Genetics of Dementia with Lewy Bodies

L.J.M. Vergouw

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Genetica van dementie met Lewy bodies

Leonie Johanna Maria Vergouw

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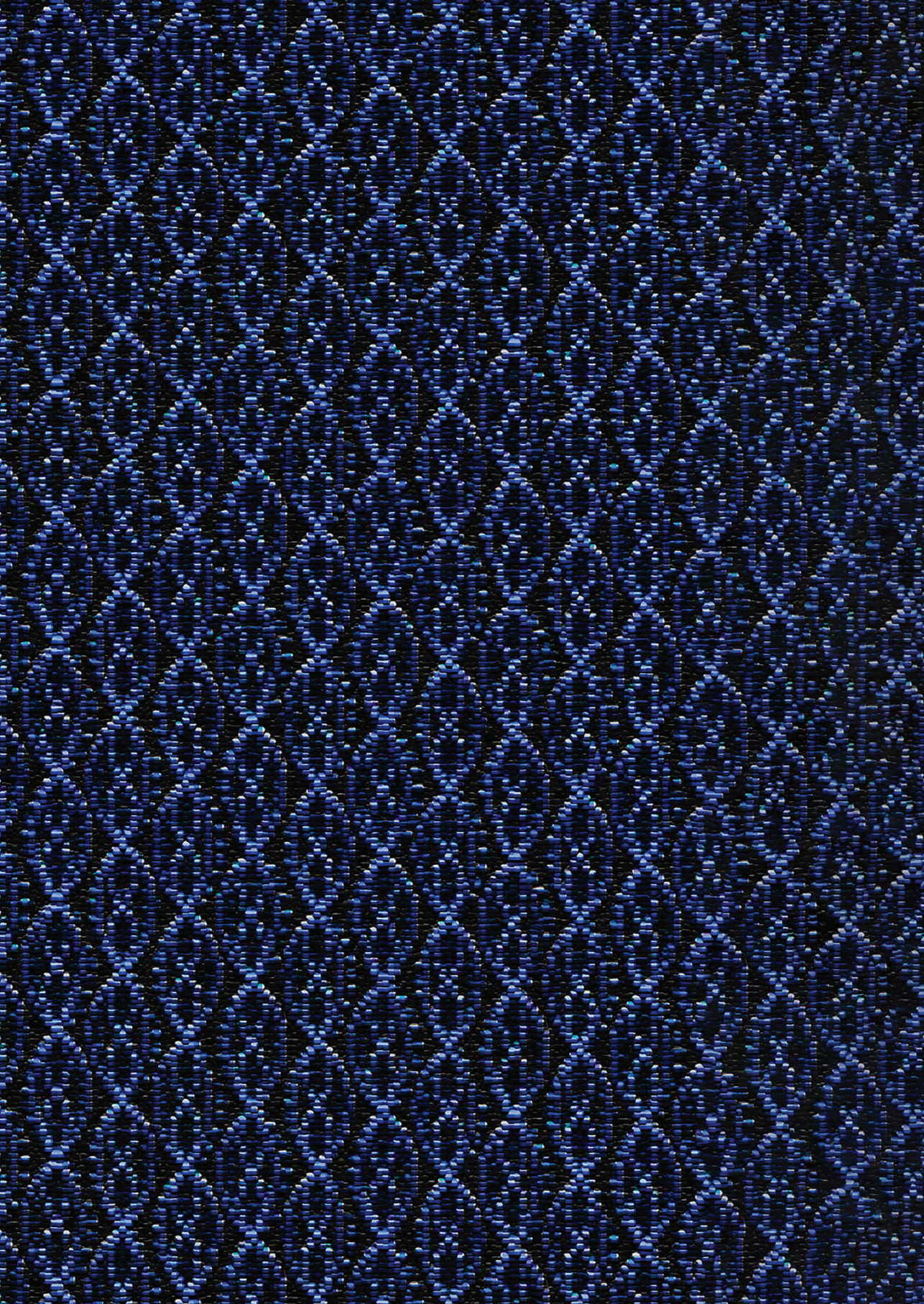

Part 1

General introduction

1.1

Chapter 1.1

Introduction to the thesis

Introduction to the thesis

Dementia with Lewy bodies (DLB) is a common neurodegenerative disease of the elderly. It accounts for approximately 5% of all patients with clinically diagnosed dementia.[1,2] However, the true prevalence is probably higher due to the fact that DLB is often overlooked and misdiagnosed.[3,4] Typical clinical features of DLB include progressive cognitive decline accompanied by parkinsonism, hallucinations, fluctuating cognition and REM-sleep behavior disorders. Other common symptoms are autonomic dysfunction, anxiety and depression.[5] Currently, there is no cure for DLB and treatment options are only available to lessen symptoms.[6] Patients with DLB have a median survival of approximately four years from diagnosis.[7] The mixture and severity of symptoms, lack of disease-modifying treatment options, and poor prognosis makes DLB a dreadful disease.

DLB shares clinical and pathological features with Parkinson's disease (PD) and Alzheimer's disease (AD).[6] Pathological hallmarks of DLB and PD are Lewy bodies and Lewy neurites.[8] This Lewy pathology is spread throughout the cortical regions of the brain in DLB, in contrast to PD where Lewy pathology is largely confined to subcortical regions, at least in the initial disease stages.[9] Additionally, AD pathology is observed in the majority of DLB patients.[10,11]

In contrast to genetic research in PD and AD, few genetic studies have been performed in DLB. Recently, a considerable genetic component has been suggested in the pathogenesis of DLB.[12] Nonetheless, only some genetic factors (the *apolipoprotein E* ε4 allele, and specific variants in the *glucocerebrosidase* and *α-synuclein* genes), previously associated with AD and PD, have also been associated with DLB.[13,14]

The aim of this thesis is to shed more light on the genetics of DLB, which could lead to a better understanding of the causes of DLB and its associated pathobiology. This knowledge could lead to the development of biomarkers, which are very important in the diagnostic and prognostic process, and may ultimately contribute to the identification of new targets for the development of disease-modifying treatments.

Chapter 1.2 provides an overview of the genetics of DLB. Little is known about possible differences between DLB patients with a positive family history of dementia or PD, as opposed to DLB patients with a negative family history of these diseases. In **Part 2** differences in phenotype between these two groups are described. Considering the overlap between DLB, AD and PD, in **Part 3** we investigated whether the known AD and PD genes are also associated with DLB. To increase the chances of finding genetic associations, we focused on two specific patient groups. We directed our analyses on DLB patients with a positive family history of dementia or PD in **Chapter 3.1** and on pathologically confirmed DLB patients with rapid disease progression (clinically suspected of Creutzfeldt-Jakob's disease) in **Chapter 3.2**. In **Part 4** and **Part 5**, the focus shifts to the search for novel genes associated with DLB. The *LRP10* gene was recently nominated as a novel gene associated with PD, PD dementia, and DLB[15], and is, therefore, the principal focus of **Part 4**. This gene was analyzed in clinically

diagnosed PD and DLB patients (**Chapter 4.1**), dementia patients with Lewy pathology, dementia patients with parkinsonism without Lewy pathology (**Chapter 4.2**), and patients with progressive supranuclear palsy (**Chapter 4.3**). In **Part 5**, a multimodal approach is used to search for novel genes. Cerebrospinal fluid (CSF) proteomic analysis in DLB patients is described in **Chapter 5.1**. This dataset was used in the **Appendix to Chapter 5.1** to combine genetic and proteomic data to find novel candidate genes in a pilot study. **Part 6** entails a general discussion of the thesis in the context of the current literature and provides suggestions for future research. Finally, **Part 7** summarizes the principal findings of the thesis.

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1.2

Chapter 1.2

An update on the genetics of dementia with Lewy bodies

Leonie J.M. Vergouw, Inger van Steenoven, Wilma D.J. van de Berg, Charlotte E. Teunissen, John C. van Swieten, Vincenzo Bonifati, Afina W. Lemstra, Frank Jan de Jong

Parkinsonism and Related Disorders 2017;43:1-8

Abstract

The genetic architecture of dementia with Lewy bodies (DLB) is increasingly taking shape. Initially, genetic research focused mainly on linkage and candidate gene studies in small series of DLB patients. More recently, association and exome sequencing studies in larger groups have been conducted, and have shown that several variants in *GBA* and the *APOE* ε4 allele are important genetic risk factors for DLB. However, genetic research in DLB is still in its infancy. So far, many genetic studies have been biased and performed in clinically and pathologically heterogeneous populations. Therefore, it is likely that multiple DLB-specific genetic determinants still have to be identified. To further our understanding of the role of genetics in DLB, future genetic studies should be unbiased and performed in large series of DLB patients, ideally with both a clinical diagnosis and pathological confirmation. The combination of genomic techniques with other research modalities, such as proteomic research, is a promising approach to identify novel genetic determinants. More knowledge about the genetics of DLB will increase our understanding of the pathophysiology of the disease and its relation with Parkinson's disease and Alzheimer's disease, and may eventually lead to the development of disease modifying treatments.

