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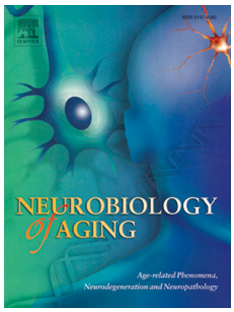

Genetic variability in *SQSTM1* and risk of early-onset Alzheimer dementia: a European Early-Onset Dementia Consortium study

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Genetic variability in *SQSTM1* and risk of early-onset Alzheimer dementia: a European Early-Onset Dementia Consortium study

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

[a] Department of Molecular Genetics, VIB, Antwerp, Belgium

[b] Institute Born-Bunge, University of Antwerp, Antwerp, Belgium

[c] Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken, Antwerp, Belgium

[d] Department of Old Age Psychiatry and Memory Clinic, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium

[e] Karolinska Institutet, Department of Neurobiology, Care sciences and society (NVS), Center for Alzheimer Research, Division of Neurogeriatrics, 14157 Huddinge, Sweden

[f] Department of Geriatric Medicine, Genetics unit, Karolinska University Hospital, Stockholm, Sweden

[g] Neurogenetics Laboratory, Division of Neurosciences, Center for Applied Medical Research, Universidad de Navarra, Pamplona, Spain

[h] Department of Neurology, Hospital Universitari Mutua de Terrassa, Terrassa, Barcelona, Spain.

[i] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas, CIBERNED, Instituto de Salud Carlos III, Madrid, Spain

[j] Neuroimaging Laboratory, Division of Neurosciences, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

[k] Department of Neurology, Clínica Universidad de Navarra, University of Navarra School of Medicine, Pamplona, Spain

[l] Department of Psychiatry and Psychotherapy, Technische Universität München, 81675 München, Germany

[m] Molecular Markers Laboratory - IRCCS Istituto Centro San Giovanni di Dio- Fatebenefratelli, Brescia, Italy

[n] Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA) University of Florence, Florence, Italy

[o] Alzheimer's disease and other cognitive disorders unit. Neurology department, Hospital Clínic, IDIBAPS, Barcelona, Spain

[p] Neurological Tissue Bank of the Biobanc - Hospital Clinic-Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

[q] Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

[r] Department of Neurology, IIB Sant Pau, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain

[s] Faculty of Medicine and Institute of Molecular Medicine, University of Lisbon, Lisbon, Portugal

[t] Neurology Unit, University of Brescia, Brescia, Italy

[u] Center of Clinical Neurosciences, Department of Neurology, First Medical Faculty, Charles University in Prague, Czech Republic

[v] Department of Pathology and Molecular Medicine, Thomayer Hospital, Prague, Czech Republic

[w] Institute of Pathology, Third Medical Faculty of Charles University in Prague, Prague, Czech Republic

[x] Memory Clinic of Fundaciò ACE, Institut Català de Neurociències Aplicades, Barcelona, Spain

[y] Hôpitaux Universitaires de Genève et Université de Genève, Geneva, Switzerland.

[z] IRCCS Fatebenefratelli, Brescia, Italy

[aa] Department of Neurological and Movements Sciences, Section of Neurology, University Hospital G.B. Rossi, University of Verona, Verona, Italy

[bb] Laboratory for Cognitive Neurology, Department of Neurology, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium

[cc] Department of Neurology and Alzheimer Research Center, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands

***Corresponding authors:**

Prof. Dr. Kristel Sleegers, MD, PhD

Neurodegenerative Brain Diseases Group

VIB Department of Molecular Genetics, University of Antwerp - CDE

Universiteitsplein 1, B-2610, Antwerp, Belgium

Phone +32 3 265 1032, Fax +32 3 265 1112

Email: kristel.sleegers@molgen.vib-ua.be

Prof. Dr. Christine Van Broeckhoven PhD DSc

Neurodegenerative Brain Diseases Group

VIB Department of Molecular Genetics, University of Antwerp - CDE

Universiteitsplein 1, B-2610, Antwerp, Belgium

Tel: +3232651102; Fax:+3232651112

Email: christine.vanbroeckhoven@molgen.vib-ua.be

ABSTRACT

Meta-analysis of existing genome-wide association studies on Alzheimer's Disease (AD) showed sub-genome wide association of an intronic variant in the Sequestosome 1 gene (*SQSTM1*) 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 non-affected individuals (n=872) to investigate the role of both common and rare *SQSTM1* variants. Results were extended to the European Early-Onset Dementia (EU EOD) cohorts (926 EOAD patients and 1,476 non-affected individuals).

Of the 61 detected exonic variants in *SQSTM1*, the majority was rare (n=57). Rare variant (MAF<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 ($OR_{p.D292=}$=1.11[95%C.I.1-1.22];*p*-value 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.

Keywords: *SQSTM1*/p62, Alzheimer's disease, rare variants, meta-analysis, European Early-Onset Dementia Consortium

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 (IGAP), identified an intronic variant in the Sequestosome 1 gene (*SQSTM1*), which showed sub-genome wide association with AD (rs72807343; OR 1.35 [1.20-1.52]; *p*-value $7x10^{-7}$) (Lambert *et al.,* 2013). *SQSTM1* encodes the p62 protein which is a stress responsive ubiquitin-binding protein commonly found in neuronal cytoplasmic inclusions in protein aggregation diseases like AD, Parkinson disease, Pick's disease, etc. (Kuusisto et al., 2001; Zatloukal et al., 2002). P62 is involved in protein degradation via the proteasome, in protein aggregation as well as 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 (FALS and SALS) was identified in a European-American population (Fecto *et al.,* 2011). Screening of additional 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; van der Zee et al., 2009; Watts et al., 2007). 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 twofold 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 non-affected individuals (n=872). Our results were extended to a European early-onset dementia (EU EOD) cohort comprising 926 EOAD patients and 1,476 non-affected individuals.

