 caused a change at the protein level, 4 in AD patients only, 9 in controls only, and 10 in both patients and controls (Fig. 1, Supplementary Tables 2 and 3). Of the 4 variants that were only identified in AD patients and excluded from the tested control population, 2 variants were never described before in the context of PDB, ALS, or FTLD: p.P29S and p.L268V (Table 3). The patient carrying the p.P29S mutation also carried a second *SQSTM1* variant (p.A117V) and a pathogenic mutation in the *PSEN1* (p.L392V) gene, which most likely explain the early onset age of 40 years. Furthermore, the AD patient who carried the p.L268V mutation also carried another mutation (p.P397L) that was also excluded from the control population but was earlier described in context of PDB.

Rare variant (MAF <0.01) burden analysis did not reveal an increased frequency of rare variants in *SQSTM1* in EOAD patients in any of the separate study populations nor when meta-analyzing all EU EOD cohorts of the consortium (OR = 1.39 [95% CI = 0.89–2.17], $p = 0.14$) (Table 2). Inclusion of the Flanders-Belgian cohort in the meta-analysis did not change the outcome (OR = 1.32 [95% CI = 0.91–1.91], $p = 0.14$) (Table 2). Furthermore, we found no evidence of predominant clustering of disease-causing alleles in specific protein domains in separate cohorts or in a meta-analysis with or without inclusion of the Flanders-Belgian cohort (data not shown). Meta-analysis of the low-frequent variant p.E274D (and p.S318=, in strong LD) in the different EU EOD cohorts did not reach statistical significance ($OR_{p.E274D} = 0.9$ [95% CI = 0.6–1.34], allelic $p = 0.59$). Inclusion of this variant in the whole-gene burden meta-analysis of the Flanders-Belgian and EU EOD cohorts (OR = 1.14 [95% CI = 0.89–1.46], allelic $p = 0.28$) did not change the observations. Remarkably, 17 of 29 synonymous variants were predicted to be "disease causing" by MutationTaster. However, inclusion of these variants in the rare variant meta-analysis did not show evidence of association with AD (OR = 1.15 [95% CI = 0.87–1.53], allelic $p = 0.32$). The common variants p.D292= and p.R312= showed association with AD ($OR_{p.D292=} = 1.11$ [95% CI = 1–1.22], nominal $p = 0.04$) (Fig. 2) but only when including the Flanders-Belgian cohort. Meta-analysis excluding the Flanders-Belgian cohort did not show evidence of association ($OR_{p.D292=} = 1.07$ [95% CI = 0.95–1.21], $p = 0.27$).