Introduction

Dementia with Lewy bodies (DLB) is a common neurodegenerative disease in the elderly.[1] DLB is characterized by progressive cognitive decline with variable combinations of fluctuating cognition, parkinsonism, visual hallucinations, neuroleptic sensitivity and rapid eye movement (REM)-sleep behavior disorders.[2] Clinical features of DLB are not specific to the disease and overlap with those of Parkinson's disease (PD) and Alzheimer's disease (AD).[1] In addition, neuropathological features also overlap between these diseases. Cortical Lewy bodies and neurites, which mainly comprise abnormal aggregated α-synuclein[3], are the pathological hallmarks of DLB, but are also observed in advanced PD and Parkinson's disease dementia (PDD)[4]. Furthermore, AD pathology is present in most DLB patients[4-6], which may aggravate the clinical manifestation of the disease and may increase the risk of mortality[7-9]. Due to these overlapping features, DLB is often considered as part of a spectrum with DLB placed between PD and AD (Figure 1).[10]

Over the last years, the genetic architecture of DLB is increasingly taking shape.[10-13] Defects in genes associated with PD (such as *α-synuclein* (*SNCA*)[14-17], *leucine-rich repeat kinase 2* (*LRRK2*)[18] and *glucocerebrosidase* (*GBA*)[19-21]) or AD (such as *presenilin 1* (*PSEN1*)[22-24], *presenilin 2* (*PSEN2*)[13,24,25], *amyloid precursor protein* (*APP*)[11,26], *apolipoprotein E* (*APOE*)[11,13,24,27-30] and *microtubule-associated protein tau* (*MAPT*)[31]) have also been associated with DLB. In addition to the clinical and pathological overlap, these findings also suggest a genetic overlap of DLB with PD and AD (Figure 1).[10]

In this article, we present a comprehensive overview of the genetics of DLB and discuss the genetic overlap of DLB with PD and AD. In addition, we describe promising genetic research methods, which in the near future will further our understanding about the pathophysiology of DLB and the clinicopathological spectrum between DLB, PD and AD.

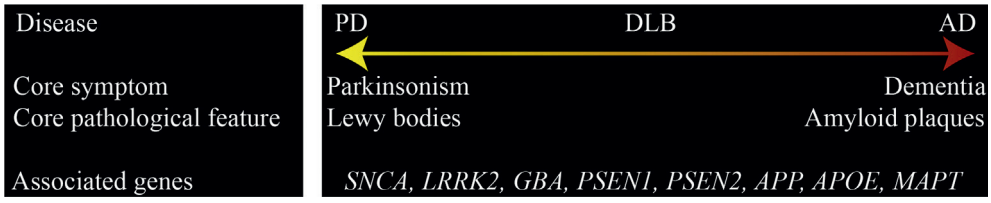

Figure 1: Disease spectrum of PD-DLB-AD (simplified representation).

Genetics of DLB

Multiple studies have been published on the genetics of DLB. The first genetic studies involving DLB patients mainly focused on families with multiple affected members with variable phenotypes ranging from DLB to PD and AD.[14-18,22-26,32-36] These studies used linkage analysis or a candidate gene approach to find rare variants (usually defined as variants with an allele frequency of less than 1% in the general population) with a large risk of disease development.[37,38] In such families, two disease-associated loci (2q35-q36 and 2p13[35-36]) and twelve disease-associated rare variants in six genes have been identified[11,13-18,22-26,32,34] (Table 1). Rare disease-associated variants have also been identified by candidate gene studies in series of unrelated DLB patients (Supplementary Table 1).[13,19-21,24,39-47] Some of these variants reside in genes previously associated with DLB in familial studies, which supports a role for these genes in DLB. Interestingly, rare disease-associated variants in *GBA* are often observed in unrelated DLB patients.[19-21,39-41,43]

Association studies in large cohorts of patients and controls generally take the approach of identifying common variants (usually defined as variants with an allele frequency of more than 1% in the general population) with a small to intermediate risk of disease development.[37,38] These studies with candidate genes have shown an association between DLB and, among others, the *APOE* ε4 allele[11,13,24,27-30] and the *MAPT* H1G haplotype.[31] A genome wide association (GWA) study, which is hypothesis free and typically identifies new disease-associated loci[37,38], has not yet been reported for DLB. Other relatively new and unbiased genetic approaches[37,38], such as whole exome and genome sequencing studies, have also not yet been reported.

Genes associated with DLB are discussed in more detail in the next sections.

Rare variants in SNCA

Several defects in *SNCA* (p.E46K, p.A53T variant and duplication) have been described in DLB patients with family members diagnosed with PD or PDD (Table 1).[14-17] A *SNCA* duplication was also found in a DLB patient without affected family members (Supplementary Table 1).[24] These defects were previously identified in multiple familial PD patients and are considered pathogenic.[14,16,48,49] Although evidence is scarce, there are some indications that specific genetic variability within *SNCA* could lead to different phenotypes in the PD-DLB spectrum. For example, the p.A30P variant[50] and duplications of *SNCA* are more often associated with PD and sometimes with PDD with a long disease course[51,52], whereas the p.E46K variant, the p.A53T variant and triplications are associated with PD and DLB with an early age of onset, severe clinical symptoms and a short survival[53]. The difference of phenotype with the type of variant may be related to the position of the variant and its impact on protein function.[14] Similarly, the kind of multiplication and genomic range of the *SNCA* multiplication may influence the clinical phenotype.[17]

Table 1: Rare disease-associated genetic variants in familial DLB.

Genetic characteristics				Clinical diagnosis	Family history				Pathological characteristics			References
Gene	**Protein**	**Chromosome location**	**Protein change**		**DLB**	**PD/PDD**	**AD or unspecified dementia**	**number of affected family members**	**Autopsy performed**	**Cortical Lewy pathology**	**AD-pathology**	
SNCA	α-synuclein	4q22.1	E46K	DLB**	no	yes	no	12	yes	yes	no	[14]
			A53T	DLB**	no	yes	no	3	yes	yes	no	[15]
				DLB	no	yes	no	4	no	NA	NA	[16]
			duplication*	DLB	no	yes	no	1	no	NA	NA	[17]
LRRK2	leucine-rich kinase 2	12q12	G2019S	DLB	no	yes	no	4	yes	yes	yes	[18]
PSEN1	presenilin 1	14q24.2	T440 deletion*	DLB**	no	yes	no	2	no	NA	NA	[22,23]
			A79V	DLB	no	no	yes	1	no	NA	NA	[24]
PSEN2	presenilin 2	1q42.13	A85V	DLB	yes	no	yes	5	yes	yes	yes	[25]
			R71W	DLB	no	no	yes	1	no	NA	NA	[24]
			D439A	DLB	no	yes	no	1	yes	yes	yes	[13]
APP	amyloid precursor protein	21q21.3	V717I	DLB/AD	UN	UN	UN	>1	yes	yes	yes	[11]
			duplication	DLB	no	no	yes	2	yes	yes	yes	[26]
SNCB	β-synuclein	5q35.2	P123H	DLB	yes	no	yes	7	yes	yes	yes	[32,34]

* Confirmed in a son with PD and dementia. ** Based on the clinical criteria by McKeith et al., 2005, no definite diagnosis was mentioned in article. DLB: dementia with Lewy bodies, PD: Parkinson's disease, PDD: Parkinson's disease dementia, AD: Alzheimer's disease, UN: unknown, NA: not available.

Rare variants in LRRK2

Mutations in *LRRK2* are an important genetic cause of PD. The most common mutation is p.G2019S, with mean frequencies ranging from 1% in sporadic, to 4% in familial PD worldwide.[54] However, the p.G2019S frequency varies significantly between populations, with higher frequencies in North African Arabs, Ashkenazi Jews and patients from the Middle East and southern Europe.[54,55] Disease-associated rare variants in *LRRK2* have rarely been found in DLB patients. Only one of 417 patients with clinical DLB and 355 patients with neuropathological confirmed Lewy body disease carried the p.G2019S mutation (Supplementary Table 1).[42] In addition, the p.G2019S mutation was only observed in one single patient with DLB from a family with several members affected with PD (Table 1).[18] This suggests that *LRRK2* disease-associated rare variants are, in contrast to PD, not a common cause of DLB.

