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Genetic screening in early-onset dementia patients with unclear phenotype: relevance for clinical diagnosis

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ABSTRACT

In a prospective study of dementia in Flanders (Belgium), we observed a substantial fraction of early-onset dementia patients who did not fulfill the criteria for a specific dementia subtype, leaving the patients without a precise clinical diagnosis. We selected 211 of these patients for genetic testing of causal genes linked to neurodegenerative brain diseases. In this group, the onset or inclusion age was 59.9 ± 8.2 years and 27.4% had a positive family history. We used a panel of 16 major genes linked to Alzheimer's disease, frontotemporal dementia, amyotrophic lateral sclerosis, Parkinson's disease, and prion diseases. In addition, we tested for the presence of a pathogenic *C9orf72* repeat expansion. We identified 13 rare variants in 15 patients, including a carrier of variants in 2 different genes. Six patients (2.84%), carried a mutation in a Mendelian causal gene, that is, *APP*, *MAPT*, *SOD1*, *TBK1*, and *C9orf72*. In the other 7 patients, 7 variants were of uncertain significance, including a frameshift mutation in *PSEN2*, p.G359Lfs*74, in 2 patients sharing a common haplotype, and in *LRRK2*, p.L2063fs*. Expression studies showed reduced PSEN2 and a near complete loss of LRRK2, in lymphoblast cells or brain material of these patients. Overall, our study underscores the relevance of genetic testing of known causal genes in early-onset patients with symptomatology of neurodegenerative dementia but an unclear clinical diagnosis. A positive genetic result can help to obtain a precise diagnosis as well as a better understanding of the presence of multiple affected relatives in the family.

1. Introduction

Dementia is a devastating disorder, representing a massive burden on patients and caregivers, as well as on the health care and social care system (Chemali et al., 2010). Most dementia patients are elderly people, but alertness for dementia in young patients is increasing (Prince and Jackson, 2009). Dementia before the age of 65 years is classified as early-onset dementia (EOD) (Lambert et al., 2014), with main clinical subtypes Alzheimer's disease (AD), frontotemporal dementia (FTD), dementia with Lewy bodies (DLB), and vascular dementia (VaD) (Masellis et al., 2013). Characteristic for EOD is the vast clinical and genetic heterogeneity, which poses great diagnostic challenges. Standard criteria for a dementia diagnosis require that the cognitive impairment is sufficiently severe to compromise the social and occupational functioning (Rossor et al., 2010). Also in research, diagnostic criteria should be adequate to allow stratification of dementia patients into homogeneous groups of patients that most likely are sharing a common biological disease mechanism. The currently used criteria for probable AD, described by the National Institute on Aging–Alzheimer's Association (McKhann et al., 2011), as well as those for probable FTD (Rascovsky et al., 2011), require the absence of any evidence of other neurodegenerative processes or non-neurological comorbidities that might have a substantial effect on cognition. Owing to these stringent exclusion criteria, the sensitivity of the current diagnostic criteria is rather low (Harris et al., 2015). In addition, overlap in symptomatology between different neurodegenerative dementia

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or other neurodegenerative brain diseases (NBD) has been documented (Fernández et al., 2017). Consequently, at initial clinical presentation and in the early stages of disease, patients may have symptoms that are insufficient or are not specific enough to comply with the diagnostic criteria, resulting in the exclusion of those patients from research studies as well as diagnostic genetic testing.

From a larger prospective study of dementia in Flanders (Belgium), initiated in 1995, (Engelborghs et al., 2003), we selected 211 index patients with symptomatology of dementia at early age. These patients had been excluded from research because of an unclear clinical diagnosis of a specific dementia subtype. At their first clinical consultation, they presented with a cognitive impairment possibly due to neurodegenerative dementia, but their symptoms did not fulfill the criteria for possible or probable dementia of a specified subtype. In this selected patient group, 27.4% of the index patients had a positive family history of disease. We screened causal genes that are frequently mutated in NBD, including AD, FTD, amyotrophic lateral sclerosis (ALS), Parkinson's disease and prion diseases. Identification of a pathogenic mutation might help establishing a more precise clinical diagnosis and might clarify the familial presentation in their family.

2. Materials and methods

2.1. Study population

In a large prospective study on neurodegenerative and VaD in Flanders-Belgian population, initiated in 1995 (Engelborghs et al., 2003), patients were ascertained at the Memory Clinic at the general hospitals Middelheim and Hoge Beuken, Hospital Network Antwerp, Antwerp, Belgium. Index patients were systematically included indifferent of their age, their gender or their clinical dementia subtype. Today, the study counts 4954 participants of whom 3202 received a clinical diagnosis in the dementia spectrum.

A cohort of 741 control individuals was included in the study (Supplementary Table S1), composed of community-dwelling volunteers or spouses of patients. Subjective memory complaints, neurologic or psychiatric antecedents, and familial history of neurodegeneration were measured by means of a face-to-face personal interview. Cognitive screening was performed using the mini-mental state examination (cutoff score $\geq$26) (Folstein et al., 1975) or the Montreal Cognitive Assessment test (cutoff score $>$25) (Nasreddine et al., 2005).