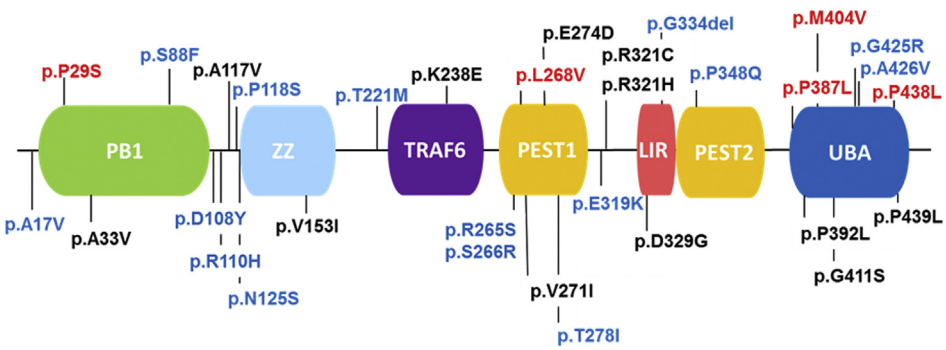

Fig. 1. Nonsynonymous sequestosome 1 (*SQSTM1*) mutations identified in Alzheimer's disease (AD) and control cohorts from Flanders-Belgian population and the European early-onset dementia consortium. Protein domains are indicated (transcript level on NM_003900.4 and protein level on the GenPept Accession Number NP_003891.1). Protein domains were assigned as described previously (van der Zee et al., 2014). PB1, PhoX and Bem1P; ZZ, zinc finger (zz type); TRAF6, tumor necrosis factor receptor–associated factor 6; PEST, regions rich in proline, glutamate, serine, and threonine; LIR, LC3-interacting region; and UBA, ubiquitin associated. Variants that were only identified in AD patients ($n = 5$) in our study are indicated in red. Variants that were only identified in control individuals ($n = 15$) in our study are indicated in blue. Variants identified in both AD patients and control individuals ($n = 12$) are indicated in black.

Table 3
SQSTM1 mutations present in patients and absent from the control cohorts that were screened for this study

Mutation	Functional domain	Origin	Gender	Clinical diagnosis	Family history	Age at onset (y)	Previously reported
p.P29S[a]	PB1	Italy	f	Definite AD	S	40	No
p.L268V	PEST1	Italy	f	Probable AD	S	58	No
p.P387L	UBA	Italy	f	Probable AD	S	58	FTLD/PDB
p.M404V	UBA	Italy	m	Probable AD	S	52	PDB
p.P438L	UBA	Belgium	f	Probable AD	F	67	SALS
			f	Probable AD	F	75	

Protein domains were assigned as described previously (van der Zee et al., 2014). More information on the AD patients carrying the mutations can be found in the columns "origin," "gender," clinical diagnosis," "family history" (sporadic [S] or familial [F]), and "age at onset." The column "previously reported" shows the variants that were previously described in context of ALS, FTLD, or PDB (Le Ber et al., 2013; Rea et al., 2013; Rubino et al., 2012). Rare variants p.L268V and p.P387L were carried by the same AD patient, originating from Italy.
Key: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; F, familial; f, female; FTLD, frontotemporal lobar degeneration; m, male; PB1, Phox and Bem1p; PDB, Paget disease of the bone; PEST1, proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST); S, sporadic; SALS, sporadic amyotrophic lateral sclerosis; *SQSTM1*, sequestosome 1; UBA, ubiquitin associated.
[a] Carried a known pathogenic mutation for AD (presenilin-1 [*PSEN1*] p.L392V).

4. Discussion

In this study, we have investigated the presence of common and rare exonic variants in *SQSTM1* in a total of 1361 early-onset and/or familial AD patients and 2348 healthy individuals from 7 countries across Europe. We detected a total of 61 variants in the exonic region of *SQSTM1*, of which the majority ($n = 57$) were rare and identified in only one or few individuals, suggesting a high genetic variability of *SQSTM1*. We identified 5 variants that were not present in our tested control population of which one (p.P438L) was earlier described in the context of ALS (Rubino et al., 2012). Two variants, (p.P29S and p.L268V) that were only identified in our AD population, were excluded from publicly available databases (Exome Variant Server, dbSNP, and Ensemble). Overall, however, rare *SQSTM1* variants were identified at equal frequencies in AD patients and control individuals across populations (cumulative frequencies ranging from 0.9% to 2.8%), suggesting no major causal role for rare *SQSTM1* variants in the pathogenesis of early-onset AD. Of note, 2 of the variants we identified in patients only are known to be pathogenic in PDB (Rea et al., 2013). Other known pathogenic mutations for PDB were identified both in AD patients and control individuals, and the frequency of these mutations corresponded to the prevalence of PDB in the general population (1%–2%) (Ralston et al., 2008). Unfortunately, our patient cohorts were not systematically screened for clinical or radiological signs of PDB, precluding further inferences.