Rare variants in PSEN1, PSEN2, APP

Mutations in *PSEN1*, *PSEN2* and *APP* are typically associated with familial AD, but have also been associated with other phenotypes including DLB.[56,57] Several defects (*PSEN1:* p.A79V variant and p.T440 deletion[22-24], *PSEN2:* p.R71W, p.A85V and p.D439A variant[13,24,25] and *APP:* p.V717I variant and duplication[11,26]) were found in families with DLB and dementia or PD (Table 1). Most of these defects (except from the *PSEN1* p.T440 deletion and *PSEN2* p.A85V variant) have previously been identified in (familial) AD and are considered pathogenic (except from *PSEN2:* p.R71W and p.D439A variant).[58-64]

There are a number of possible reasons for finding defects in *PSEN1, PSEN2* and *APP* in DLB patients. First, patients may have been misdiagnosed as having DLB instead of AD, as neuropathological confirmation was not always available. Second, in addition to AD pathology, Lewy pathology is frequently observed in patients with familial AD. Lewy pathology is found (especially in the amygdala) in more than 60% of the familial AD cases, and approximately 30% of *PSEN1* or *PSEN2* mutation carriers have cortical Lewy pathology.[65,66] Previous studies have suggested that specific genetic defects in *PSEN1* and *PSEN2* influence the amount of coincidental Lewy pathology in AD patients, which may lead to a more DLB-like phenotype in patients with a higher Lewy pathology load.[65,67,68]

Rare (and common) variants in GBA

The frequency of *GBA* variants in DLB patients varies between populations, ranging from 3.5% in a cohort of neuropathological confirmed DLB cases from the United States[20] to 33% in a clinical DLB cohort of Ashkenazi Jews, a population in which variants in *GBA* are overrepresented[21,69]. Variations in this frequency are due to differences in population and diagnostic criteria (clinically diagnosed patients versus pathologically diagnosed patients), as well as differences in research methods (e.g. genotyping of specific variants versus whole coding region) and selection criteria of identified variants (e.g. inclusion of all variants

versus rare pathogenic variants). Currently, the largest multicenter study has reported a disease-associated rare variant frequency of 7.5% in 721 clinically diagnosed DLB patients, compared to 0.97% in 1962 controls.[19]

Recent studies show that DLB patients carrying disease-associated *GBA* variants may have a different clinical disease course than those without such variants.[19,21,43] A study among Ashkenazi Jews with DLB has shown more severe motor complaints, REM-sleep behavior disorders, and cognitive dysfunction in carriers than in non-carriers.[21] Furthermore, several studies have shown an earlier age of disease onset and death in DLB patients carrying a rare *GBA* variant than in non-carriers.[19,21,43]

Many rare and common disease-associated variants in *GBA* have been found in DLB and PD patients.[19-21,39-41,43,70] The risk of disease or a particular phenotype is dependent on the type of disease-associated variant.[69,71]

Rare variants in SNCB

The *SNCB*: p.P123H variant has been found in a family with DLB[32,34] (Table 1) and the p.V70M variant in a DLB patient without affected family members[32] (Supplementary Table 1). The clinical diagnosis of DLB was pathologically confirmed in the patient with the p.P123H variant, but did not fully cosegregate in his family. The p.P123H and p.V70M variants have not been found in other DLB patients, PD patients, or in 331 control individuals from the population of the affected patients.[32] Possible pathogenicity is supported by the location of the variants, as they reside in highly conserved regions.[32] It is also supported by research in transgenic mice expressing the p.P123H variant, where progressive neurodegeneration was observed.[72] Further studies are necessary to replicate these findings.

Other rare variants with unclear pathogenicity

Other genes harboring rare variants with unclear pathogenicity which have been associated with DLB are *parkin* (*PARK2*)[13,24], *PTEN induced putative kinase 1* (*PINK1*)[24], *granulin* (*GRN*)[24,44,45], *triggering receptor expressed on myeloid cells 2* (*TREM2*)[45], *charged multivesicular body protein 2B* (*CHMP2B*)[13], *sequestosome* (*SQSTM1*)[13], *microtubule-associated protein tau* (*MAPT*)[24] and *prion protein* (*PRNP*)[46] (Supplementary Table 1). Defects in these genes have previously been associated with other neurodegenerative diseases, such as PD and frontotemporal dementia (FTD), but have only sporadically been found in DLB patients. Rare variants in *coiled-coil-helix-coiled-coil-helix domain containing 2* (*CHCHD2*)[47], *eukaryotic translation initiation factor 4 gamma 1* (*EIF4G1*)[13] and *GRB10 interacting GYF protein 2* (*GIGYF2*)[13] have also incidentally been associated with DLB (Supplementary Table 1). However, the role of these genes in neurodegeneration has not yet been conclusively established.

Common variants in APOE

The *APOE* ε4 allele has repeatedly been associated with DLB.[11,13,24,27-30] Its frequency in DLB patients varies between studies, but is approximately 30% in Caucasian DLB patients[11,13,24,27,28,30] in comparison to 14% in Caucasian controls free of neurodegenerative and neuropsychiatric diseases[73].

The effect of the *APOE* ε4 allele on different levels of Lewy and AD pathology was studied in 640 patients with dementia and 269 cognitively normal controls. This study showed that *APOE* ε4 allele carriers have an increased risk of both AD and Lewy pathology: *APOE* ε4 allele carriers had a 13-fold increased risk of developing a dementia with Lewy and AD pathology, a 10-fold increased risk of developing a dementia with only AD pathology, and a 6-fold increased risk of developing a dementia with Lewy pathology in comparison with non-carriers.[28] These findings suggest that the *APOE* ε4 allele may be a larger risk factor for dementia with both Lewy and AD pathology than for dementia with AD pathology only, and may contribute to the development of dementia through mechanisms unrelated to AD pathology.[28] Studies investigating the influence of the *APOE* ε4 allele on disease course report that DLB patients carrying an *APOE* ε4 allele have a shorter survival than non-carriers.[11,13] In contrast to *APOE* ε4 allele carriers, *APOE* ε2 allele carriers have a reduced risk of developing DLB in comparison with non-carriers.[74]

Common variants in MAPT

Recently, an association study was performed in which *MAPT* haplotypes were investigated in clinically diagnosed DLB patients (n=431), patients with Lewy pathology and a high likelihood of clinical DLB (n=347), and in individuals without dementia or movement disorders (n=1049). The H1G haplotype was associated with a higher risk of DLB in comparison with controls (2.8% vs. 1.0%, OR=2.2). In line with findings in PD, the H2 haplotype was associated with a lower risk of DLB in comparison with controls (20.9% vs. 23.6%, OR=0.8).[31,75] Other *MAPT* haplotypes (e.g. H1C and H1P) have also been linked to PD, PDD or AD[76-78], which suggests that different haplotypes may increase the risk of a specific phenotype[31]. Replication of findings in larger cohorts of patients is necessary to validate these genetic associations.

Other common variants

The largest association study to date not only showed an association between the *APOE* locus, but also between the *SNCA* and *scavenger receptor class B member 2* (*SCARB2*) loci and DLB. This study investigated 54 genomic regions, that were previously implicated in PD or AD, and was conducted in 788 clinically diagnosed DLB cases, of which 85% were neuropathologically confirmed, and 2624 controls.[12] Interestingly, the associations observed in this study for the *SNCA* and *SCARB2* loci were different than those previously found for PD.[12] This suggests that these loci may play a subtle different role in these diseases. In addition, a common variant in *butyrylcholinesterase* (*BuChE*) has been associated with a decreased risk of DLB (n=174) in comparison with controls (n=86) in a recent study.[79]

Genetic overlap between DLB, AD and PD

Genetic research in DLB has mainly focused on genes associated with PD and AD. Because of this biased approach, nearly all disease-associated variants found in DLB overlap with those associated with PD and AD (Figure 2). To assess the genetic overlap in a more unbiased way, an analysis of genetic correlation of DLB, PD and AD was performed.[80] In this study, a genome-wide genotyping was conducted on 788 clinically diagnosed DLB cases of which 85% were neuropathologically confirmed, 804 PD cases, and 959 clinically diagnosed AD cases. The proportion of variance explained by all single nucleotide polymorphisms (SNPs) for DLB was 0.31, for AD 0.60 and for PD 0.28. When comparing DLB with PD and DLB with AD for these SNPs, a correlation of 0.36 and 0.58 was found, respectively. No genetic correlation between PD and AD was found. Limitations of this study were the relatively small sample size, the inclusion of common risk loci only, and possible selection bias of the array. Nevertheless, the results of this study are interesting. First, the study suggests a larger overlap between DLB and AD than between DLB and PD. Secondly, the absence of an association between AD and PD indicates that the mechanisms underlying the association of DLB with AD and with PD are completely different. Finally, this study also suggests that, although genetic factors overlap with AD and PD, it is likely that DLB-specific genetic factors exist.[80]

Taken together, studies in DLB show that identical genetic defects are associated with several phenotypes in the PD-DLB-AD spectrum. This suggests that these diseases share some underlying mechanisms, but that other genetic or non-genetic factors may also play a role. There are also indications that specific genetic defects within an identical gene are linked to different clinical and pathological features in the PD-DLB-AD spectrum. This in turn suggests that the underlying mechanisms may be similar, but are, for example, dependent on the severity of the defect, which could be reflected in the level of pathology and clinical symptoms. Only a few studies indicate a role of DLB-specific genetic factors in the development of the disease.

However, a number of limitations to these current genetic studies may influence their quality. These, and the outlook for future research are discussed in the next two sections.

Limitations of current genetic studies

When interpreting genetic findings in DLB, a number of aspects have to be taken into account. First, genetic studies have often been performed in clinically and pathologically heterogeneous groups of patients. In the past, different diagnostic and pathologic criteria and nomenclature for DLB were used, which has made comparison of studies problematic. Applying the revised consensus diagnostic criteria of McKeith et al., 2005[2] led to greater homogeneity of diagnosis. Although these criteria have a high specificity (90%), the low sensitivity (54%)[81] is the main reason why a definite DLB diagnosis can only be made after autopsy. However, accurate clinical information is still required to differentiate between DLB

and late stage PD or PDD, as these diseases cannot be reliably distinguished based on the neuropathology alone.[82] Currently, the differentiation between DLB and PDD is based on the 'one year rule', in which DLB is diagnosed when dementia presents before or within a year after the onset of parkinsonism.[2] PDD is diagnosed when dementia starts more than one year after an established PD diagnosis. This is a somewhat artificial rule for differing between two conditions on the same spectrum of Lewy body diseases.[83] However, the rule is still applied as it increases the homogeneity of the study population and research comparability.[2] Only a selected group of genetic studies have been performed on patients with both a clinical and a neuropathological diagnosis of DLB. Misdiagnosis is possible in those studies with a lack of either neuropathological confirmation or detailed clinical information.