2.1.1. Ethical assurances

We obtained informed consent of patients or their legal guardians and of control persons for their participation in clinical and genetic studies. The study protocols were approved by the ethics committee of the Antwerp University Hospital and the University of Antwerp as well as the hospitals that contributed to the clinical diagnosis and sampling.

2.2. Mutation screening

We extracted genomic DNA from whole blood using standard laboratory procedures and performed massive parallel sequencing of the coding sequences of a multiamplicon gene panel of 16 NBD genes, 15 Mendelian genes and the risk gene *APOE* (Supplementary Table S2) (Perrone et al., 2017). Rare variants with a minor allele frequency $<$ 1% and predicted to affect the protein sequence were selected and validated by Sanger sequencing. Sanger sequencing was also used for exon 5 of *PSEN2*, which is located in a complex genomic region. The *C9orf72* hexanucleotide (G_4C_2) repeat expansion mutation was determined using repeat-primed PCR analysis (Gijselinck et al., 2012). The allele frequencies of the rare variants, observed in the patient group, were assessed in the control cohort. For patients carrying the same rare variant, we tested for relatedness by examining allele sharing of flanking short tandem repeat markers located in the *PSEN2* and the *APP* loci (Supplementary file).

2.3. Transcript and protein analyses

To generate cDNA, we extracted total RNA from lymphoblast cells of the *PSEN2* p.G359Lfs*74 and *LRRK2* p.Leu2063fs* carriers, and 3 control individuals that were NBD mutation negative, using the RiboPure kit as previously described (Perrone et al., 2017). For the *PSEN2* p.K82Ifs*42 carrier (DR1427), total RNA was isolated from fresh frozen brain tissue of the frontal cortex BA10 and hippocampus.

To inhibit nonsense-mediated mRNA decay, the lymphoblast cells of the *LRRK2* p.L2063fs* carrier and 3 controls were treated with cycloheximide or vehicle at 37 °C for 4 hours, followed by extraction of total RNA, cDNA amplification, and Sanger sequencing (Supplementary file). Protein lysates were prepared from lymphoblast cells of the *PSEN2* p.G359Lfs*74 and *LRRK2* p.L2063fs* carriers and 13 NBD mutation-negative control persons (Supplementary file). For *PSEN2* p.K82Ifs*42 carrier, protein lysates were prepared from fresh frozen brain tissue of the frontal cortex BA10 and hippocampus and from the same brain regions of 6 control brains. We analyzed protein expression by western blotting, using monoclonal antibodies against the PSEN2 C-terminal fragment (CTF) and LRRK2, to quantify against glyceraldehyde 3-phosphate dehydrogenase. Results were visualized by electrochemiluminescence detection. To inhibit the ubiquitin proteasome system, we treated cells with MG-132 or vehicle at 37 °C for 4 hours (Supplementary file).

3. Results

3.1. Selected study group

We selected 211 unrelated early-onset patients, with signs of neurodegenerative dementia but lacking a diagnosis of a specific dementia subtype, leaving them with an unclear clinical diagnosis. The DNA samples and associated clinical diagnoses of those patients were obtained between 1995 and 2015 in the frame of the prospective study on neurodegenerative and VaD in Flanders-Belgian population (Engelborghs et al., 2003), before the use of amyloid positron emission tomography imaging. Most patients were included between 2005 and 2015 (164/211; 77.72%) (Supplementary Figure S1). In case of lack of age at onset data of the patients, we used a cutoff age at inclusion of 70 years because we observed in the Flanders-Belgian dementia patients pathogenic mutations in AD genes up to an onset age of 70 years (Brouwers et al., 2008). In the study group, mean onset age or inclusion age was 59.9 $\pm$ 8.2 years (range 33–70), and 27.4% (58/211) had a positive family history (Supplementary Table S1), meaning that they had at least 1 affected first-degree relative.

Data from clinical history of patients and family, neurological examination, and neuroimaging were collected. Of the rare variant carriers, we summarized in Table 1 the data of the clinical examination and the technical examinations, available in the prospective study at initial neurological consultation.

3.2. Mutation screening

Overall, we observed 13 different rare variants in NBD genes in 15 of the 211 unrelated EOD patients (Tables 2 and 3). Six EOD patients (2.84%, 6/211) carried a known pathogenic mutation in 5 genes, that is, *APP*, *C9orf72*, *MAPT*, *SOD1*, and *TBK1* (Table 2). After

Table 1
Initial clinical features and structural and functional imaging data of carriers