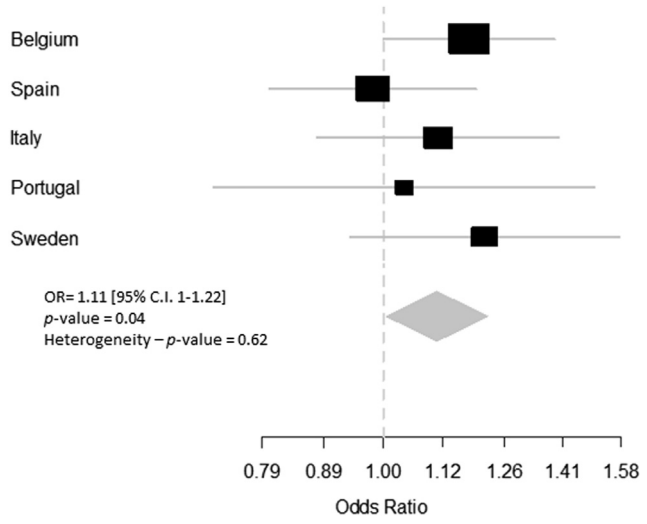

Fig. 2. Common variant meta-analysis of the Flanders-Belgian and European early-onset dementia cohorts: p.D292=.

Two AD patients harbored multiple rare variants in *SQSTM1*, and 2 patients carried both a *PSEN1* and a *SQSTM1* mutation. Double *SQSTM1* mutations were described earlier in the context of PDB (Collet et al., 2007) and ALS (Shimizu et al., 2013). This could imply that individual mutation burden of *SQSTM1* could modify disease susceptibility; however, additional systematic screening efforts are required to investigate this further. Of note, 2 control individuals also carried several *SQSTM1* variants.

Resequencing of the full coding region of *SQSTM1* revealed only 4 variants at individual frequencies >1%. Two common synonymous variants, which are in strong pairwise LD, showed marginal evidence of association with AD. These variations exert no obvious effect on protein, but in silico predictions (MutationTaster, Schwarz et al., 2014) suggest that they might introduce a splice site. Both SNPs are in pairwise LD with the GWAS top SNP rs72807343 ($D' = 1$), although a large difference in frequency of occurrence was found ($r^2 = 0.011$). However, the observed association appeared limited to the Flanders-Belgian population and would not have survived correction for multiple testing. Moreover, although the GWAS top SNP was not covered by the genotyping assays in the present study because of its localization outside the coding sequence of *SQSTM1*, it had previously been genotyped by custom Illumina SNP chip in the replication stage of an AD GWAS meta-analysis in part of our Flanders-Belgian late-onset AD cohort (887 AD patients and 674 control individuals; overlap with the patient cohort described here $n = 343$) (Lambert et al., 2013). In this subset of the Flanders-Belgian population, rs72807343 did not reveal statistical association with AD (OR = 0.83 [95% CI = 0.45–1.54], allelic $p = 0.56$).

One low-frequent missense variant, p.E274D (MAF 2%), showed a trend toward association in the Flanders-Belgian AD cohort. Interestingly, this variant showed tentative evidence of association in the International Genomics of Alzheimer's Project exome chip data analysis, which is performed on late-onset AD patients and control individuals (S. van der Lee and C.M. van Duijn, personal communication 2014). Nevertheless, when meta-analyzing the EU EOD cohort, this trend toward association disappeared. Of note, the Flanders-Belgian patient group had a higher average onset age than the EOD cohorts because of inclusion of familial AD patients with onset >65 years. Conceivably, this might explain why we cannot confirm the Flanders-Belgian trend toward association between *SQSTM1* variants and AD in the EU EOD cohort, which should have sufficient statistical power (>90%) to detect a risk allele with MAF 2% and OR 1.67 at alpha level of 0.05. In line with this, the GWAS association at *SQSTM1* was predominantly based on late-onset AD (Lambert et al., 2013).

In conclusion, in this European study on AD patients with early-onset and/or positive family history, thus likely to have an augmented genetic risk profile, we observed 61 variants in the exonic region of *SQSTM1* (comprising only 8 exons), both in patients and in cognitively healthy individuals, suggesting a high genetic variability of the gene. We cannot exclude a role of *SQSTM1* genetic variability in late-onset AD, but our data indicate that common and rare coding variations in *SQSTM1* do not play a major role in the etiology of early-onset AD.