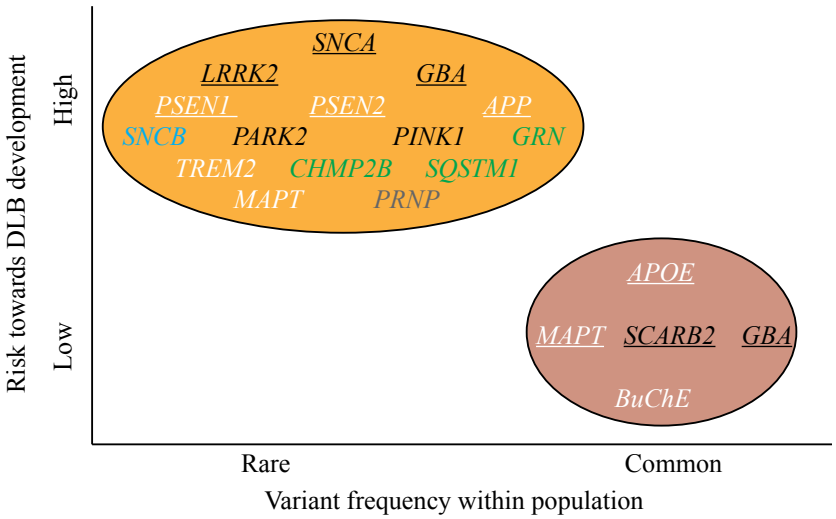

Figure 2: Variant risk versus variant frequency for DLB.
Genes previously associated with PD, AD, FTD and Creutzfeldt-Jacob's disease, and no other neurodegenerative disease are depicted in black, white, green, grey, and blue respectively. Evidence for the association between genes and DLB is stronger for those genes that are underlined than not underlined.

Secondly, only a few genetic findings from family studies have been reported. In addition, in these studies segregation of the variant of interest was not always studied and genetic analysis was not always performed in the DLB patients, but instead in affected relatives.

Thirdly, the pathogenicity of many of the identified variants is unclear, which leads to the question whether these variants are directly related to DLB or are just coincidental findings. Support of pathogenicity can, for example, be obtained from well-designed studies in which the prevalence of a specific variant is significantly higher in affected individuals in comparison with controls. Well-established *in vitro* or *in vivo* functional studies on specific variants can also support the claim of pathogenicity.[84] Genetic studies with large sample sizes

of affected and control patients are scarce for DLB. Furthermore, very few functional and replication studies have been performed in DLB.

Future research

To increase our understanding of the role of genetics in DLB, we believe that future genetic studies should focus on the optimization of conventional research methods, on the implementation of next generation sequencing technologies, and on the combination of different research modalities.

Optimization of conventional genetic research methods

Ideally, future genetic studies should focus on DLB patients with both a clinical and pathological diagnosis of DLB, while taking the amount of coincidental pathological findings (especially AD pathology) into account. For this, multicenter studies are essential to ensure substantial patient numbers. A systematic analysis of genes previously associated with neurodegenerative diseases in a large group of these patients can lead to a better understanding of the role of these genes in DLB. Another interesting, but biased, approach is to select genes that play a role in pathways related to PD and AD. In addition to single nucleotide variants, structural variants, such as copy-number variants and inversions, must also be taken into account.[85] However, to find new genetic determinants, unbiased research is necessary. Unbiased linkage studies may in general be a powerful tool in the search for new disease-causing defects, but these are not feasible for the identification of new genetic determinants in DLB given the rarity and often small size of DLB-families. GWA studies may be a source of unbiased information about new loci containing common variants with small to moderate effect sizes. Yet, functional and replication studies are still necessary when resolving the role of genetic defects with unclear pathogenicity in DLB.

Next generation sequencing

Whole exome or genome sequencing studies which provide the opportunity to screen simultaneously for genetic variants in the entire exome or genome[37,38], have not yet been reported for DLB. Applying these techniques in homogeneous, well-phenotyped groups, such as families, patients with a similar disease course or identical amount of (coincidental) pathology, may increase the chances of finding new genetic variants for DLB. Whole exome and genome sequencing may especially help in the identification of genetic variants with a low frequency and intermediate effect size that are hard to detect with conventional research techniques.[37]

Multimodal approach

Applying combinations of different research modalities, such as genomics, transcriptomics and proteomics, may further increase the chance of finding new

genetic determinants for DLB. A challenge when using these techniques is the selection of the variable of interest from a large amount of data. However, combining these techniques can reduce the number of potential disease-associated genetic variants. The application of whole exome sequencing in combination with proteomic research has been successful in identifying new genetic variants in several diseases.[86,87] For example, Wong et al., 2015[86] reported a rare autosomal dominant neurodegenerative disorder comprising of parkinsonism and dementia in a large family, and found the causal mutation in *protein kinase cAMP-dependent type I regulatory subunit beta* (*PRKAR1B*) by combining linkage analysis, whole exome sequencing, and proteomics.

Currently, four proteomic studies[88-91] and one transcriptomic study[92] have been performed in DLB patients (Supplementary Table 2). The results have not directly led to a reduction in the number of potential disease-associated genetic variants, as the proteomic profile found in these studies varies substantially because of the use of different inclusion criteria, research techniques, and different specimens (e.g. blood versus brain tissue) at different stages of the disease.

In future, multimodal research, the combination of unbiased genomic analysis and proteomics may prove particularly valuable for researching diseases like DLB, in which easily discernable and large pathological inclusions may provide a large amount of material for proteomic analyses.

Conclusion

To date, rare variants in *GBA* and the *APOE* ε4 allele are the strongest known risk factors for DLB. Defects in other genes have also been found in DLB patients. However, the risk profile of many of these defects has yet to be determined. Most of the genes associated with DLB overlap with genes associated with PD and AD, which suggests common neurobiological mechanisms for these diseases. However, because of the different phenotypes, other genetic and non-genetic factors may also play a role. Because no large unbiased genetic studies have been performed, it is likely that multiple DLB-specific determinants still have to be identified. The combination of different research modalities, such as next generation sequencing and proteomics may help in the identification of these determinants. The search for DLB-specific genetic determinants is important as it will give us a better understanding of the pathophysiology of DLB and its relation with PD and AD. This in turn could ultimately lead to the development of disease modifying treatments.

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Supplementary Table 1: List of rare disease-associated variants identified in unrelated DLB patients.

Gene	Protein change	Observed in number of DLB patients	Number of DLB patients in study	Diagnosis based on clinical criteria	Diagnosis based on pathological criteria	Observed in number of control population	Number of control patients in study	Reference
SNCA	duplication	1	99	99	1	NA	NA	[24]
LRRK2	p.G2019S	1	772	417	355	0	1790	[42]
GBA	several	54	721	721	443	19	1962	[19]
		18	95	0	95	1	32	[39]
		14	301	0	301	3	381	[20,40]
		8	35	0	35	NA	NA	[41]
		11	33	33	0	NA	NA	[21]
		5	97	97	50	0	115	[43]
PARK2	p.A46S, p.R275W	2	99	99	1	NA	NA	[24]
	p.R275W/p.G430D	1	91	91	91	NA	NA	[13]
PINK1	p.P138L, p.M318L, p.S499C	3	99	99	1	NA	620	[24]
GRN	p.L261I/p.R433W	1	99	99	1	NA	620	[24]
	Leu271LeufsX10	1	45	45	0	0	120	[44]
	several	7	58	58	58	12	380	[45]
TREM2	p.R62H	7	58	58	58	5	368	[45]
CHMP2B	p.I29V	1	91	91	91	NA	NA	[13]
SQSTM1	p.A33V, p.P27L	2	91	91	91	NA	NA	[13]
MAPT	p.R221Q	1	99	99	1	0	620	[24]
PRNP	p.M232R	1	1	0	1	NA	NA	[46]
Not confirmed genes								
SNCB	p.V70M	1	43	43	20 + 9*	0	331	[32]
CHCHD2	several	6	610	>1	610	1	717	[47]
EIF4G1	p.M1134V	1	91	91	91	NA	NA	[13]
GIGYF2	p.S1029C, p.S66T	2	91	91	91	NA	NA	[13]

* Lewy pathology in at least the patient or a family member with DLB. DLB: dementia with Lewy bodies, NA: not available, as genetic analysis in a control group was not performed or reported.

Supplementary Table 2: Overview of proteomic and transcriptomic studies in DLB.