Patient id	YAI	Symptoms and signs	NPE deficient domains	Structural imaging	Functional imaging	Diagnosis
DR1330	2004	Naming; irritable; discrete ↑DTR Le	Weak logical memory for age	(CT) normal	(SPECT) ↓ FP bilat → P bilat	MCI, poss
DR1331	2005	Memory, attention	-	(MRI) early cortical atrophy, discrete CVWML	(SPECT) ↓ FP bilat Ri > Le	MCI, poss
DR1332	2005	Memory				MCI
DR1336	2012	STM, irritable, aggression, depressed, suicidal, alcohol abuse	Imprinting, retention (visual), STM, attention, confrontation naming, depressed	(MRI) cerebellar and cerebral atrophy, limited CVWML F bilat	(fSPECT) remarkable ↓ medioF bilat; mild ↓ FP cortical	md aMCI
DR1341	2012	Alcohol abuse, anosognosia	Imprinting, retention, recognition, time orientation, attention, naming, fluency, executive function	(MRI) diffuse cerebral atrophy, minimal CVWML	(SPECT) diffuse, severe, heterogeneous ↓ cortical	Korsakoff vs. AD
DR1342	2015	Memory, word retrieval, reading, orthography, sad	Word retrieval (verbal paraphasia, anomia), memory, concentration, executive function	(MRI) early cortical atrophy and mild CVWML	(FDG-PET) compatible with AD (NFD)	md aMCI
DR1333	2005	STM, spatial orientation, less spontaneous speech	Memory: imprinting + retention	(MRI) CVWML	(SPECT) ↓ PT bilat	aMCI
DR1334	2003	-	Time orientation, memory: imprinting	(CT) normal	(SPECT) ↓ PT bilat → P bilat, asymmetric BG	aMCI
DR1335	2005	-	Imprinting, auditive attention, word retrieval	(MRI) moderate CVWML, mainly posterior	(SPECT) normal	aMCI
DR654	2005	CD, resting tremor, rigidity, dysarthria, ↓ocular range of motion, epilepsy, flexion contractures, ↓ DTR, epilepsy	-	(MRI) early cortical atrophy, CVWML: few periventricular, more peripheral	-	poss Dementia
DR1337	1999	Gait, speech, bradypsychia, dysarthria, hypomimia, CW rigidity UL Ri>Le, UVGP, symmetric paraparesis, amyotrophy, hypotonia LL, dysmetria Le UL, weak DTR Ri>Le	-	(CT) ischemia Le ACM region; (MRI) cortical atrophy	(SPECT) severe ↓ F bilat → PT bilat, Le > Ri	PSPS
DR1338	1998	Word retrieval, STM, sad, irritable		-	-	Dementia
DR1340	2007	STM, naming, attention; tremor	Compatible with aMCI	(MRI) mild atrophy HC, Le<Ri	(SPECT) ↓ PT bilat	aMCI

The carriers of a known pathogenic mutation are listed first, followed by the carriers of a VUS.
Key: ACM, arteria cerebri media; AD, Alzheimer's disease; aMCI, amnestic MCI; BG, basal ganglia; bilat, bilateral; CD, cognitive deterioration; CSF, cerebrospinal fluid; CVWML, chronic vascular white matter lesions; CW, cog wheel; DTR, deep tendon reflexes; F, frontal; HC, hippocampi; Id, identifier; incl, inclusive; Le, left; LL, lower limbs; MCI, mild cognitive impairment; md aMCI, multiple domain aMCI; NPE, neuropsychological examination; P, parietal; PET, positron emission tomography; poss, possible; PSPS, progressive supranuclear palsy syndrome; Ri, right; STM, short-term memory; T, temporal; UL, upper limbs; UVGP, upper vertical gaze palsy; YAI, year at inclusion.

the genetic screening, we performed a clinical follow-up of the carriers of a pathogenic mutation by contacting the referring neurologist to obtain clinical records and results of technical investigations (Table 4).

The remaining 7 rare variants were labeled as variant of uncertain significance (VUS) and were found in 5 NBD genes, that is, *PSEN1*, *PSEN2*, *GRN*, *PRNP*, and *LRRK2* (Table 3). Two VUS were novel, a nonsynonymous mutation, p.M134I, in *PRNP*, and an out-frame deletion mutation (c.1073-2delA) in *PSEN2* leading to a frameshift and premature stop codon in *PSEN2*, p.G359Lfs*74. The p.V198L in *PSEN1* (Supplementary Figure S2), was absent from 741 Belgian control persons, but segregation and functional data are missing. The novel p.M134I variant falls among 3 known neutral *PRNP* variants (Human Prion Mutation Database), and it was observed in patient DR1337, diagnosed with progressive supranuclear palsy syndrome (Table 1). The other variant in *PRNP*, p.V209M, was previously reported in a British patient diagnosed with and treated for a psychotic depression, co-occurring with a second variant *PRNP* p.P105L (Beck et al., 2010). The authors considered p.V209M a rare polymorphism, which was found coincidentally. In our study, p.V209M was present in 3 siblings who received a diagnosis of mental retardation, epilepsy, and parkinsonism. Taking the heterogeneous phenotypes of the 3 carriers, a causal role for p.V209M is unlikely.