Disclosure statement

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors are grateful to the personnel of the Genomic Service Facility and of the Bioinformatics Unit of the VIB Department of Molecular Genetics for their support of the genetic analyses and to Dr Johan Goeman, Hospital Network Antwerp (ZNA) Memory Clinic, Antwerp, Belgium. The data generation for this article was in part funded by the Belgian Science Policy Office Interuniversity Attraction Poles program (http://www.belspo.be/), the Alzheimer Research Foundation (S#13023) (http://alzh.org/), the Flemish Government Initiated Methusalem Excellence Program to CVB, the Research Foundation Flanders (G043211N) (FWO, http://www.fwo.be/), the Agency for Innovation by Science and Technology, Flanders (111477, IWT), the University Research Fund, the Medical Research Foundation Antwerp, Belgium, the Flemish Government initiated Flanders Impulse Program on Networks for Dementia Research, the MetLife Foundation Research Award to CVB, and the EU FP7 project AgedBrainSYSBIO under grant agreement N° 305299 (http://ec.europa.eu/research/fp7). EC is a PhD fellow of the IWT, and KB is a postdoctoral fellow of the FWO. RV is a senior clinical investigator of the FWO. The Barcelona IDIBAPS site (RS, AL, and EG) was partially financed by a grant to AL (PI11/00234, ISCIII, Cofinancia Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, Otra manera de hacer Europa). They are indebted to the Neurological Tissue Bank of the IDIBAPS Biobanc in Barcelona, Spain, for sample and data procurement and to brain donors and relatives for generous donation for research. The Barcelona Sant Pau site (JC, AL, and JF) was partially supported by grants from Instituto de Salud Carlos III (PI12/01311). The Barcelona ACE site (AR) thanks the controls who participated in this project. We are indebted to Trinitat Port-Carbó and her family who are supporting Fundació ACE research programs. AR is supported by grant PI13/02434 (Acción Estratégica en Salud Instituto de Salud Carlos III (ISCIII) Ministerio de Economía y Competitividad, Spain) and Obra Social "La Caixa" (20131209) (Barcelona, Spain). The Prague site (RM and ZR) was partly supported by grant IGA NT12094-5 from Grant Agency of Ministry of Health and Charles University Project PRVOUK P26/1/4. The Lisbon site (AM and MM) was supported by the Fundação para a Ciência e a Tecnologia (SFRH/BPD/29354/2006 to MM) and thank Gabriel Miltenberger-Miltényi and Mafalda Matos for helpful comments and technical support. The Brescia IRCCS Fatebenefratelli site was funded by the Ricerca Corrente, Italian Ministry of Health. From the Florence site, BN is funded by Cassa di Risparmio di Pistoia e Pescia (CRPT 2013/0347). SS is funded by Cassa di Risparmio di Firenze (CRF 2013/0199) and from Ministry of Health n° RF-2010-2319722.M. The Sweden site (CG, HT, and HC) acknowledges the financial support by Swedish Brain Power, Swedish Research Council (grant number 521-2010-3134, A031340), the King Gustaf V and Queen Victoria's Foundation of Freemasons, and the Foundations of Marianne and Marcus Wallenberg, Knut and Alice Wallenberg, Gun and Bertil Stohne, Gamla tjänarinnor, Demensfonden, Swedish Alzheimer Foundation (N° 462081), and StratNeuro at Karolinska Institute (KI). Furthermore, they thank Jenny Björkström, Anne Kinhult Ståhlbom, and Marie Fallström (Department of Geriatric Medicine, Genetics Unit, Karolinska University Hospital, Stockholm, Sweden); Charlotte Forsell, Lena Lilius, and Lukas Graff (Department of Neurobiology, Care Sciences and Society [NVS], Center for Alzheimer Research, Division of Neurogeriatrics, Karolinska Institutet, Huddinge, Sweden); and Laura Fratiglioni (Aging Research Center, Department of Neurobiology, Care Sciences and Society [NVS], Karolinska Institutet and Stockholm University, Stockholm, Sweden).