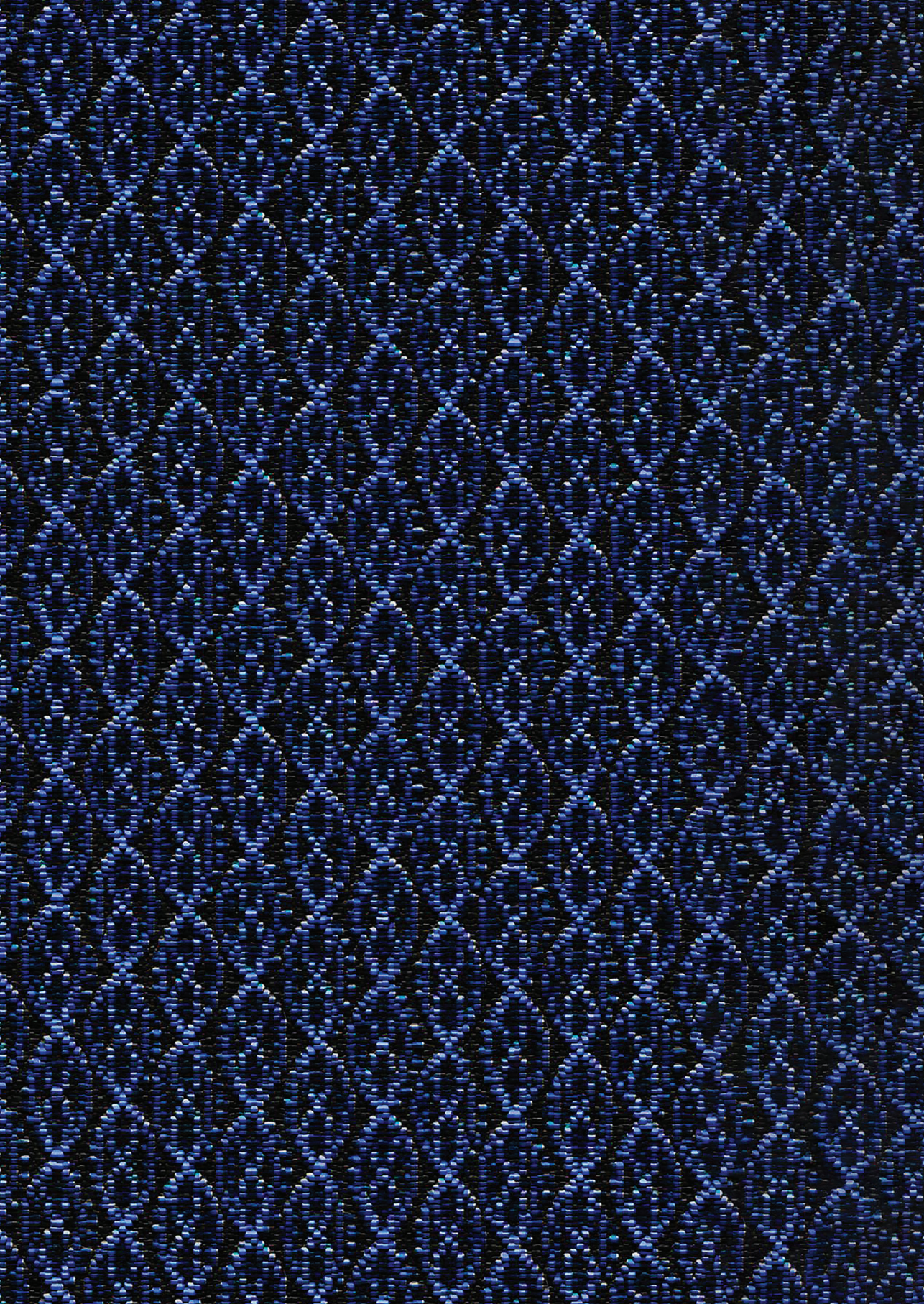
Proteomics

Patients	Based on clinical diagnosis	Pathologically confirmed	Control group	Specimen	Method	Number of proteins	Number of differentially expressed proteins	Validated proteins	Reference
dementia (n=6)	yes	cortical Lewy bodies	no	2500 Lewy bodies (pooled)	laser dissection microscopy, liquid chromatography-tandem mass spectrometry	296	n.a.	heat shock cognate 71 kDA	[88]
DLB (n=5), PD (n=10), AD (n=10)	yes	only DLB, AD	n=10 (age matched, healthy volunteers)	CSF	isobaric tagging for relative and absolute protein quantification, multidimensional chromatography, tandem mass spectrometry	1539	380 (DLB versus controls, proteomic changes >50% as compared to controls)	apoC1, t-cadherin	[89]
DLB/PDD (n=10)	yes	no	n=15 (age matched, not further specified)	CSF	label-free liquid chromatography-tandem mass spectrometry	>1000	22 (DLB/PDD vs controls with fold change >2)	osteopontin, ubiquitin carboxy-terminal hydrolase L1, chitinase-3-like protein 1	[90]
DLB (n=30), AD (n=30)	yes	no	n=28 (healthy controls, age differed significantly with disease groups)	serum	matrix-assisted laser desorption/ionization time of flight mass spectrometry	146 peptides	14 (DLB vs controls with fold change >1,5)	none	[91]

Transcriptomics

Patients	Clinical diagnosis	Pathological confirmed	Control group	Biomaterial	Method	Number of differentially expressed genes	Reference
DLB (n=8)	yes	yes	n=10	anterior cingulate cortex	gene expression profiling	367 downregulated	[92]

DLB: dementia with Lewy bodies, PD: Parkinson's disease, AD: Alzheimer's disease, PDD: Parkinson's disease dementia, CSF: cerebrospinal fluid, n.a.: not applicable.



Part 2

Familial aggregation in dementia with Lewy bodies

2.1

Chapter 2.1

Family history is associated with phenotype in dementia with Lewy bodies

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Abstract

It is currently unknown whether patients with dementia with Lewy bodies (DLB) with relatives with dementia or Parkinson's disease (PD) (familial DLB patients) have a different phenotype than sporadic DLB patients. In this study, we aimed to examine disease onset, rate of cognitive decline, survival and Alzheimer's disease (AD) biomarkers in patients with familial DLB (n=154) and sporadic DLB (n=137), using linear mixed model analysis and Cox regression analysis, among others. Familial patients had a shorter survival (8.0 years) and more often elevated CSF AD biomarkers (47%) than sporadic patients (9.0 years; p=<0.001; 30%, p=0.037). Our findings suggest that genetic factors are important in DLB and that the identification of new genetic factors will probably improve the prediction of prognosis.

Introduction

Dementia with Lewy bodies (DLB) is one of the most common forms of degenerative dementia in the older population.[1] DLB is diagnosed when dementia is accompanied by at least two of the following four core clinical features: parkinsonism, visual hallucinations, fluctuating cognition, and rapid eye movement (REM)-sleep behavior disorders (RBD). DLB can also be diagnosed based on dementia with one core clinical feature, in the presence of reduced dopamine transporter uptake in the basal ganglia, abnormal ^{123}iodine-MIBG myocardial scintigraphy, or polysomnographic confirmation of RBD.[2] Symptoms of DLB are not specific to the disease, but overlap with clinical features of Parkinson's disease (PD), PD dementia (PDD) and Alzheimer's disease (AD).[1] The distinction between DLB and PDD is most challenging, and based on differences in time between onset of dementia and parkinsonism. In PDD, dementia occurs in the context of well-established PD as opposed to DLB, in which dementia occurs before or concurrently with parkinsonism.[2] In addition, pathological and genetic features are also shared between DLB, PD(D) and AD.[1,3,4] For example, Lewy bodies containing the α-synuclein protein are the pathological hallmark of DLB, but are also observed in PD(D)[1], and genetic factors, such as the *APOE* ε4 allele and *GBA* variants, are risk factors for DLB as well as for AD and PD(D), respectively[3,4]. However, genetic risk factors for AD and PD seem to explain a part of the total phenotypic variance in DLB only.[5-7]

Recent studies have indicated that genetic factors play an important role in DLB. The heritable component of DLB has even been estimated at 60%.[7] Families with multiple DLB patients have rarely been described.[3,8] However, it has been reported that siblings of DLB patients are at higher risk of developing DLB compared to siblings of AD patients.[9] Furthermore, DLB patients more often have a family history of PD or dementia than controls.[10,11] This finding supports the notion that DLB, PD and dementia share, at least partially, the same genetic factors. This in turn might lead to shared molecular pathways and possibly similar phenotypes.

The *APOE* ε4 allele has been associated with a shorter survival in DLB[12-14] and disease-associated genetic variants in *GBA* have been associated with an earlier age of onset and death in DLB[15-17]. However, it is currently unknown whether DLB patients with relatives with dementia or PD (familial DLB) have a different phenotype than sporadic DLB patients. The main aim of this study is to examine the role of family history, used as a proxy of genetic factors, in relation to disease onset, rate of cognitive decline, survival and AD biomarkers. The secondary aim of this study is to explore the aforementioned features in DLB patients with relatives with dementia or PD to examine if their phenotype is more similar to AD or PD.

Materials and methods

Patients and study design

This is a retrospective study in which demographic and clinical data were collected from patients' medical records. Information on the occurrence of dementia and/or PD in first-degree relatives was based on medical records (41%) or a standardized assessment (59%), using a questionnaire or an additional patient/family member interview. A nation-wide registration system containing demographic data about all Dutch citizens was consulted to obtain information about dates of death (collected until December 2018).