2. Material and methods

2.1 Study population

2.1.1 Flanders-Belgian cohort

We selected 435 AD patients with early-onset age (AAO <65 years) and/or familial disease (at least one first degree relative with the disease) (mean age of onset (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 (P.P.D.D. and S.E.) (Engelborghs et al., 2003; Engelborghs et al., 2006) and the Memory Clinic of the University Hospitals of Leuven (UHL), Leuven, Belgium (M.V., R.V.) (Table 1). Consensus diagnosis of possible and probable AD was given by at least two neurologists based on the National Institute of Neurological and Communication Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984; McKhann et al., 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 and neurological or psychiatric antecedents as well as a familial history of neurodegeneration were ruled out by means of an interview. Cognitive screening was performed using the Mini Mental State examination (MMSE cutoff ≥ 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 European Early-Onset Dementia cohort

Patients and control individuals ascertained through the EU EOD consortium were included as replication cohort (van der Zee *et al.,* Human Mutation 2013; van der Zee *et al.,* Acta Neuropathologica 2014). For this study, DNA and medical/demographic 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; Supplementary material and methods). Patients were diagnosed following the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group international criteria (McKhann et al., 1984; McKhann et al., 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), *PSEN1* (n = 248), *PSEN2* (n = 225), *GRN* (n = 11), *MAPT* (n = 11) (Supplementary material and methods). This revealed 3 *APP*, 14 *PSEN1*, 5 *PSEN2* missense mutations and 1 *GRN* frameshift mutation. Genotyping of *APOE* was performed in the total patient population.

As control group, we sequenced 1,476 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 MMSE 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 supplement (Supplementary Table 1, Supplementary material and methods).

***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, California).

Resequencing of the full *SQSTM1* exonic DNA 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) 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 (MPS) after multiplex amplicon enrichment. To this end, we designed a target enrichment assay based on MASTR^{TM} technology (Multiplicom, Niel, Belgium) covering *SQSTM1* coding exons 2 - 8, flanking intron-exon boundaries and UTR regions. *SQSTM1* exon 1 was screened by Sanger sequencing as described above. 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 prior to bridge amplification and sequencing on an Illumina MiSeq platform, using the Illumina reagent kit v2, generating 250bp paired-end reads. A subset of the control cohort (n=707) was screened using both MASTR MPS 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 (BWA)(Li et al., 2009). Variant calling and annotation was performed using GATKv2.2 (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, 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 PolyPhen-2 (Polymorphism Phenotyping v2; http://genetics.bwh.harvard.edu/pph2/), SIFT (Sorting Intolerant From Tolerant; 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 greater than 0.5, the variation is predicted 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 Analysis

For common *SQSTM1* variants with MAF >1%, deviations from Hardy-Weinberg equilibrium (HWE) were assessed using an exact HWE test (www.pharmgat.org/IIPGA2/Bioinformatics/exacthweform), and allele frequencies were compared between AD patients and healthy control individuals using $\chi 2$ statistics. Odds ratios (OR) (calculated relative to the common genotype) and 95% confidence intervals (95%C.I.) were calculated using a logistic regression model, using SPSS 20.0 Version for Windows (IBM SPSS Inc., Chicago, IL), 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, 7 control individuals) and German (patients only) cohorts were not included in the association analysis. Mantel-Haenszel summary odds ratio and Woolf's 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 non-synonymous variant alleles with MAF <1% either spanning the full exonic region of *SQSTM1* or affecting different protein domains using χ2 statistics. As for the common variants, meta-analysis (Mantel-Haenszel) of rare variant alleles was performed, following the same procedures as described above. 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 non-synonymous (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 two AD patients with onset ages of 67 and 75 years. The two AD patients shared a second non-synonymous 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 non-synonymous variant, which is the other variant that was absent from control individuals, i.e. p.A33V. This variant is located in the first exon of *SQSTM1*, encoding the Phox and Bem1p domain (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 75 years, a diploic skull was noted. Further, two synonymous variants (p.P232= and p.S361=) were found in patients only, located in TNFR-associated factor 6 (TRAF6) and proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST2) domain. Sixteen variants were observed in control individuals only, of which 9 non-synonymous.

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% C.I. 0.57-1.55]; allelic *p*-value 0.8). 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% C.I. 0.91-3.05]; allelic *p*-value 0.096). Inclusion of this variant in the whole gene burden analysis (RR=1.16 [95% C.I. 0.91-1.49]; allelic *p*-value 0.23) or analysis of the PEST1 domain (RR=1.2 [95% C.I. 0.72-2.16]; allelic *p*-value 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% C.I. 1.01-1.46]; allelic *p*-value 0.037) and p.R312= (OR=1.23 [95% C.I. 1.02-1.48]; allelic *p*-value 0.03) which are in strong pairwise linkage disequilibrium (HapMap D' = 0.915 in 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% C.I. 1.01-1.47]; nominal allelic *p*-value 0.04) or the presence of rare alleles (OR=1.23 [95% C.I. 1.02-1.49]; nominal allelic *p*-value 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 1,476 control individuals originating from Spain, Portugal, Italy, Sweden, Germany and Czech Republic. In total, 48 variations, both synonymous and non-synonymous, 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 (Figure 1, Supplementary table 2-3). 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 *Presenilin-1* (*PSEN1* p.L392V) gene, which most likely explains the early onset age of 40 years. Further 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% C.I. 0.89-2.17]; p-value 0.14) (Table 2). Inclusion of the Flanders-Belgian cohort in the meta-analysis did not change the outcome (OR= 1.32 [95% C.I. 0.91-1.91]; p-value 0.14)(Table 2). Further 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% C.I. 0.6-1.34]; allelic *p*-value 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% C.I. 0.89-1.46]; allelic *p*-value 0.28) did not change the observations. Remarkably, 17 out 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% C.I. 0.87-1.53]; allelic *p*-value 0.32). The common variants p.D292= and p.R312= showed association with AD ($OR_{p.D292=}$= 1.11 [95% C.I. 1 -1.22]; nominal *p*-value 0.04) (Figure 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% C.I. 0.95-1.21]; *p*-value 0.27).

4. Discussion

In this study we have investigated the presence of common and rare exonic variants in *SQSTM1* in a total of 1,361 early-onset and/or familial AD patients and 2,348 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) was rare and identified in only one or few individuals, suggesting a high genetic variability of *SQSTM1*. We identified five 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 (EVS), 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, two 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.

Two AD patients harbored multiple rare variants in *SQSTM1*, and two 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, two control individuals also carried several *SQSTM1* variants.

Resequencing of the full coding region of *SQSTM1* revealed only four 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 current 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 mega-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% C.I. 0.45-1.54] allelic *p*-value 0.56).