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2015.02.014.

References

Bettens, K., Brouwers, N., Van Miegroet, H., Gil, A., Engelborghs, S., De Deyn, P.P., Vandenberghe, R., Van Broeckhoven, C., Sleegers, K., 2009. Follow-up study of susceptibility loci for Alzheimer's disease and onset age identified by genome-wide association. J. Alzheimers Dis. 19, 1169–1175.

Bjorkoy, G., Lamark, T., Johansen, T., 2006. p62/*SQSTM1*: a missing link between protein aggregates and the autophagy machinery. Autophagy 2, 138–139.

Collet, C., Michou, L., Audran, M., Chasseigneaux, S., Hilliquin, P., Bardin, T., Lemaire, I., Cornelis, F., Launay, J.M., Orcel, P., Laplanche, J.L., 2007. Paget's disease of bone in the French population: novel *SQSTM1* mutations, functional analysis, and genotype-phenotype correlations. J. Bone Miner. Res. 22, 310–317.

Engelborghs, S., Dermaut, B., Goeman, J., Saerens, J., Marien, P., Pickut, B.A., van den Broeck, M., Serneels, S., Cruts, M., Van Broeckhoven, C., De Deyn, P.P., 2003. Prospective Belgian study of neurodegenerative and vascular dementia: APOE genotype effects. J. Neurol. Neurosurg. Psychiatry 74, 1148–1151.

Engelborghs, S., Dermaut, B., Marien, P., Symons, A., Vloeberghs, E., Maertens, K., Somers, N., Goeman, J., Rademakers, R., van den Broeck, M., Pickut, B., Cruts, M., Van Broeckhoven, C., De Deyn, P.P., 2006. Dose dependent effect of APOE epsilon4 on behavioral symptoms in frontal lobe dementia. Neurobiol. Aging 27, 285–292.

Fecto, F., Yan, J., Vemula, S.P., Liu, E., Yang, Y., Chen, W., Zheng, J.G., Shi, Y., Siddique, N., Arrat, H., Donkervoort, S., Ajroud-Driss, S., Sufit, R.L., Heller, S.L., Deng, H.X., Siddique, T., 2011. *SQSTM1* mutations in familial and sporadic amyotrophic lateral sclerosis. Arch. Neurol. 68, 1440–1446.

Folstein, M.F., Folstein, S.E., McHugh, P.R., 1975. "Mini-mental state." A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. 12, 189–198.

Hirano, M., Nakamura, Y., Saigoh, K., Sakamoto, H., Ueno, S., Isono, C., Miyamoto, K., Akamatsu, M., Mitsui, Y., Kusunoki, S., 2013. Mutations in the gene encoding p62 in Japanese patients with amyotrophic lateral sclerosis. Neurology 80, 458–463.

Johnson-Pais, T.L., Wisdom, J.H., Weldon, K.S., Cody, J.D., Hansen, M.F., Singer, F.R., Leach, R.J., 2003. Three novel mutations in *SQSTM1* identified in familial Paget's disease of bone. J. Bone Miner. Res. 18, 1748–1753.

Kimonis, V.E., Fulchiero, E., Vesa, J., Watts, G., 2008. VCP disease associated with myopathy, Paget disease of bone and frontotemporal dementia: review of a unique disorder. Biochim. Biophys. Acta 1782, 744–748.

Kuusisto, E., Salminen, A., Alafuzoff, I., 2001. Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. Neuroreport 12, 2085–2090.