A total of 291 patients with probable DLB according to the criteria of Mckeith et al. (2005)[18] were enrolled from three hospitals in the Netherlands (Elisabeth-TweeSteden Hospital, Tilburg; Erasmus Medical Center, Rotterdam; Amsterdam University Medical Center, Amsterdam). Patients visited the outpatient clinics of the Neurology departments between 2000 and 2018 and were diagnosed by expert neurologists. Dopamine transporter uptake scans were performed in 128 patients, and were used in the diagnostic process. Familial patients were defined as patients with at least one first-degree relative with dementia or PD. Sporadic patients were defined as patients without first-degree relatives with dementia or PD. Patients were excluded from the study when no dementia or PD was diagnosed in relatives together with no information on the occurrence of the other disease in relatives (e.g. no relatives with PD and no information of the occurrence of dementia, or vice versa), and when no information on family history was available at all. The distribution of the different groups according to family history is depicted in Supplementary Figure 1.

The study was performed according to the ethical principles of the Declaration of Helsinki and was approved by the local ethics committees (Elisabeth-TweeSteden Hospital: MEC-2016-608, L0318.2016; Erasmus Medical Center: MEC-2015-304, MEC-2016-608; Amsterdam University Medical Center: MEC-2016-061, MEC-2017-2116).

Outcome measures

Several clinical features and AD biomarkers (i.e. the presence of medial temporal lobe atrophy (MTA)[19], a CSF tau/amyloid-$\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) ratio of >0.52[20] and ≥1 *APOE* ε4 allele(s)[21]), were explored with respect to family history in DLB.

Age of onset and type of first symptom were based on anamnestic information from the patient or family members. We categorized the type of first symptom into cognitive decline, parkinsonism and hallucinations. Cognitive decline included descriptions of memory impairment and executive function impairment. Parkinsonism was based on bradykinesia with muscular rigidity, rest tremor or postural instability[22] or when parkinsonism was noted in the medical files in absence of more specific information. Rate of cognitive decline was based on the available Mini-Mental State Examination (MMSE) scores[23]. Survival was defined as the time between age of onset and death. Magnetic resonance imaging (MRI)

scans were evaluated visually and rated according to the MTA scale[19] by radiologists for clinical purposes. CSF was collected by lumbar puncture in polypropylene tubes (Starstedt, Nümbrecht, Germany). Levels of $A\beta_{1-42}$, total tau and p-tau were measured with commercially ELISAs (Innotest®, Fujirebio, Gent, Belgium). *APOE* genotyping was performed using the LightCycler *APOE* mutation detection method (Roche Diagnostics GmbH, Mannheim, Germany) after genomic DNA extraction.

Data analysis

Differences in sex, age of onset, type of first symptom and *APOE* genotype between familial and sporadic DLB were explored using the χ^2 test, Fisher's Exact Test, Fisher-Freeman-Halton test, independent Student t test or Mann-Whitney U test, where appropriate. We analyzed the MTA score and CSF tau/$A\beta_{1-42}$ ratio of >0.52 (CSF AD biomarkers) between the groups using linear and logistic regression, respectively, with time between age of onset and date of MRI or lumbar puncture as covariate to correct for possible confounding by disease stage. Linear mixed model analysis were performed to assess changes in MMSE levels over time, while accounting for the correlation between the repeated measurements of each patient. The model included time since first MMSE, family history and an interaction effect between time and family history to assess differences in the course of cognitive decline between groups. All MMSEs which were administered within 6 months from the previous MMSE were removed, because of possible learning effects. In the random effect structure, covariance type was set on unstructured and a random intercept was included. Differences in survival between familial and sporadic DLB were explored using Kaplan-Meier analysis and Cox regression analysis. We included sex, age of onset and study center to correct for possible confounding

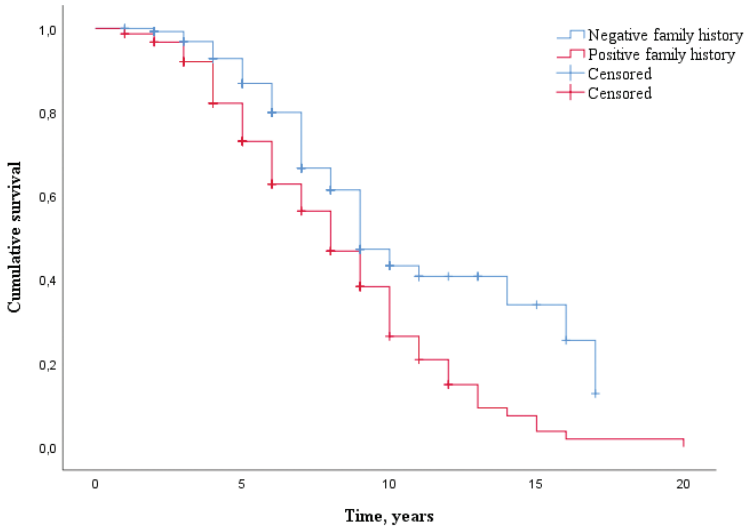

Figure 1: Survival distribution (Kaplan-Meier curve) of DLB patients with a negative family history as compared to DLB patients with a positive family history.

in the linear mixed model analysis and the Cox regression analysis.

In addition, differences in phenotype between DLB patients with a family history of either dementia or PD were explored. Statistical analyses were performed as described above.

Statistical significance for all tests was established at $p<0.05$ (two-tailed). The data was analyzed using SPSS software (Version 24).

Results

Differences in phenotype between patients with familial and sporadic DLB

The 154 familial DLB patients and the 137 sporadic DLB patients did not differ in sex, age of onset, type of first symptom (Table 1) or rate of cognitive decline (Table 2). Significantly more familial DLB patients had elevated CSF biomarkers for AD (47%), compared to those with sporadic DLB (30%, $p=0.039$). This finding remained significant after correction for time between age of onset and date of lumbar puncture ($p=0.037$, Table 1). In addition, a borderline significant result was found concerning the *APOE* ε4 allele, with a higher frequency of this allele in familial patients (65%) compared to sporadic patients (51%, $p=0.069$). MTA score was not different between the groups (Table 1).

Differences in survival between patients with familial and sporadic DLB

A total of 154 patients (53.1%) were deceased with a median survival of 7.0 years (IQR 5.0–9.0). Uncorrected survival analysis showed that patients with familial DLB had a significantly shorter survival (median 8.0 years, SE 0.51) compared to patients with sporadic DLB (median 9.0 years, SE 0.63, $p=<0.001$, Table 2 and Figure 1). This finding remained significant after correction for sex, age of onset and study center (HR 1.64, 95% CI: 1.16-2.31, $p=0.005$, Table 2).

Differences in phenotype between DLB patients with a family history of either dementia or PD

A total of 84 patients had a family history of either dementia (n=69) or PD (n=15) (Supplementary Figure 1). Parkinsonism was more prevalent as first symptom in DLB patients with a family history of PD (33%) compared to the patients with a family history of dementia (6%, $p=0.008$). These groups did not differ in sex, age of onset (Supplementary Table 1) or rate of cognitive decline (Supplementary Table 2).

In addition, there were no significant differences in the presence of the *APOE ε4* allele, CSF AD biomarkers and MTA score between the groups (Supplementary Table 1).

Differences in survival between DLB patients with a family history of either dementia or PD

A total of 43 patients (51.2%) were deceased with a median survival of 8.0 years (IQR 5.0–10.0). There were no differences in survival between DLB patients with relatives with dementia as compared to DLB patients with relatives with PD (Supplementary Table 2).

Table 1: Demographic features, clinical features and biomarkers in the total study group, and group differences.

	Total (n=291)	Familial DLB (n=154)	Sporadic DLB (n=137)	*p* value
Study center				0.011*
Elisabeth-TweeSteden Hospital	35 (12%)	24 (16%)	11 (8%)	
Erasmus Medical Center	54 (19%)	20 (13%)	34 (25%)	
Amsterdam University Medical Center	202 (69%)	110 (71%)	92 (67%)	
Sex, male	232 (80%)	122 (79%)	110 (80%)	0.82
Age of onset, years	66.0 (7.6)	66.6 (8.4)	65.3 (6.7)	0.14
First symptom				
Cognitive decline	245 (84%)	133 (86%)	112 (82%)	0.28
Parkinsonism	29 (10%)	14 (9%)	15 (11%)	0.60
Hallucinations	17 (6%)	7 (5%)	10 (7%)	0.32
MTA score (average of right and left) (n=199[98;101])	1 (0.5-1.5)	1 (0.5-1.5)	1 (0-1.1)	0.10[a]
CSF tau/$A\beta_{1\text{-}42}$ ratio >0.52 (n=169[83;86])	65 (38%)	40 (47%)	25 (30%)	0.037[a]*
APOE ε4 carrier (n=160[76;84])	94 (59%)	55 (65%)	39 (51%)	0.069

Values are presented as mean (SD), median (IQR) or n (%). DLB: dementia with Lewy bodies, PD: Parkinson's disease, CSF:cerebrospinal fluid, MTA: medial temporal lobe atrophy, $A\beta_{1\text{-}42}$: amyloid-$\beta_{1\text{-}42}$,[a] corrected for time between age of onset and date of MRI or date of lumbar puncture, * $p<0.05$.