Patient DR1336 carried 2 rare variants, the pathogenic p.R406W mutation in *MAPT* and the p.R71W nonsynonymous variant in

Table 2
Patients carrying a pathogenic mutation in a causal NBD gene

Patient identifier	AAO	Year at inclusion	Diagnosis at inclusion	FH	*APOE* genotype	Gene	cDNA	Protein	Reference
DR1330	67	2004	MCI	+	33	*APP*	c.2137G>A	p.A713T	(Carter et al., 1992)
DR1331	48	2005	MCI	+	33	*C9orf72*		expansion	(Gijselinck et al., 2012)
DR1332	66	2005	D	+	23	*C9orf72*		expansion	(Gijselinck et al., 2012)
DR1336	64	2012	MCI	+	44	*MAPT*	c.1216C>T	p.R406W	(Rademakers et al., 2004; Van Mossevelde et al., 2017c)
DR1341	53	2012	Korsakoff		33	*SOD1*	c.341T>C	p.I114T	(Rosen et al., 1993)
DR1342	61	2015	MCI	+	33	*TBK1*	c.379C>T	p.R127*	(van der Zee et al., 2017)

The carriers of a pathogenic mutation are listed. *C9orf72*, expansion means that the G_4C_2 repeat is >80 repeat units. See Supplementary file for cDNA and protein numbering and for the given frequencies and scores.
Key: AAO, age at onset; NBD, neurodegenerative brain diseases; MCI, mild cognitive impairment; D, dementia unspecified; FH, family history.

Table 3
Patients carrying a VUS in a causal NBD gene

Patient identifier	AAO/AAI	Year at inclusion	Diagnosis at inclusion	FH	*APOE* genotype	Gene	cDNA	Protein	dbSNP iD	gnomAD (NFE)	CADD PHRED score
DR1333	58	2005	MCI		24	*GRN*	c.1604G>A	p.R535Q	rs753857985	0.0044	18.41
DR1334	64	2003	MCI	+	34	*GRN*	c.1294C>T	p.R432C	rs63750130	0.0094	23.7
DR1335	63	2005	MCI		34	*LRRK2*	c.6187_6191delCTCTA	p.L2063fs*	rs111739194	-	36
DR654	33	2005	E+P+MR	+	23	*PRNP*	c.625G>A	p.V209M	rs758820257	0.0031	20.2
DR1337	62	1999	PSP poss		23	*PRNP*	c.402G>A	p.M134I	-	-	23.5
DR1338	59	1998	D	+	24	*PSEN1*	c.592G>C	p.V198L	rs201375628	0.0015	9.41
DR1340	55	2007	MCI		33	*PSEN2*	c.1073-2delA	p.G359Lfs*74	-	-	23.9

The carriers of a VUS are listed. *C9orf72*, expansion means that the G_4C_2 repeat is >80 repeat units. See Supplementary file for cDNA and protein numbering and for the given frequencies and scores.
Key: +, positive FH; AAI, age at inclusion; AAO, age at onset; D, dementia unspecified; E+P+MR, epilepsy + parkinsonism + mental retardation; f, female; FH, family history; m, male; MCI, mild cognitive impairment; ps, psychiatric disease; PSP, progressive supranuclear palsy syndrome; VUS, variant of uncertain significance.

PSEN2, which was reported as likely benign (ACMG-AMP recommendations; Richards et al., 2015), and was also present in patient DR1339. Allele-sharing analysis was indicative for a shared haplotype of 10.86 cM between patients DR1336 and DR1339. The p.R71W variant was observed in 1 control person (1/741, 0.13%), who did not share the haplotype of the patients.

3.3. *Expression analysis of the* PSEN2 *and* LRRK2 *frameshift mutations*

Prediction analysis indicated that the 1-bp deletion (delA) in *PSEN2* (c.1073-2delA; p.G359Lfs*74), abolished the canonical splice acceptor site of exon 12 leading to exon 12 skipping and a frameshift resulting in a termination codon that falls in the 3′UTR. Amplification of cDNA showed 2 bands matching in size with the wild type and the predicted mutant transcripts (Fig. 1A). Quantification of the *PSEN2* mutant transcript revealed a 4.4-fold reduced expression compared with wild type (Fig. 1B). Protein expression analysis showed a reduction of PSEN2 in the mutation carrier compared with controls (Fig. 2). The inhibition of the proteasome with MG-132 induced a partial accumulation of the PSEN2 in the lymphoblast cells of the mutation carrier, indicating that the mutant PSEN2 protein is translated but unstable and subjected to degradation (Supplementary Figure S3). However, the mutated protein could not be discriminated from the wild type on proteasome inhibition; in fact, the predicted molecular weight was 15 kDa for the mutated PSEN2 CTF and 16.58 kDa for the wild-type PSEN2 CTF. Screening of the Belgian AD patient cohort identified the same *PSEN2* variant in another patient (DR1409). Genotyping of flanking polymorphic markers, in the *PSEN2* locus, evidenced a common haplotype shared by the 2 patients (Supplementary Table S3), but absent in 192 Belgian control individuals. Analysis of in-house NGS data from 849 patients, revealed a low-frequency *PSEN2* frameshift variant (gnomAD frequency 0.0004062%), located in the same splice acceptor site of exon 12 (*PSEN2* c.1073−2_1073-1delAG), predicted also to lead to exon 12 skipping and to the same protein change p.G359Lfs*74. The carrier of this variant had Moroccan origin and received an ALS diagnosis. Furthermore, screening of the FTD research cohort (n=286) identified another novel *PSEN2* frameshift variant (p.K82Ifs*42, c.245_246delAA) in a patient (DR1427) with FTD and autopsy confirmed Pick Disease (FTLD-tau). Transcript analysis on cDNA obtained from brain tissue of the carrier revealed the presence of the mutated transcript (Supplementary Figure S4A), and protein expression analysis on both frontal cortex BA10 and hippocampus showed a reduction of PSEN2 protein in the mutation carrier compared with controls (Supplementary Figure S4B).