Lambert, J.C., Ibrahim-Verbaas, C.A., Harold, D., Naj, A.C., Sims, R., Bellenguez, C., Jun, G., DeStefano, A.L., Bis, J.C., Beecham, G.W., Grenier-Boley, B., Russo, G., Thornton-Wells, T.A., Jones, N., Smith, A.V., Chouraki, V., Thomas, C., Ikram, M.A., Zelenika, D., Vardarajan, B.N., Kamatani, Y., Lin, C.F., Gerrish, A., Schmidt, H., Kunkle, B., Dunstan, M.L., Ruiz, A., Bihoreau, M.T., Choi, S.H., Reitz, C., Pasquier, F., Hollingworth, P., Ramirez, A., Hanon, O., Fitzpatrick, A.L., Buxbaum, J.D., Campion, D., Crane, P.K., Baldwin, C., Becker, T., Gudnason, V., Cruchaga, C., Craig, D., Amin, N., Berr, C., Lopez, O.L., De Jager, P.L., Deramecourt, V., Johnston, J.A., Evans, D., Lovestone, S., Letenneur, L., Moron, F.J., Rubinsztein, D.C., Eiriksdottir, G., Sleegers, K., Goate, A.M., Fievet, N., Huentelman, M.J., Gill, M., Brown, K., Kamboh, M.I., Keller, L., Barberger-Gateau, P., McGuinness, B., Larson, E.B., Green, R., Myers, A.J., Dufouil, C., Todd, S., Wallon, D., Love, S., Rogaeva, E., Gallacher, J., St George-Hyslop, P., Clarimon, J., Lleo, A., Bayer, A., Tsuang, D.W., Yu, L., Tsolaki, M., Bossu, P., Spalletta, G., Proitsi, P., Collinge, J., Sorbi, S., Sanchez-Garcia, F., Fox, N.C., Hardy, J., Naranjo, M.C., Bosco, P., Clarke, R., Brayne, C., Galimberti, D., Mancuso, M., Matthews, F., Moebus, S., Mecocci, P., Del, Z.M., Maier, W., Hampel, H., Pilotto, A., Bullido, M., Panza, F., Caffarra, P., Nacmias, B., Gilbert, J.R., Mayhaus, M., Lannfelt, L., Hakonarson, H., Pichler, S., Carrasquillo, M.M., Ingelsson, M., Beekly, D., Alvarez, V., Zou, F., Valladares, O., Younkin, S.G., Coto, E., Hamilton-Nelson, K.L., Gu, W., Razquin, C., Pastor, P., Mateo, I., Owen, M.J., Faber, K.M., Jonsson, P.V., Combarros, O., O'Donovan, M.C., Cantwell, L.B., Soininen, H., Blacker, D., Mead, S., Mosley Jr., T.H., Bennett, D.A.,

Harris, T.B., Fratiglioni, L., Holmes, C., de Bruijn, R.F., Passmore, P., Montine, T.J., Bettens, K., Rotter, J.I., Brice, A., Morgan, K., Foroud, T.M., Kukull, W.A., Hannequin, D., Powell, J.F., Nalls, M.A., Ritchie, K., Lunetta, K.L., Kauwe, J.S., Boerwinkle, E., Riemenschneider, M., Boada, M., Hiltunen, M., Martin, E.R., Schmidt, R., Rujescu, D., Wang, L.S., Dartigues, J.F., Mayeux, R., Tzourio, C., Hofman, A., Nothen, M.M., Graff, C., Psaty, B.M., Jones, L., Haines, J.L., Holmans, P.A., Lathrop, M., Pericak-Vance, M.A., Launer, L.J., Farrer, L.A., van Duijn, C.M., Van, B.C., Moskvina, V., Seshadri, S., Williams, J., Schellenberg, G.D., Amouyel, P., 2013. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat. Genet. 45, 1452–1458.

Le Ber, I., Camuzat, A., Guerreiro, R., Bouya-Ahmed, K., Bras, J., Nicolas, G., Gabelle, A., Didic, M., De Septenville, A., Millecamps, S., Lenglet, T., Latouche, M., Kabashi, E., Campion, D., Hannequin, D., Hardy, J., Brice, A., 2013. *SQSTM1* mutations in French patients with frontotemporal dementia or frontotemporal dementia with amyotrophic lateral sclerosis. JAMA Neurol. 70, 1403–1410.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., Stadlan, E.M., 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34, 939–944.