Table 2: Statistical models regarding rate of cognitive decline and survival in the total study group.

Rate of cognitive decline		**β**	**SE**		**95% CI**	***p* value**	
Uncorrected (Linear mixed model analysis)	Baseline	-0.47	0.59		-1.63-0.70	0.43	
	Change over time	-0.0090	0.017		-0.041-0.024	0.59	
With correction for sex, age of onset, and study center (Linear mixed model analysis)	Baseline	-0.34	0.59		-1.50-0.81	0.56	
	Change over time	-0.0091	0.016		-0.042-0.023	0.59	
Survival		**Median[a]**	**SE**		**95% CI**	***p* value**	
Uncorrected (Kaplan-Meier analysis)	Sporadic DLB	9.0	0.63		7.77-10.23	<0.001*	
	Familial DLB	8.0	0.51		7.00-9.00		
		B	**SE**	**HR**	**95% CI**	***p* value**	**Z-score**
With correction for sex, age of onset, and study center (Cox regression analysis)		0.50	0.18	1.64	1.16-2.31	0.005*	5.71

[a]Time between age of onset and death,* $p<0.05$.

Discussion

The main finding of this study is that patients with familial DLB have a shorter survival than patients with sporadic DLB. We also found a higher percentage of familial DLB patients with elevated AD biomarkers in their CSF compared to sporadic DLB patients. Several longitudinal studies in DLB showed that concomitant AD pathology is associated with a

higher mortality.[24-26] The shorter survival in familial DLB compared to sporadic DLB may be due to concomitant AD pathology in familial DLB, which is reflected in a higher CSF tau/ $A\beta_{1-42}$ ratio in familial patients compared to sporadic patients. Genetic factors, such as the *APOE* ε4 allele, contribute to the presence of concomitant AD pathology.[25,27] Interestingly, although only borderline statistically significant, a higher frequency of the *APOE* ε4 allele in familial patients compared to sporadic patients was seen. This suggests that genetic factors are associated with survival, possibly by influencing neuropathology.

Previous studies have shown several possible risk factors for a shorter survival in DLB, such as a the presence of a fluctuating cognition, hallucinations at onset and a low MMSE score.[14,26] Other variables have also been reported to be associated with survival, but are contradictory between different studies (e.g female and male sex, early and late age of onset).[14,26] There were no significant differences in these clinical characteristics between familial and sporadic DLB patients in our study (data not shown for a fluctuating cognition and MMSE score). This might indicate that family history has an effect on survival independent of these clinical characteristics.

In addition, we found parkinsonism to be a more frequent presenting symptom in patients with a positive family history of PD than in patients with a positive family history of dementia. This might be based on more 'pure' Lewy pathology in patients with relatives with PD, and mixed pathology (Lewy pathology and AD pathology) in patients with relatives with dementia. However, this finding could also be based on recall bias due to familiarity with PD symptoms. Other AD or PD features were equally distributed between patients with a positive family history of dementia or PD, respectively. These results should be interpreted with caution due to the relative small number of patients in the group with relatives with PD and the presence of non-AD dementias in the group with relatives with dementia.

About half of the DLB patients in this study had at least one first-degree relative with dementia or PD. This is in line with previous studies, in which a positive family history for dementia was observed in 39-44% and for PD in 10-24% of patients with DLB.[10,11] Nonetheless, the percentages that we found could be an overestimation as patients with insufficient information on family history were not taken into account. These patients might be less likely to have relatives with relevant diseases. However, the most important limitation of this study is its retrospective character. Furthermore, information on family history and disease onset was based on anamnestic information, and may have introduced a recall bias. In addition, the patients in this study were predominantly male (80%), which may not be a good representation of the general DLB population.[28] However, our main finding (familial DLB patients have a shorter survival than sporadic DLB patients) stayed significant after correction for sex. At last, the DLB diagnoses in this study were not pathologically confirmed.

Strengths of this study include its large sample size and the enrollment of patients from university medical centers as well as from a general hospital. The latter increases the generalizability of our findings.

In conclusion, we are the first to report that DLB patients with a positive family history

of dementia or PD have a shorter survival than DLB patients with a negative family history of these diseases. This suggests that genetic factors contribute to disease course, possibly by influencing the amount of concomitant AD pathology, which is supported by our data. Future studies are necessary to identify which genetic and other contributing factors are accountable for our findings. This knowledge will lead to a better understanding of the pathophysiology of the disease and the overlap with AD and PD(D), and will probably improve the prediction of prognosis.

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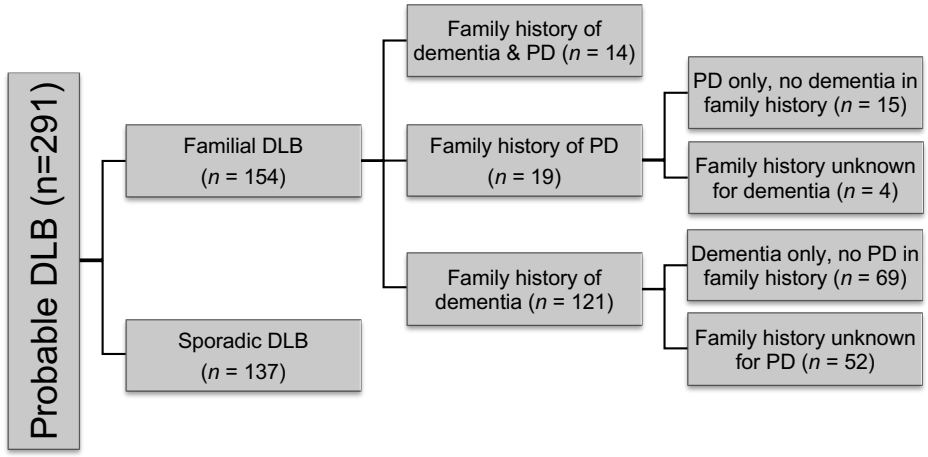
Supplementary Information



Supplementary Figure 1: Distribution of different groups according to family history. Family members with PDD were assigned to the subcategory 'Family history of dementia & PD', and family members with DLB were assigned to the subcategory 'Family history of dementia'. DLB: dementia with Lewy bodies, PD: Parkinson's disease, PDD: Parkinson's disease dementia.

Supplementary Table 1: Differences in demographic features, clinical features and biomarkers between DLB patients with either a family history of dementia or a family history of PD.

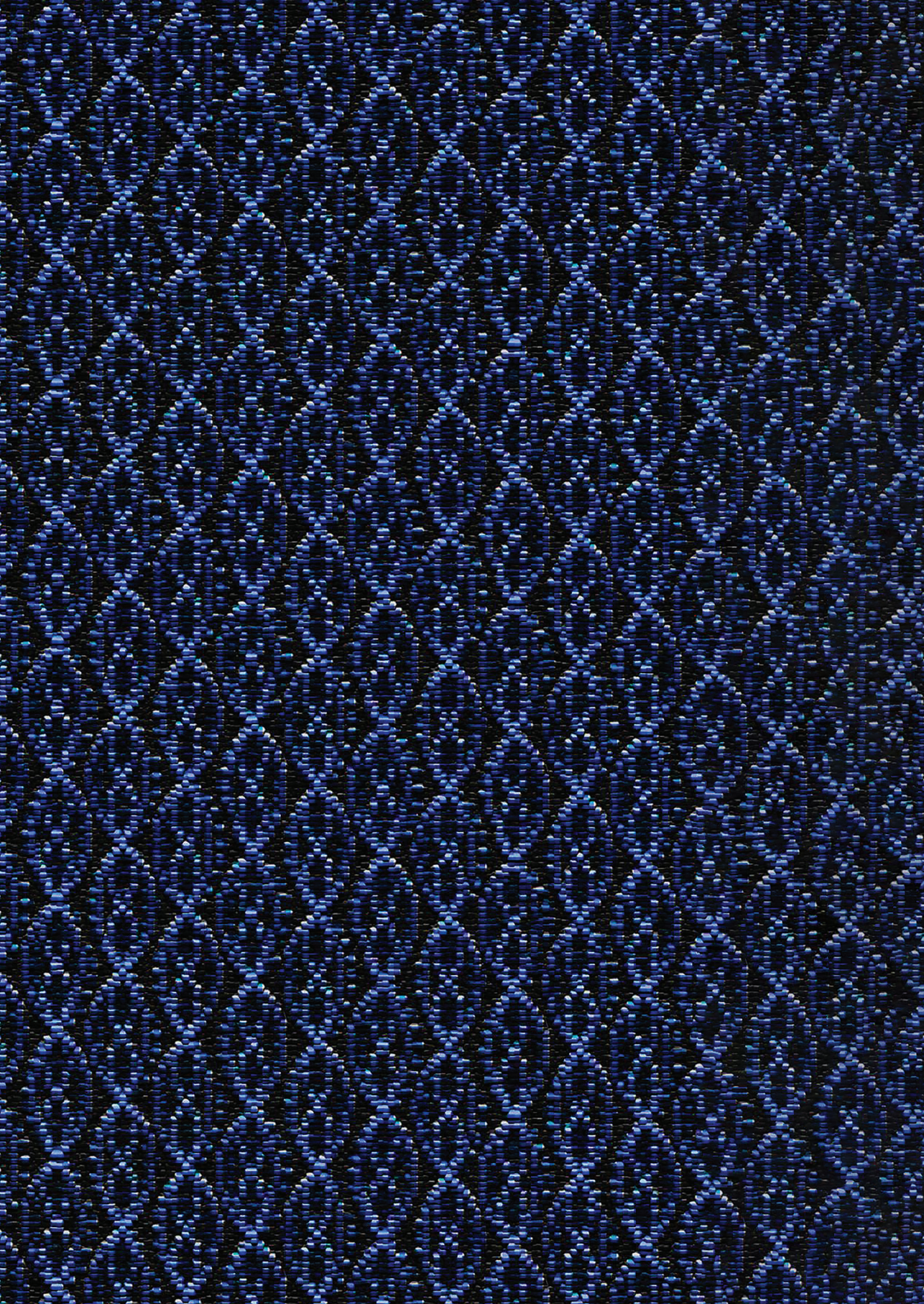
	Family history of dementia only (n=69)	**Family history of PD only (n=15)**	***p* value**
Study center			0.72
Elisabeth-TweeSteden Hospital	11 (16%)	3 (20%)	
Erasmus Medical Center	7 (10%)	2 (13%)	
Amsterdam University Medical Center	51 (74%)	10 (67%)	
Sex, male	55 (80%)	13 (87%)	0.73
Age of onset, years	64.6 (8.0)	64.5 (8.7)	0.99
First symptom			
Cognitive decline	60 (87%)	10 (67%)	0.12
Parkinsonism	4 (6%)	5 (33%)	0.008*
Hallucinations	5 (7%)	0 (0%)	0.58
MTA score (average of right and left) (n=51;9)	1 (0.5-1.5)	1 (0-1.3)	0.24[a]
CSF tau/Aβ_{1-42} ratio >0.52 (n=43;6)	22 (51%)	1(17%)	0.13[a]
APOE ε4 carrier (n=43;6)	28 (65%)	4 (67%)	1.00