The 5-bp deletion in *LRRK2* (c.6187_6191delCTCTA; p.L2063fs*) predicted a frameshift and a premature termination codon after amino acid residue 2063. However, cDNA sequencing identified the mutant transcript (Fig. 3B). Quantification of the mutant and wild-type transcripts revealed a 1.9-fold reduction (Fig. 3A). Blocking nonsense-mediated mRNA decay by cycloheximide treatment showed an increase of the LRRK2 mutant transcript (Fig. 3B).

Table 4
Clinical follow-up data of carriers of a pathogenic mutation

Patient id	Mutation	Symptoms and signs	NPE deficient domains	Structural imaging	Functional imaging	CSF biomarkers	Diagnosis
DR1330	APP p.A713T	-	Word retrieval	-	-	-	MCI
DR1331	C9orf72 Expansion	-	Memory (auditive < visual): imprinting, working memory; attention	-	-	-	aMCI
DR1332	C9orf72 Expansion	-	Angry, aggression; depressed, loss of initiative, anxious, delusions, shadowing, sundowning	(MRI) biT and HC	(SPECT) diffuse moderate ↓	-	AD + DLB
DR1336	MAPT p.R406W	Increasingly aggressive, anosognosia, delusions; dyspraxia, anxiety	2013: significant ↓: Memory, spatial problem solving, word retrieval, attention	(MRI) severe T atrophy incl HC	(FDG-PET) moderate ↓ medioF bilat	Compatible with AD	AD + DLB
DR1341	SOD1 p.I114T	-	Memory, executive function	-	-		Korsakoff
DR1342	TBK1 p.R127*	Alexia, agraphia, dyspraxia	2016: md aMCI	-	-	Compatible with AD	AD

The carriers of a known pathogenic mutation are listed. There was no additional information for the VUS carriers.
Key: AD, Alzheimer's disease; aMCI, amnestic MCI; CSF, cerebrospinal fluid; DLB, Dementia with Lewy bodies; HC, hippocampi; id, identifier; incl, inclusive; MCI, mild cognitive impairment; md, multiple domain; NPE, neuropsychological examination; PET, positron emission tomography; T, temporal; UL, upper limbs; VUS, variant of uncertain significance; YAI, year at inclusion.