One low-frequent missense variant, p.E274D (MAF 2%), showed a trend towards association in the Flanders-Belgian AD cohort. Interestingly, this variant showed tentative evidence of association in the IGAP exome chip data analysis, which is performed on late-onset AD patients and control individuals (S. van der Lee – C.M. van Duijn, personal communication). Nevertheless, when meta-analyzing the EU EOD cohort, this trend towards association disappeared. Of note, the Flanders-Belgian patient group had a higher average onset age than the EOD cohorts due to inclusion of familial AD patients with onset >65 years. Conceivably, this might explain why we cannot confirm the Flanders-Belgian trend towards 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 as well as rare coding variations in *SQSTM1* do not play a major role in the etiology of early-onset AD.

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Disclosure statement

The authors declare that they have no conflicts of interest.

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

	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). – denotes not applicable. Total allele count for the Belgian population is 870 AD alleles and 1,744 control alleles. A comparison of frequencies with those in Exome Variant Server (EVS) in European samples (at least 7,388 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. OR: Odds ratio; Freq: frequency.

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 non-synonymous 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).

Table 2. Whole gene rare variant burden analysis per country.

Country	Rare alleles/total alleles AD patients	Rare alleles/total alleles control individuals	Fisher's Exact (*p*-value)
Belgium	14/870 (1.6%)	24/1,744 (1.4%)	0.61
Spain	15/658 (2.3%)	21/968 (2.2%)	0.87
Italy	10/420 (2.4%)	11/1,036 (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	**50/2,512 (2%)**	**68/4,682 (1.5%)**	**OR= 1.32 [95% C.I. 0.91-1.91]** ***p*-value = 0.14** **Heterogeneity – *p*-value = 0.6**

All non-synonymous rare alleles were taken into account to perform the burden analysis. Fisher's Exact two-tailed *p*-values are shown for the individual populations. Mantel-Haenszel summary odds ratio and Woolf's test for heterogeneity are shown for the meta-analysis of the 5 cohorts.

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 (years)	Previously Reported
p.P29S[#]	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	

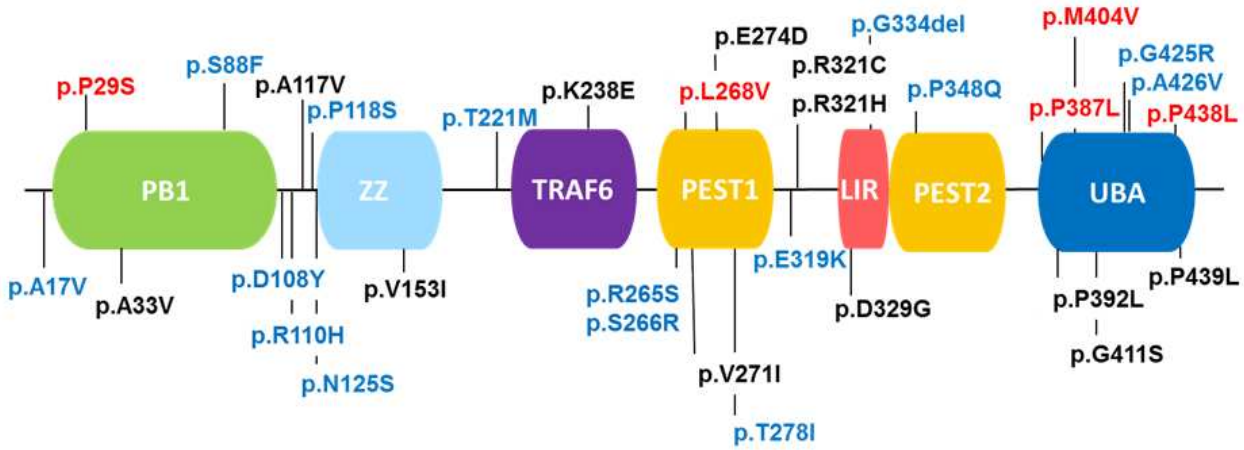
Protein domains were assigned as described previously (van der Zee *et al.,* 2014). More information on the AD patients carrying the mutations can be found in the columns 'Origin', 'Gender', 'Clinical Diagnosis', 'Family History' (Sporadic (S) or Familial (F)) and 'Age at Onset'. The column 'Previously 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. [#]Carried a known pathogenic mutation for AD (*PSEN1* p.L392V).

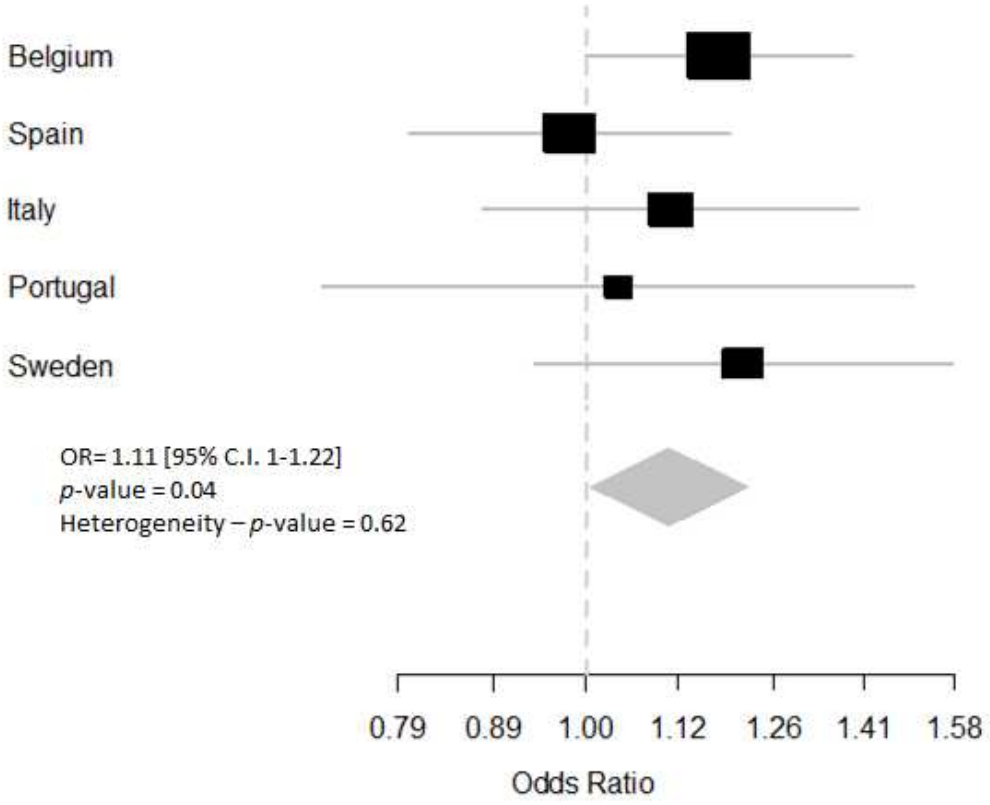
FIGURES

Figure 1. Non-synonymous *SQSTM1* mutations identified in AD and control cohorts from Flanders-Belgian population and the European EOD 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 Bem 1P. 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. 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.