McKhann, G.M., Knopman, D.S., Chertkow, H., Hyman, B.T., Jack Jr., C.R., Kawas, C.H., Klunk, W.E., Koroshetz, W.J., Manly, J.J., Mayeux, R., Mohs, R.C., Morris, J.C., Rossor, M.N., Scheltens, P., Carrillo, M.C., Thies, B., Weintraub, S., Phelps, C.H., 2011. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers. Dement. 7, 263–269.

Montine, T.J., Phelps, C.H., Beach, T.G., Bigio, E.H., Cairns, N.J., Dickson, D.W., Duyckaerts, C., Frosch, M.P., Masliah, E., Mirra, S.S., Nelson, P.T., Schneider, J.A., Thal, D.R., Trojanowski, J.Q., Vinters, H.V., Hyman, B.T., 2012. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. Acta Neuropathol. 123, 1–11.

Ralston, S.H., Langston, A.L., Reid, I.R., 2008. Pathogenesis and management of Paget's disease of bone. Lancet 372, 155–163.

Rea, S.L., Walsh, J.P., Layfield, R., Ratajczak, T., Xu, J., 2013. New insights into the role of sequestosome 1/p62 mutant proteins in the pathogenesis of Paget's disease of bone. Endocr. Rev. 34, 501–524.

Reumers, J., De, R.P., Zhao, H., Liekens, A., Smeets, D., Cleary, J., Van, L.P., Van Den Bossche, M., Catthoor, K., Sabbe, B., Despierre, E., Vergote, I., Hilbush, B., Lambrechts, D., Del-Favero, J., 2011. Optimized filtering reduces the error rate in detecting genomic variants by short-read sequencing. Nat. Biotechnol 30, 61–68.

Rubino, E., Rainero, I., Chio, A., Rogaeva, E., Galimberti, D., Fenoglio, P., Grinberg, Y., Isaia, G., Calvo, A., Gentile, S., Bruni, A.C., St George-Hyslop, P.H., Scarpini, E., Gallone, S., Pinessi, L., 2012. *SQSTM1* mutations in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Neurology 79, 1556–1562.

Schwarz, J.M., Cooper, D.N., Schuelke, M., Seelow, D., 2014. MutationTaster2: mutation prediction for the deep-sequencing age. Nat. Methods 11, 361–362.

Seibenhener, M.L., Babu, J.R., Geetha, T., Wong, H.C., Krishna, N.R., Wooten, M.W., 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell. Biol. 24, 8055–8068.

Shimizu, H., Toyoshima, Y., Shiga, A., Yokoseki, A., Arakawa, K., Sekine, Y., Shimohata, T., Ikeuchi, T., Nishizawa, M., Kakita, A., Onodera, O., Takahashi, H., 2013. Sporadic ALS with compound heterozygous mutations in the *SQSTM1* gene. Acta Neuropathol. 126, 453–459.

Teyssou, E., Takeda, T., Lebon, V., Boillee, S., Doukoure, B., Bataillon, G., Sazdovitch, V., Cazeneuve, C., Meininger, V., Leguern, E., Salachas, F., Seilhean, D., Millecamps, S., 2013. Mutations in *SQSTM1* encoding p62 in amyotrophic lateral sclerosis: genetics and neuropathology. Acta Neuropathol. 125, 511–522.