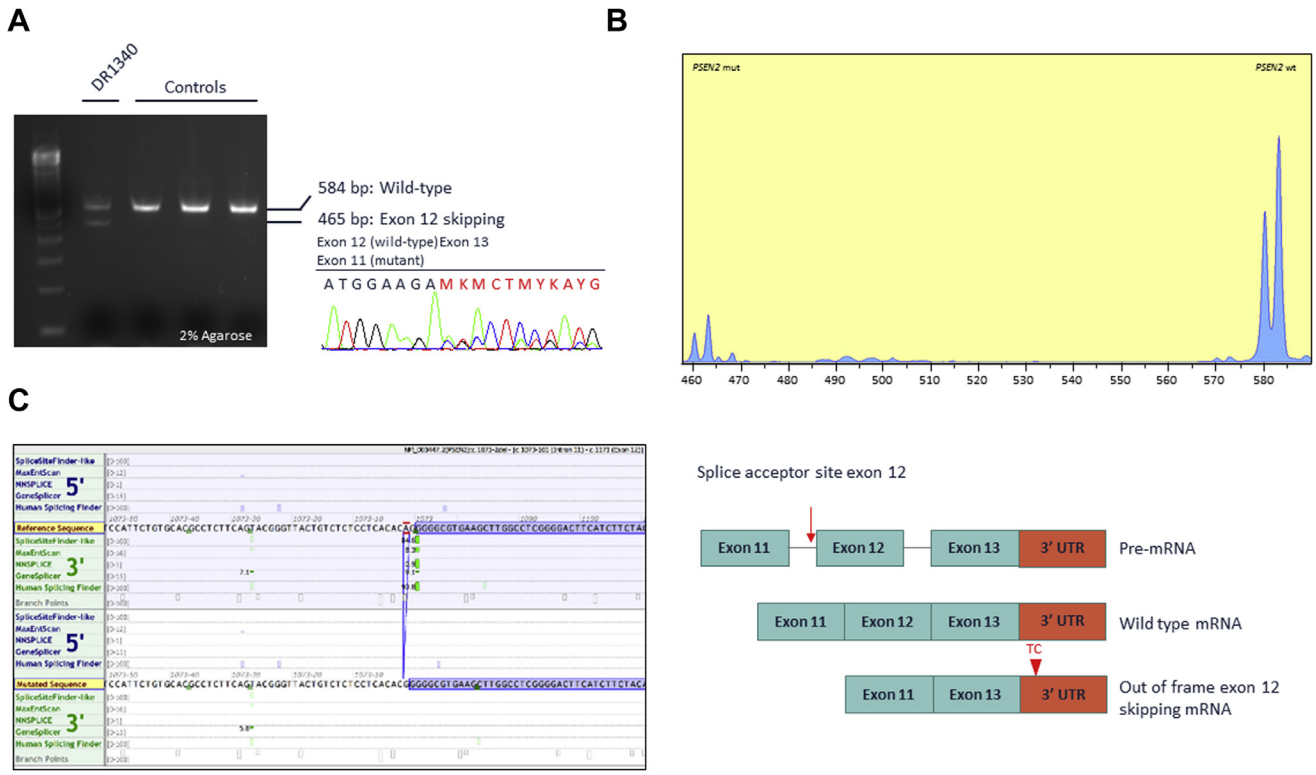

Fig. 1. Transcript analysis of PSEN2 p.G359Lfs*74. Exon counting based on RefSeq NM_000447. (A) Agarose gel picture shows the results of the exon 9–13 fragments amplification, which was carried out using a primer in exon 9 (5′-AAAGGGCCTCTGAGAATGCT-3′) and a reverse primer located in exon 13 (5′-CCAATGAAAATTCCCTGCAGC-3′) used for sequencing. The wild-type amplicon is 584 bp in size and, in case of exon 12 skipping (119 bp), the expected amplicon length is 466 bp. In the patient carrier, the electropherogram, obtained after sequencing of the fragments amplification, shows both the wild-type and the mutant alleles. (B) Capillary electrophoresis results of the wild-type and mutant transcripts. The mutant transcript is 4.4-fold less expressed than the wild type. There is a difference of 4 nucleotides in exon 11 between isoform NM_000447 and isoform NM_012486, explaining the double peaks for each transcript. (C) Visualized prediction of the PSEN2 splice acceptor site loss with Alamut. On the right is the representation of PSEN2 exon 12 skipping mechanism. The splicing mechanism produced 2 alternative transcripts wild type and exon 12 skipping. A red triangle in the 3′UTR of the aberrant transcript indicates the introduction of a termination codon (TC). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Protein expression analysis in the lymphoblast cells of the patient carrier and mutation negative controls, showed a variable LRRK2 expression (Fig. 3C and Supplementary Fig. S5). Inhibition of the proteasome did not alter LRRK2 protein expression in the mutation carrier (Supplementary Figure S3).

4. Discussion

We aimed at examining the genetic etiology of patients with clinical symptoms reminiscent of central nervous system neurodegeneration even though insufficient to fulfill the current strict inclusion criteria for the different dementia subtypes. These patients with unclear clinical diagnoses were excluded from our cohorts of possible or probable dementia patients that we are using in genetic research. For this pilot study, we analyzed a group of 211 early-onset patients.

4.1. Pathogenic mutations

We identified 6 carriers of a reported pathogenic mutations in 5 NBD genes that are associated with AD (*APP*), FTD, and/or ALS (*MAPT, C9orf72, TBK1, SOD1*) resulting in a carrier frequency of 2.84%

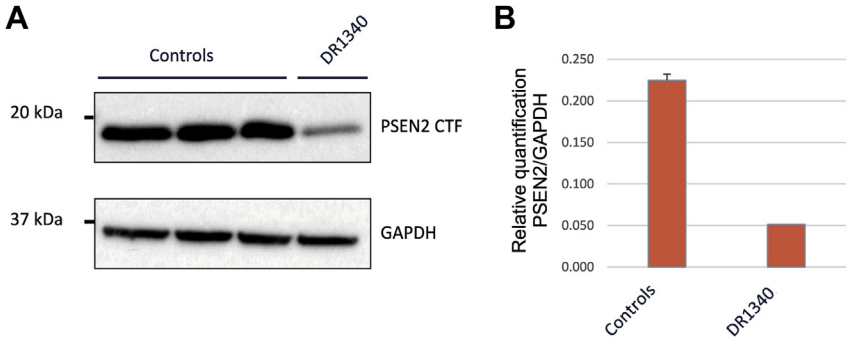

Fig. 2. Protein analysis of PSEN2 p.G359Lfs*74. (A) Western blot analysis of protein extracts from lymphoblast cells of the patient carrier compared with control individuals without mutation. The upper band represents the c-terminal fragment (CTF) of PSEN2 (17 kDa) and the lower band the reference protein GAPDH (37 kDa). (B) The graph shows the quantification in control samples and the patient carrier of the PSEN2 signal normalized to the signal of GAPDH. Error bar represent the standard deviation. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