Figure 2. Common variant meta-analysis of the Flanders-Belgian and EU EOD cohorts: p.D292=.





Highlights

- Targeted resequencing of *SQSTM1* gene in early-onset Alzheimer dementia is presented
- *SQSTM1* shows a high genetic variability
- Rare *SQSTM1* variants are not overrepresented in EOAD compared to controls

SUPPLEMENTARY MATERIAL AND METHODS

Genetic variability in *SQSTM1* and risk of early-onset Alzheimer dementia: a European Early-Onset Dementia Consortium study

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

[a] Department of Molecular Genetics, VIB, Antwerp, Belgium
[b] Institute Born-Bunge, University of Antwerp, Antwerp, Belgium
[c] Department of Neurology and Memory Clinic, Hospital Network Antwerp Middelheim and Hoge Beuken, Antwerp, Belgium
[d] Department of Old Age Psychiatry and Memory Clinic, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium
[e] Karolinska Institutet, Department of Neurobiology, Care sciences and society (NVS), Center for Alzheimer Research, Division of Neurogeriatrics, 14157 Huddinge, Sweden
[f] Department of Geriatric Medicine, Genetics unit, Karolinska University Hospital, Stockholm, Sweden
[g] Neurogenetics Laboratory, Division of Neurosciences, Center for Applied Medical Research, Universidad de Navarra, Pamplona, Spain
[h] Department of Neurology, Hospital Universitari Mutua de Terrassa, Terrassa, Barcelona, Spain.
[i] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas, CIBERNED, Instituto de Salud Carlos III, Madrid, Spain
[j] Neuroimaging Laboratory, Division of Neurosciences, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain
[k] Department of Neurology, Clínica Universidad de Navarra, University of Navarra School of Medicine, Pamplona, Spain
[l] Department of Psychiatry and Psychotherapy, Technische Universität München, 81675 München, Germany
[m] Molecular Markers Laboratory - IRCCS Istituto Centro San Giovanni di Dio- Fatebenefratelli, Brescia, Italy
[n] Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA) University of Florence, Florence, Italy
[o] Alzheimer's disease and other cognitive disorders unit. Neurology department, Hospital Clínic, IDIBAPS, Barcelona, Spain
[p] Neurological Tissue Bank of the Biobanc - Hospital Clinic-Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
[q] Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[r] Department of Neurology, IIB Sant Pau, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain
[s] Faculty of Medicine and Institute of Molecular Medicine, University of Lisbon, Lisbon, Portugal
[t] Neurology Unit, University of Brescia, Brescia, Italy

[u] Center of Clinical Neurosciences, Department of Neurology, First Medical Faculty, Charles University in Prague, Czech Republic
[v] Department of Pathology and Molecular Medicine, Thomayer Hospital, Prague, Czech Republic
[w] Institute of Pathology, Third Medical Faculty of Charles University in Prague, Prague, Czech Republic
[x] Memory Clinic of Fundaciò ACE, Institut Català de Neurociències Aplicades, Barcelona, Spain
[y] Hôpitaux Universitaires de Genève et Université de Genève, Geneva, Switzerland.
[z] IRCCS Fatebenefratelli, Brescia, Italy
[aa] Department of Neurological and Movements Sciences, Section of Neurology, University Hospital G.B. Rossi, University of Verona, Verona, Italy
[bb] Laboratory for Cognitive Neurology, Department of Neurology, University of Leuven and University Hospitals Leuven Gasthuisberg, Leuven, Belgium
[cc] Department of Neurology and Alzheimer Research Center, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands

***Corresponding authors:**
Prof. Dr. Kristel Sleegers, MD, PhD
Neurodegenerative Brain Diseases Group
VIB Department of Molecular Genetics, University of Antwerp - CDE
Universiteitsplein 1, B-2610, Antwerp, Belgium
Phone +32 3 265 1032, Fax +32 3 265 1112
Email: kristel.sleegers@molgen.vib-ua.be

Prof. Dr. Christine Van Broeckhoven PhD DSc
Neurodegenerative Brain Diseases Group
VIB Department of Molecular Genetics, University of Antwerp - CDE
Universiteitsplein 1, B-2610, Antwerp, Belgium
Tel: +3232651102; Fax:+3232651112
Email: christine.vanbroeckhoven@molgen.vib-ua.be

Detailed description of the European Early-Onset Dementia cohort

For the *Pamplona cohort*, patients (n = 171, mean onset age 59y (±SD 5.47y), 50.68% familial, 61.4% women) were ascertained in an out-clinics hospital-based recruitment. Patients were diagnosed with sporadic or familial EOAD (disease onset ≤65y) and fulfilled clinical criteria for probable AD (McKhann et al., 1984). Familial AD was considered when the proband had a first degree relative/s clinically diagnosed with dementia. Mutation screening of known dementia genes (*PSEN1*, *PSEN2* and *APP* genes) was performed in 50% of the patients. Control individuals (n = 234, 62.8% women) consisted of spouses of out-clinic patients with neurodegenerative disease with no family history of neurological or psychiatric diseases and apparently with normal cognition. The study was approved by the Ethics Committee of the "Clinica Universidad de Navarra" and informed written consent was obtained for all participants.

For the *Barcelona Hospital Clinic- IDIBAPS cohort*, patients (n = 69, mean onset age 57y (±SD 4.48), 45.16% familial, 57.97% women) were ascertained in a University hospital-based study. Patients were diagnosed following the National Institute of Aging-Alzheimer's association criteria for probable AD with high level of evidence of AD pathophysiological process (McKhann et al., 2011). Mutation screening of *PSEN1* and *APP* genes were performed only if the patient referred familial history of EOAD. Control individuals (n = 47, mean age at inclusion 60y (±SD 12.12), 63.83% women) consisted of both community-dwelling volunteers and family members, mostly spouses, of participants. Individuals were selected for normal cognition according to age and education in a comprehensive cognitive battery. Family history of neurodegenerative or psychiatric disease was not considered an exclusion criteria for control individuals. The study was approved by the Ethics Committee of the Barcelona Hospital Clinic and informed consent was obtained for all participants.