van der Zee, J., Gijselinck, I., Dillen, L., Van Langenhove, T., Theuns, J., Engelborghs, S., Philtjens, S., Vandenbulcke, M., Sleegers, K., Sieben, A., Bäumer, V., Maes, G., Corsmit, E., Borroni, B., Padovani, A., Archetti, S., Perneczky, R., Diehl-Schmid, J., de Mendonça, A., Miltenberger-Miltenyi, G., Pereira, S., Pimentel, J., Nacmias, B., Bagnoli, S., Sorbi, S., Graff, C., Chiang, H.H., Westerlund, M., Sanchez-Valle, R., Llado, A., Gelpi, E., Santana, I., Almeida, M.R., Santiago, B., Frisoni, G., Zanetti, O., Bonvicini, C., Synofzik, M., Maetzler, W., Vom Hagen, J.M., Schöls, L., Heneka, M.T., Jessen, F., Matej, R., Parobkova, E., Kovacs, G.G., Ströbel, T., Sarafov, S., Tournev, I., Jordanova, A., Danek, A., Arzberger, T., Fabrizi, G.M., Testi, S., Salmon, E., Santens, P., Martin, J.J., Cras, P., Vandenberghe, R., De Deyn, P.P., Cruts, M., Van Broeckhoven, C., 2013. A pan-European study of the C9orf72 repeat associated with FTLD: geographic prevalence, genomic instability, and intermediate repeats. Hum. Mutat. 34, 363–373.

van der Zee, J., Pirici, D., Van Langenhove, T., Engelborghs, S., Vandenberghe, R., Hoffmann, M., Pusswald, G., van den Broeck, M., Peeters, K., Mattheijssens, M., Martin, J.J., De Deyn, P.P., Cruts, M., Haubenberger, D., Kumar-Singh, S., Zimprich, A., Van, B.C., 2009. Clinical heterogeneity in 3 unrelated families linked to VCP p.Arg159His. Neurology 73, 626–632.

van der Zee, J., Van Langenhove, T., Kovacs, G.G., Dillen, L., Deschamps, W., Engelborghs, S., Matej, R., Vandenbulcke, M., Sieben, A., Dermaut, B., Smets, K., Van, D.P., Merlin, C., Laureys, A., van den Broeck, M., Mattheijssens, M., Peeters, K., Benussi, L., Binetti, G., Ghidoni, R., Borroni, B., Padovani, A., Archetti, S., Pastor, P., Razquin, C., Ortega-Cubero, S., Hernandez, I., Boada, M., Ruiz, A., de Mendonica, A., Miltenberger-Miltenyi, G., do Couto, F.S., Sorbi, S., Nacmias, B., Bagnoli, S., Graff, C., Chiang, H.H., Thonberg, H., Perneczky, R., Diehl-Schmid, J., Alexopoulos, P., Frisoni, G.B., Bonvicini, C., Synofzik, M., Maetzler, W., Vom Hagen, J.M., Schols, L., Haack, T.B., Strom, T.M., Prokisch, H., Dols-Icardo, O., Clarimon, J., Lleo, A., Santana, I., Almeida, M.R., Santiago, B., Heneka, M.T., Jessen, F., Ramirez, A., Sanchez-Valle, R., Llado, A., Gelpi, E., Sarafov, S., Tournev, I., Jordanova, A., Parobkova, E., Fabrizi, G.M., Testi, S., Salmon, E., Strobel, T., Santens, P., Robberecht, W., De, J.P., Martin, J.J., Cras, P., Vandenberghe, R., De Deyn, P.P., Cruts, M., Sleegers, K., Van Broeckhoven, C., 2014. Rare mutations in *SQSTM1* modify susceptibility to frontotemporal lobar degeneration. Acta Neuropathol. 128, 397–410.

Watts, G.D., Thomasova, D., Ramdeen, S.K., Fulchiero, E.C., Mehta, S.G., Drachman, D.A., Weihl, C.C., Jamrozik, Z., Kwiecinski, H., Kaminska, A., Kimonis, V.E., 2007. Novel VCP mutations in inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia. Clin. Genet. 72, 420–426.

Weckx, S., Del-Favero, J., Rademakers, R., Claes, L., Cruts, M., De Jonghe, P., Van Broeckhoven, C., De Rijk, P., 2005. novoSNP, a novel computational tool for sequence variation discovery. Genome Res. 15, 436–442.

Zatloukal, K., Stumptner, C., Fuchsbichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A., Denk, H., 2002. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. Am. J. Pathol. 160, 255–263.