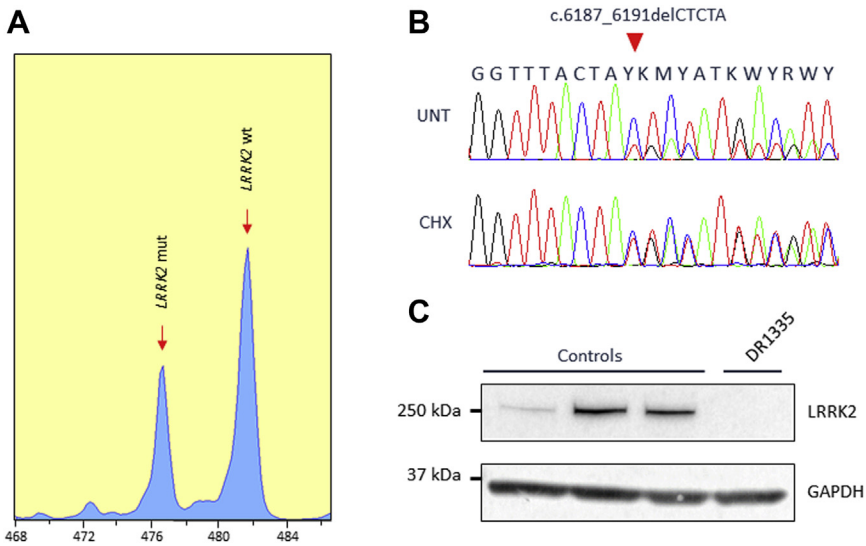

Fig. 3. Transcript and protein analysis of LRRK2 p.L2063fs*. (A) Capillary electrophoresis results of the wild-type and mutant transcripts. The mutant transcript is 1.9-fold less expressed than the wild type. (B) Electropherogram shows the results of the cDNA sequencing of the carrier not treated (UNT) and treated with CHX. The cDNA was amplified and Sanger sequenced using a forward primer in exon 41 (5′-CACTGTATCCCAATGCTGCC-3′) and a reverse primer located in exon 44 (5′-CATTCCTGCTGTTGTGATGTG-3′). (C) Western blot analysis of protein extracts from lymphoblast cells of the patient carrier compared with controls without mutation. The upper band represents LRRK2 (286 kDa) and the lower band the reference protein GAPDH (37 kDa). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(6/211). In comparison, in our Belgian patient cohorts of clinical diagnosed EOAD, FTD, and ALS patients, mutations in known AD (*APP*, *PSEN1*, *PSEN2*), FTD (*MAPT*, *GRN*, *C9orf72*, *TBK1*), and ALS (*C9ORF72*, *VCP*, *SOD1*, *TARDBP*, *FUS*, *ATXN2*) genes accounted for 5% (Cacace et al., 2016), 18% (Gijselinck et al., 2015), and 17% (Gijselinck et al., 2012). Two of the pathogenic mutations are observed at high frequencies in the Flanders-Belgian population, the *C9orf72* repeat expansion in FTD and ALS (Van Mossevelde et al., 2017b), and the *MAPT* missense mutation p.R406W, the latter is frequently found in families with a clinical AD-like phenotype who are all sharing the same disease haplotype (Rademakers et al., 2003). The Flanders-Belgian population has a high degree of genetic homogeneity, with several extended pedigrees and founder mutations identified (Van Broeckhoven et al., 1987; Cruts et al., 2006; Brouwers et al., 2007; Gijselinck et al., 2015). *C9orf72* repeat expansions are the most common genetic cause of familial FTD and familial ALS. Up to now, we documented 36 extended families segregating a *C9orf72* repeat expansion (Van Mossevelde et al., 2017b).

In this study, patients DR1331 and DR1332 carried a pathogenic repeat expansion in *C9orf72*. Patient DR1331 was diagnosed at age 48 years and patient DR1332 at age 66 years with MCI (Table 1). Follow-up clinical data indicated that patient DR1332 was later diagnosed with AD and DLB (Table 4). DR1332 developed aggressive behavior, comparable to other *C9orf72* expansion carriers (Van Mossevelde et al., 2017a) and delusions, characteristic of a DLB phenotype. Rare *C9orf72* repeat expansions have been observed in patients clinically diagnosed with AD (Cacace et al., 2013), as well as pathologically confirmed patients (Kohli et al., 2013). Patient DR1336 carrying the founder *MAPT* p.R406W mutation (Rademakers et al., 2004; Van Mossevelde et al., 2017c) received an initial clinical diagnosis of amnestic MCI (Table 1) but at follow-up was diagnosed with AD (Table 4), supported by CSF biomarkers (Van Mossevelde et al., 2017c). In addition to the pathogenic *MAPT* mutation, patient DR1336 also carried the benign variant *PSEN2* p.R71W.