For the *Barcelona Sant Pau cohort*, patients (n = 51, mean onset age 58y (±SD 3.22), 51.06% familial, 58.8% women) were ascertained in a hospital-based study. All subjects were diagnosed by neurologists with expertise in neurodegenerative diseases from a specialized Memory Unit, and undergone formal cognitive evaluation using a comprehensive neuropsychological battery. Diagnosis of AD was established according to the National Institute on Neurological Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINDS-ADRDA) guidelines (McKhann et al., 1984). Mutations in Mendelian AD genes (*PSEN1*, *PSEN2* and *APP*) were discarded in 15 individuals by means of Sanger sequencing of the respective coding sequences for *PSEN1* and *PSEN2* and exons 16 and 17 for the *APP* gene. The study was approved by the Sant Pau Hospital Ethics Committee and informed consent was obtained for all participants.

For *the Barcelona IDIBAPS Brain bank cohort*, pathology confirmed patients (n = 40), mean onset age 55y (±SD 4.99), 50% familial, 37.5% women) were ascertained from the Barcelona IDIBAPS biobank. Patients were diagnosed following currently accepted diagnostic criteria (Montine et al., 2012). In cases with positive family history, screening for mutations in *PSEN1*, *PSEN2* and /or *APP* genes was performed. The study was approved by the Ethics Committee of the Hospital Clínic de Barcelona (ref: 2011/ 6450) and informed consent was obtained for all participants.

For the *Barcelona Alzheimer Treatment and Research Center cohort*, control individuals (n = 209, mean age at inclusion 67.75y (±SD 8.55), 70.81% women) were neurologically normal elderly controls. All of them were screened for the absence of cognitive impairment by a structured interview including neurological mental status examination, category fluency test, and Folstein MMSE (Ramirez-Lorca et al., 2009). The study was approved by the Ethical Committee of the Hospital Clinic i Provincial (Barcelona, Spain) and informed consent was obtained for all participants.

For the *Brescia IRCCS Fatebenefratelli cohort*, patients (n = 95, mean onset age 59y (±SD 6.79), 65.26% familial, 65.26% women) were ascertained in a hospital-based study. Patients were diagnosed following the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Work Group international

criteria (McKhann et al., 1984; McKhann et al., 2011). Mutation screening of known dementia genes, was done in 10.5% of patients. Control individuals (n = 228, 60.09% women) consisted of volunteers (mainly spouses and unrelated caregivers of patients). Family history of neurodegenerative or psychiatric diseases was not excluded. The study was approved by the local ethical committee (Comitato Etico delle Istituzioni Ospedaliere Cattoliche CEIOC – Brescia, Italy) and informed consent was obtained for all participants.

For the *Florence cohort, patients* (n = 94, mean onset age 54y (±SD 7.62), 25.53% familial, 68.09% women) were ascertained in a hospital-based study. Clinical assessment was done according to published guidelines, and the AD diagnosis fulfilled the Diagnostic and Statistical Manual of Mental Disorders criteria (DSM-IV) (The Dementia Study Group of the Italian Neurological Society 2000). Control individuals (n = 146, mean age at inclusion 63y (±SD 9.12), 60.27% women) were recruited from the same region and they were carefully assessed by means of a rigorous diagnostic evaluation, so as to exclude any possible neurological disorder. The local ethical committee approved the protocol and written consent was obtained from all subjects or, where appropriate, their caregivers.

For the *Brescia University cohort*, patients (n = 21, mean onset age 56y (±SD 7.06), 57.89% familial, 80.95% women) were ascertained in a hospital-based study. Patients were diagnosed following current clinical diagnostic criteria. Control individuals (n = 81, 61.73% women) consisted of community-dwelling volunteers and spouses. The study was approved by the Ethics Committee of the Brescia University Hospital and informed consent was obtained for all participants.

The *Brescia IRCCS Fatebenefratelli LENITEM cohort* contributed 56 control individuals (mean age at inclusion 66.8y (±SD 8.15), 53.57% women). The study was approved by the Ethics Committee and informed consent was obtained for all participants.

The *Verona cohort* contributed 7 control individuals (42.86% women). The study was approved by the Ethics Committee and informed consent was obtained for all participants.

For the *Lisbon cohort*, patients (n = 43, mean onset age 55y (±SD 6.84), 83.78% familial, 55.81% women) were ascertained in a hospital and memory-clinic based study. Patients were diagnosed following the NINDS-ADRDA guidelines (McKhann et al., 1984). Control individuals (n = 121) consisted of community-dwelling healthy volunteers with normal cognitive test, GDepressionS, and iADL. The study was approved by the local Ethics Committee and informed consent was obtained for all participants.

For the *Coimbra cohort,* patients (n = 62), mean onset age 57y (SD±6.62), 64.52% women) were ascertained in a hospital-based study. Patients were diagnosed following the NINDS-ADRDA diagnostic criteria (McKhann et al., 1984; McKhann et al., 2011). The study was approved by the local Ethics Committee and informed consent was obtained for all participants.

For the *Prague Brain bank cohort*, pathology confirmed patients (n = 7, mean onset age 56y (±SD 10.57), 25% familial, 28.57% women) were diagnosed following contemporary neuropathological criteria for definite AD (Hyman et al., 2012; The National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease 1997). Screening for *APP*, *PSEN1*, *PSEN2*, *MAPT*, *GRN* and *TARDBP* gene mutations was negative in early-onset cases with positive family history. Control individuals (n = 7, mean age at inclusion 59.71y (±SD 8.38), 28.57% women) were selected from archived group of living individuals without any known neurological or psychiatric disorder and negative family history.

For the *Munich cohort*, patients (n = 98, mean onset age 58y (±SD 4.75), 54.08% women) were ascertained in a hospital-based study. Patients were diagnosed following the NINDS-ADRDA guidelines (McKhann et al., 1984). The study was approved by the Ethics Committee of the Technische Universität München and informed consent was obtained for all participants.

For the *Stockholm cohort*, patients (n = 175, mean onset age 58y (±SD 4.81y), 2.86% familial, 63.43% women) were ascertained in at the Department of Geriatric Medicine, Karolinska University Hospital,

Stockholm, Sweden. Patients were evaluated and diagnosed according to the NINDS-ADRDA guidelines (McKhann et al., 1984; McKhann et al., 2011). The control individuals (n = 340, mean age at inclusion 64y (±SD 5.29y), 61.47% women) consisted of individuals from the population study on persons over 60 years who live in the area of Kungsholmen, Stockholm, Sweden (http://www.snac-k.se/)(Lagergren et al., 2004) and were selected based on an MMSE ≥ 28 and absence of following neurological diseases: frontotemporal dementia (FTD/FTLD), semantic dementia (SD), primary progressive aphasia/progressive non-fluent Aphasia (PPA/PNFA), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS). The study was approved by the local ethics committee in Stockholm and informed consent was obtained for all participants.

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Supplementary

Table 1. Characteristics of the Flanders-Belgian and EU EOD study populations.

	AD Patients					Control individuals			
	Total n	**Familial (%)**	**Disease onset (years)**	**± SD**	**%women**	**Total n**	**Age at inclusion (years)**	**± SD**	**%women**
Flanders-Belgium Cohort	435	52.18	67.7	8.2	62.2	872	66	12.7	55.6
EU EOD consortium									
Spain									
Pamplona Cohort, Spain (P.P.)	171	50.68	59	5.47	61.4	234	N.A.	N.A.	62.82
Barcelona IDIBAPS Cohort, Spain (R.S.)	69	45.16	57	4.48	57.97	47	60.27	12.12	63.83
Barcelona Sant Pau Cohort, Spain (J.C.)	51	51.06	58.59	3.22	58.8	N.A.	N.A.	N.A.	N.A.
Barcelona IDIBAPS Brain bank Cohort, Spain (E.G.)	40	50	55	4.99	37.5	N.A.	N.A.	N.A.	N.A.
Barcelona Alzheimer Treatment and Research Center cohort, Spain (A.R.)	N.A.	N.A.	N.A.	N.A.	N.A.	209	67.75	8.55	70.81
Italy									
Brescia IRCCS Fatebenefratelli cohort, Italy (L.B.)	95	65.26	59	6.79	65.26	228	N.A.	N.A.	60.09
Florence Cohort, Italy (B.N.)	94	25.53	54	7.62	68.09	146	63.23	9.12	60.27
Brescia University Cohort, Italy (B.B.)	21	57.89	56	7.06	80.95	81	N.A.	N.A.	61.73
IRCCS Fatebenefratelli LENITEM cohort, Italy (G.B.F.)	N.A.	N.A.	N.A.	N.A.	N.A.	56	66.8	8.15	53.57
Verona Cohort, Italy (G.M.F.)	N.A.	N.A.	N.A.	N.A.	N.A.	7	N.A.	N.A.	42.86
Portugal									
Lisbon Cohort, Portugal (A.M.)	43	83.78	55	6.84	55.81	121	N.A.	N.A.	N.A.
Coimbra Cohort, Portugal (M.R.A.)	62	N.A.	57	6.62	64.52	N.A.	N.A.	N.A.	N.A.
Czech Republic									
Prague Brain bank, Czech (R.M.)	7	25	56	10.57	28.57	7	59.71	8.38	28.57
Germany									
Munich Cohort, Germany (J.D.-S.)	98	N.A.	58	4.75	54.08	N.A.	N.A.	N.A.	N.A.
Sweden									
Stockholm Cohort, Sweden (C.G.)	175	2.86	58	4.81	63.43	340	64.08	5.29	61.47

Table 2. All variants (n=48) identified in the CDS of *SQSTM1* in the EU EOD cohorts.

				Spain				Italy				Portugal				Sweden			
Position				AD		C		AD		C		AD		C		AD		C	
Protein	dbSNP138	Transcript	Protein Domain	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq	Minor allele	Freq
p.A17V	rs141502868	c.C50T	<PB1	0	0	1	0.001	0	0	0	0	0	0	1	0.004	0	0	0	0
p.P29=	-	c.C87G	PB1	0	0	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
p.P29S	-	c.C85T	PB1	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0
p.A33=	-	c.G99A	PB1	0	0	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
p.A33V	rs200396166	c.C98T	PB1	2	0.003	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.A57=	-	c.G171A	PB1	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0
p.G61=	-	c.C183T	PB1	1	0.002	1	0.001	0	0	2	0.002	0	0	0	0	0	0	6	0.009
p.R68=	-	c.C204G	PB1	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.D80=	-	c.C240T	PB1	0	0	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
p.S88F	-	c.C263T	PB1	0	0	1	0.001	0	0	0	0	0	0	0	0	0	0	2	0.003
Total non-synonymous variants (MAF < 1%) in PB1 domain				**2**		**1**		**1**		**1**		**0**		**0**		**0**		**2**	
p.D108Y	-	c.G322T	PB1-ZZ	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.A117V	rs147810437	c.C350T	PB1-ZZ	1	0.002	1	0.001	1	0.003	0	0	0	0	0	0	5	0.014	3	0.004
p.P118S	rs200152247	c.C352T	PB1-ZZ	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.G133=	-	c.G399C	ZZ	0	0	0	0	0	0	0	0	0	0	1	0.004	1	0.003	0	0
p.S152=	rs145037913	c.C456T	ZZ	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0
Total non-synonymous variants (MAF < 1%) in ZZ domain				**0**		**0**		**0**		**0**		**0**		**0**		**0**		**0**	
p.L166=	rs372518286	c.C498T	ZZ-TRAF6	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0
p.S180=	rs370203737	c.G540A	ZZ-TRAF6	0	0	0	0	0	0	0	0	0	0	0	0	1	0.003	0	0
p.T221M	-	c.C662T	ZZ-TRAF6	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.K238E	rs11548633	c.A712G	TRAF6	4	0.006	6	0.006	3	0.007	4	0.004	1	0.005	1	0.004	1	0.003	0	0
Total non-synonymous variants (MAF < 1%) in TRAF6 domain				**4**		**6**		**3**		**4**		**1**		**1**		**1**		**0**	
p.L268V	-	c.C802G	PEST1	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0