Patient DR1330, carrying the *APP* p.A713T mutation (Table 3), was diagnosed with MCI at age 67 years (Table 1), which did not change at clinical follow-up due to lack of neurological consultations (Table 4). This mutation was also present in 1 control person DR1422, who was 73 years old at inclusion, scored 27/30 in the MMSE, and had no history of dementia. Allele sharing suggested a shared haplotype of 2.14 cM. So far, 24 carriers in 11 families have been identified (Sherrington et al., 1996; Armstrong et al., 2004; Rossi et al., 2004; Conidi et al., 2015; Lanoiselé et al., 2017). The *APP* p.A713T carriers present with highly variable onset ages from 52 to 82 years (Rossi et al., 2004), which is reminiscent of a risk allele of variable penetrance or a modifier. Patient DR1342 carrying the *TBK1* p.R127* mutation had memory problems at 61 years (Table 1) and received a diagnosis of AD at clinical follow-up supported by CSF biomarkers (Table 4). This *TBK1* mutation was first described in a German patient with sporadic ALS at the age of 70 years (van der Zee et al., 2017). The *SOD1* p.I114T mutation was identified in patient DR1341 with an initial diagnosis of Korsakoff syndrome (Table 1) and little extra information at clinical follow-up (Table 4). This *SOD1* mutation was first identified in 2 ALS families (Rosen et al., 1993) and later in an extended ALS family showing a high variability of onset age, clinical manifestations, disease progression, and penetrance (Lopate et al., 2010). Patient DR1341 had no motor impairment and there are no case reports of patients with Korsakoff syndrome carrying a *SOD1* mutation.

4.2. Variants of uncertain significance

We identified among the 7 VUS, 5 missense variants in causal NBD genes—*PSEN1* (n=1), *PRNP* (n=2), and *GRN* (n=2) (Tables 2 and 3)—of which contribution to causing disease remains doubtful based on published data as well as our data obtained in this study.

We also observed 2 VUS carriers of a frameshift mutation, 1 in *PSEN2* (p.G359Lfs*74; c.1073-2delA) in patient DR1340 and 1 in *LRRK2* (p.L2063fs*; c.6187_6191delCTCTA) in patient DR1335 (Table 3). In our Belgian AD research cohort, we found a second carrier of the same *PSEN2* frameshift mutation in patient DR1409, who shared a common haplotype of 7.57 cM with DR1340. Patient DR1340 was diagnosed with a multidomain amnestic MCI (Table 1), whereas patient DR1409 presented at age 78 years with memory and concentration complaints, which progressed to a global cognitive deterioration most compatible with AD. We also found 2 additional *PSEN2* frameshift mutations by analyzing our in-house

available NGS data. In the literature, 3 frameshift mutations were described in *PSEN2* (El Kadmiri et al., 2014; Jayadev et al., 2010), 2 of which are debatable (El Kadmiri et al., 2014). In our study, the carriers of *PSEN2* frameshift mutations each had a different clinical phenotype making it unlikely that these mutations might have a role in the pathophysiology of the patients.

The *LRRK2* frameshift mutation p.L2063fs*, was reported in a Parkinson's disease patient and in 2 control individuals (Ross et al., 2011). The 5-bp deletion occurs in exon 42 of *LRRK2*, encoding the kinase domain, in which nearly all dominant pathogenic mutations are located (Crosiers et al., 2011). We measured highly variable LRRK2 protein levels in control individuals, with some showing near null LRRK2 expression, comparable to the LRRK2 loss we observed for the patient carrying of *LRRK2* p.L2063fs*. Together, the expression data indicate that this *LRRK2* frameshift mutation is unlikely pathogenic.

5. Conclusion

In this pilot study, we demonstrated that genetic screening of patients with an unclear phenotype might reveal pathogenic mutations in known NBD causal genes, and provides valuable information that can help clinical diagnosis to obtain a more precise clinical subtype. In addition, the genetic information can improve our understanding of disease etiology and might help formulating a genetic-based diagnosis. Genetic diagnosis in patients is also relevant for the families of the carriers.

Our results support the hypothesis of the genetic overlap among diverse NBD (Fernández et al., 2017). Using exome sequencing rather than targeted gene-sequencing is expected to further improve the detection of pathogenic mutations in different NBD genes, although pathogenic copy number variations might not be detected. Contrary, exome sequencing will likely produce many more VUS, of which the contribution to the disease risk and penetrance remains unknown. In this study, we identified rare VUS, including frameshift mutations, of which the contribution to disease etiology remains unclear and need further functional studies. In addition, the finding of VUS in genetic testing might lead to a dilemma on whether or not these results should be communicated (Van Cauwenberghe et al., 2016). Nonetheless, our study underscores the importance of massive parallel screening of NBD genes in patients with early-onset of disease independent on their clinical diagnosis to clarify their genetic and clinical etiology.

Disclosure statement

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The authors have no actual or potential conflicts of interest with regard to the reported findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2018.04.015

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