p.T269=	-	c.C807T	PEST1	0	0	0	0	0	0	1	0.001	0	0	1	0.004	0	0	0	0
p.V271I	rs376283809	c.G811A	PEST1	1	0.002	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
p.E274D	rs55793208	c.G822C	PEST1	10	0.015	19	0.019	17	0.042	50	0.048	3	0.015	6	0.024	6	0.017	8	0.012
p.T278I	rs200445838	c.C833T	PEST1	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
p.D292=	rs4935	c.C876T	PEST1	341	0.518	510	0.523	240	0.594	605	0.584	105	0.509	127	0.500	196	0.560	348	0.512
p.P293=	-	c.C879T	PEST1	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
Total non-synonymous variants (MAF < 1%) in PEST1 domain				1		1		1		1		0		0		0		0	
p.G302=	rs11548642	c.C906T	PEST1-LIR	1	0.002	1	0.001	0	0	2	0.002	0	0	0	0	0	0	0	0
p.A308=	rs139482113	c.G924A	PEST1-LIR	0	0	0	0	0	0	3	0.003	0	0	0	0	1	0.003	0	0
p.R312=	rs4797	c.G936A	PEST1-LIR	322	0.489	467	0.479	223	0.552	585	0.565	101	0.490	118	0.465	199	0.569	356	0.524
p.S318=	rs56092424	c.C954T	PEST1-LIR	10	0.015	15	0.015	17	0.042	50	0.048	4	0.019	6	0.024	6	0.017	8	0.012
p.R321C	rs140226523	c.C961T	LIR	2	0.003	2	0.002	0	0	0	0	1	0.005	1	0.004	0	0	0	0
p.R321H	-	c.G962A	LIR	0	0	0	0	0	0	0	0	1	0.005	1	0.004	0	0	0	0
p.S328=	rs146164139	c.G984A	LIR	6	0.009	4	0.004	6	0.015	3	0.003	3	0.014	3	0.012	1	0.003	6	0.009
p.D329G	rs148294622	c.A986G	LIR	1	0.002	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
p.G334del		c.1001_1003 delGAG	LIR	0	0	0	0	0	0	1	0.001	0	0	0	0	0	0	0	0
Total non-synonymous variants (MAF < 1%) in LIR domain				**3**		**3**		**0**		**1**		**2**		**2**		**0**		**0**	
p.V346=	rs150470670	c.G1038A	PEST2	1	0.002	2	0.002	0	0	0	0	0	0	0	0	0	0	0	0
p.P348Q	-	c.C1043A	PEST2	0	0	2[A]	0.002	0	0	0	0	0	0	0	0	0	0	0	0
p.S361=	rs201591177	c.C1083T	PEST2	1	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total non-synonymous variants (MAF < 1%) in PEST2 domain				**0**		**2**		**0**		**0**		**0**		**0**		**0**		**0**	
p.K378=	-	c.G1134A	PEST2-UBA	1	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p.P387L	-	c.C1160T	UBA	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0
p.P392L	rs104893941	c.C1175T	UBA	3	0.005	4	0.004	1	0.003	1	0.001	2	0.009	2	0.008	0	0	0	0
p.P392=	rs75700262	c.G1176A	UBA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.002
p.M404V	-	c.A1210G	UBA	0	0	0	0	1	0.003	0	0	0	0	0	0	0	0	0	0

p.G410=	-	c.C1230T	UBA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.002
p.G411S	rs143511494	c.G1231A	UBA	0	0	1	0.001	1	0.003	0	0	0	0	0	0	0	0	0	0
P.A426V	-	c.C1277T	UBA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0.002
p.A426=	rs143977783	c.G1278A	UBA	1	0.002	0	0	1	0.003	0	0	0	0	2	0.008	0	0	0	0
p.P439L	rs199854262	c.C1316T	UBA	1	0.002	1	0.001	0	0	0	0	0	0	0	0	0	0	0	0
Total non-synonymous variants (MAF < 1%) in UBA domain				**4**		**6**		**4**		**1**		**2**		**2**		**0**		**1**	

Protein and transcript numbering is based on NM_003900.4 and NP_003891.1. Total allele count for the Spanish population is 658 AD alleles and 968 control alleles; for the Italian population is 420 AD alleles and 1,036 control alleles; for the Portuguese population is 214 AD alleles; and 254 control alleles and for the Sweden population is 350 AD alleles and 680 control alleles. [A]Homozygous variant.

Of note: Rare variant p.L268V and p.P387L were carried by the same AD patient (Italy, female, AAO 58 years). Rare variants p.P29S and p.A117V were carried by the same AD patient originating from Italy (female, AAO 40 years). This patient also carried a third, known pathogenic mutation for AD (*PSEN1* p.L392V). The Italian AD patient, carrying the p.P392L mutation, also carried a known pathogenic mutation for AD (*PSEN1* p. M146L). The Spanish control individual (female, AAI 68 years) carried both p.R321C and p.V271I.

Table 3. In silico predictions of *SQSTM1* exonic variants in the Flanders-Belgian and EU EOD Consortium cohort.

Protein position	dbSNP138	Transcript	Protein Domain	Polyphen-2	SIFT[A]	SNPs&Go			MutationTaster	
						Prediction	Reliability index	Probability	Prediction	Probability
p.A17V	rs141502868	c.C50T	<PB1	Benign (0.143)	Tolerated(0.66)	Disease	0	0.5	Polymorphism	0.99
p.P29=	-	c.C87G	PB1						Polymorphism	0.9
p.P29S	-	c.C85T	PB1	Benign (0.074)	Tolerated(0.07)	Disease	1	0.6	Polymorphism	0.87
p.A33=	-	c.G99A	PB1						Polymorphism	0.99
p.A33V	rs200396166	c.C98T	PB1	Benign (0.008)	Tolerated(0.23)	Neutral	9	0	Polymorphism	0.99
p.A57=	-	c.G171A	PB1						Disease causing	0.75
p.G61=	-	c.C183T	PB1						Disease causing	0.96
p.R68=	-	c.C204G	PB1						Disease causing	1
p.D80=	-	c.C240T	PB1						Disease causing	0.99
p.S88F	-	c.C263T	PB1	Benign (0.136)	Damaging(0.03)	Disease	4	0.7	Disease causing	0.92
p.D108Y	-	c.G322T	PB1<>ZZ	Possible Damaging (0.662)	Damaging(0.01)	Neutral	0	0.5	Disease causing	0.99
p.R110H	-	c.G329A	PB1<>ZZ	Benign (0.037)	Damaging (0.11)	Disease	3	0.7	Disease causing	0.99
p.A117V	rs147810437	c.C350T	PB1<>ZZ	Benign (0)	Tolerated(0.34)	Neutral	4	0.3	Polymorphism	0.99
p.P118S	rs200152247	c.C352T	PB1<>ZZ	Benign (0.009)	Tolerated(0.54)	Disease	2	0.6	Disease causing	0.99
p.N125S	-	c.A374G	PB1<>ZZ	Benign (0.086)	Tolerated (0.29)	Neutral	8	0.1	Disease causing	0.89
p.G133=	-	c.G399C	ZZ						Disease causing	1
p.S152=	rs145037913	c.C456T	ZZ						Polymorphism	0.99
p.V153I	rs145056421	c.G457A	ZZ	Benign (0.011)	Tolerated (0.11)	Neutral	9	0	Polymorphism	0.99
p.L166=	rs372518286	c.C498T	ZZ<>TRAF6						Disease causing	0.96
p.S180=	rs370203737	c.G540A	ZZ<>TRAF6						Polymorphism	0.99
p.T221M	-	c.C662T	ZZ<>TRAF6	Benign (0.448)	Tolerated(0.06)	Neutral	4	0.3	Polymorphism	0.99
p.T221=	-	c.G663A	ZZ<>TRAF6						Polymorphism	0.99
p.P232=	rs145688323	c.G696A	TRAF6						Disease causing	0.99
p.K238E	rs11548633	c.A712G	TRAF6	Possible Damaging (0.717)	Damaging(0.04)	Disease	7	0.8	Disease causing	0.99
p.R265S	-	c.A795C	PEST1	Possibly Damaging (0.605)	Damaging (0.01)	Neutral	6	0.2	Disease causing	0.99

p.S266R	-	c.A796C	PEST1	Benign (0.022)	Tolerated (0.4)	Neutral	7	0.1	Disease causing	0.99
p.L268V	-	c.C802G	PEST1	Benign (0.004)	Tolerated(1)	Neutral	8	0.1	Polymorphism	0.82
p.T269=	-	c.C807T	PEST1						Disease causing	0.91
p.V271I	rs376283809	c.G811A	PEST1	Benign (0.001)	Tolerated(0.18)	Neutral	7	0.1	Polymorphism	0.99
p.E274D	rs55793208	c.G822C	PEST1	Benign (0)	Tolerated(0.61)	Neutral	7	0.1	Polymorphism	0.99
p.T278I	rs200445838	c.C833T	PEST1	Benign (0.013)	Tolerated(0.18)	Neutral	8	0.1	Polymorphism	0.99
p.D292=	rs4935	c.C876T	PEST1						Polymorphism	0.99
p.P293=	-	c.C879T	PEST1						Polymorphism	0.94
p.P296=	rs148984239	c.G888A	PEST1<>LIR						Polymorphism	0.99
p.G302=	rs11548642	c.C906T	PEST1<>LIR						Polymorphism	0.99
p.A308=	rs139482113	c.G924A	PEST1<>LIR						Disease causing	0.78
p.R312=	rs4797	c.G936A	PEST1<>LIR						Polymorphism	0.93
p.S318=	rs56092424	c.C954T	PEST1<>LIR						Polymorphism	0.99
p.E319K	rs61748794	c.G955A	PEST1<>LIR	Benign (0.007)	Tolerated (0.91)	Neutral	8	0.1	Polymorphism	0.99
p.R321C	rs140226523	c.C961T	LIR	Benign (0.206)	Damaging(0.05)	Neutral	4	0.3	Polymorphism	0.99
p.R321H	-	c.G962A	LIR	Benign (0.007)	Tolerated(0.12)	Neutral	7	0.2	Polymorphism	0.99
p.S328=	rs146164139	c.G984A	LIR						Disease causing	0.99
p.D329G	rs148294622	c.A986G	LIR	Benign (0)	Tolerated(0.61)	Neutral	0	0.5	Polymorphism	0.92
p.G334del		c.1001_1003 delGAG	LIR	-	Tolerated(0.22)				Disease causing	0.99
p.K344=	-	c.A1032G	LIR<>PEST2						Disease causing	1
p.V346=	rs150470670	c.G1038A	PEST2						Disease causing	1
p.P348Q	-	c.C1043A	PEST2	Probable Damaging (1)	Damaging(0.03)	Disease	3	0.7	Disease causing	0.99
p.P348=	rs10058037	c.G1044A	PEST2						Polymorphism	-
p.S361=	rs201591177	c.C1083T	PEST2						Disease causing	0.99
p.K378=	-	c.G1134A	PEST2<>UBA						Disease causing	0.99
p.P387L	-	c.C1160T	UBA	Possible Damaging (087)	Damaging(0.01)	Disease	4	0.7	Disease causing	0.99
p.P392L	rs104893941	c.C1175T	UBA	Probable Damaging (0.988)	Damaging(0)	Disease	5	0.8	Disease causing	0.99
p.P392=	rs75700262	c.G1176A	UBA						Disease causing	1
p.M404V	-	c.A1210G	UBA	Possible Damaging (0.819)	Damaging(0)	Disease	4	0.7	Disease causing	0.99

p.G410=	-	c.C1230T	UBA						Disease causing	0.99
p.G411S	rs143511494	c.G1231A	UBA	Probable Damaging (1)	Damaging(0)	Disease	6	0.8	Disease causing	0.99
p.G425R	-	c.C1273T	UBA	Probably Damaging (1)	Damaging (0)	Neutral	6	0.2	Disease causing	0.99
P.A426V	-	c.C1277T	UBA	Probable Damaging (1)	Damaging(0.05)	Disease	1	0.5	Disease causing	0.99
p.A426=	rs143977783	c.G1278A	UBA						Disease causing	0.99
p.P438L	-	c.C1313T	UBA<>	Probably Damaging (1)	Damaging (0)	Neutral	9	0	Polymorphism	0.88
p.P439L	rs199854262	c.C1316T	UBA<>	Probable Damaging (0.997)	Tolerated(0.08)	Neutral	4	0.3	Polymorphism	0.97

Genomic position in base pairs according to hg19 (GRCh37). – denotes not applicable. [A] Based on ENSP00000374455.

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