Data are presented as mean (SD), median (IQR) or n (%). DLB: dementia with Lewy bodies, PD: Parkinson's disease, CSF: cerebrospinal fluid, MTA: medial temporal lobe atrophy, Aβ_{1-42}: amyloid-β_{1-42}, [a] corrected for time between age of onset and date of MRI or date of lumbar puncture, * p<0.05.

Supplementary Table 2: Statistical models regarding rate of cognitive decline and survival in the group of DLB patients with either a family history of dementia or a family history of PD.

Rate of cognitive decline		**β**	**SE**		**95% CI**	***p* value**	
Uncorrected	Baseline	0.67	1.51		-2.33-3.66	0.66	
(Lineair mixed model analysis)	Change over time	-0.022	0.045		-0.11-0.067	0.62	
With correction for sex, age of onset, and study center (Linear mixed model analysis)	Baseline	0.34	1.50		-2.63-3.31	0.82	
	Change over time	-0.015	0.045		-0.10-0.074	0.73	
Survival		**Median**[a]	**SE**		**95% CI**	***p* value**	
Uncorrected (Kaplan-Meier analysis)	Family history of dementia only	10.0	0.65		8.72-11.28	0.97	xx
	Family history of PD only	10.0	1.98		6.12-13.88		xx
		B	**SE**	**HR**	**95% CI**	***p* value**	**Z-score**
With correction for sex, age of onset, and study center (Cox regression analysis)		0.20	0.46	1.23	0.50-3.03	0.66	2.17

[a] Time between age of onset and death, * $p<0.05$.



Part 3

Known genes associated with dementia with Lewy bodies

3.1

Chapter 3.1

Familial dementia with Lewy bodies: a comprehensive analysis of genes involved in Parkinson's or Alzheimer's disease

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Abstract

Introduction

The genetic architecture of dementia with Lewy bodies (DLB) is poorly understood, but overlaps with Parkinson's disease (PD) and Alzheimer's disease (AD). Here, we performed a comprehensive analysis of genes known to be involved in PD or AD in a series of familial DLB patients, to gain more insight into their involvement in DLB.

Methods

We included twenty clinically diagnosed DLB patients with a positive family history. Neuropathological confirmation was available in 55% of the patients. By whole exome sequencing, we investigated variants in all the genes known to be involved in PD and AD. Copy number variants in selected genes, the *C9orf72* repeat expansion and the *APOE* risk allele were also investigated. Last, genotype-phenotype correlations of *GBA* variants and the *APOE* ε4 allele were explored.

Results

We identified five *GBA* variants (p.D140H, p.E326K, p.T369M, p.N370S, and p.R463C) in 35% of our patients. Furthermore, 20% of the patients carried a rare variant with unknown significance in *LRRK2*, *PARK2*, *ABCA7* or *SORL1*. The *APOE* ε4 allele frequency was 38%.

Conclusion

GBA variants and the *APOE* ε4 allele were frequently found. This confirms the importance of these two genes in DLB and the genetic overlap between DLB, PD and AD. Future genetic stratification may help to predict disease course and to select patients for disease-modifying clinical trials.

Introduction

Dementia with Lewy bodies (DLB) is one of the most prevalent types of dementia[1] and is characterized by cognitive decline in combination with parkinsonism, visual hallucinations, cognitive fluctuations, and REM-sleep behavior disorders[2,3].

Although its heritable component has been estimated to be 36% in a recent cohort of 1743 DLB patients[4], only few large association or exome sequencing studies have been reported[4-9]. These studies have shown that specific variants in *glucocerebrosidase* (*GBA*) and the *apolipoprotein E* (*APOE*) risk allele, which have previously been associated with Parkinson's disease (PD)[10] and Alzheimer's disease (AD) respectively[11,12], are also prominent risk factors for DLB. Moreover, variants in genes previously associated with PD, such as *α-synuclein* (*SNCA*) and *leucine-rich repeat kinase 2* (*LRRK2*), or in genes previously associated with AD, such as *presenilin 1* (*PSEN1*), *presenilin 2* (*PSEN2*) and *amyloid precursor protein* (*APP*), have been identified in some patients with DLB by family studies.[13] However, large families with multiple DLB patients are rarely reported, and genetic research has mainly been conducted in families with different phenotypes ranging within the DLB-PD-AD spectrum. Studies based on highly-selected series of pathologically-confirmed familial DLB patients, might facilitate the identification of novel genetic variants involved in the disease etiology.[13]

In this study, we aimed to further elucidate the genetic underpinnings of DLB and its overlap with PD and AD by studying a series of familial DLB patients.

Materials and Methods

Participants

Two groups of patients with clinically and/or pathologically diagnosed DLB and a positive family history (at least one first- or second-degree relative with DLB, PD or dementia) were studied.

The first group includes all patients (n=10) who visited the outpatient clinic of the Erasmus Medical Center in Rotterdam or the VU University Medical Center in Amsterdam between 2015 and 2017, who received a clinical diagnosis of probable DLB[2], and also had at least one first-degree relative affected with DLB, PD or dementia available for genetic studies. Pathological confirmation of the disease was available in one index patient. When possible, affected and non-affected relatives were also included in the study.

The second group includes all patients (n=10) from the Netherlands Brain Bank (NBB), who donated their brain between 1999 and 2013, had pathologically confirmed DLB (Braak α-synuclein stage: >4[14]; Braak neurofibrillary tangle stage: <4[15]), a retrospective clinical diagnosis of probable DLB[2] and a positive family history. Nine of these patients had at least one first-degree relative affected with DLB, PD or dementia, whereas one patient had one second-degree affected relative.

Clinical features were collected and genetic analyses were performed in all the 20 index patients. When possible, co-segregation studies were also carried out.

This study was approved by the relevant Medical Ethical Authorities, and all patients or their legal representative signed informed consent for use of clinical records, DNA, and pathological data for research purposes.

Sample preparation, exome capture and exome sequencing

Genomic DNA was isolated from blood in group 1 and from blood or cerebellar tissue in group 2 using standard methods. Whole exome sequencing (WES) was performed using the Nimblegen SeqCap EZ Exome v.2.0 44Mb kit (Roche Nimblegen, Inc., Madison, WI) on a HiSeq2000 sequencer (paired-end 2x100). Reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler alignment[16] tool and processed using Picard (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK)[17] following standard procedures[18]. Single nucleotide variations were determined using GATKs Haplotype Caller and annotated using ANNOVAR[19].

Filtering

Non-synonymous, stop-gain or stop-loss variants in exons, and variants near splice sites in genes with a well-established involvement in PD or AD were extracted from the WES data (Supplementary Table 1). Subsequently, variants were selected based on a minor allele frequency (MAF) of <1% in the ExAC-NFE (Exome Aggregation Consortium-Non Finnish Europeans) database and the GoNL (Genome of the Netherlands) database. For *GBA*, variants were followed up regardless the allele frequency reported in public databases to include both rare and common variants.

Sanger sequencing

Variants that fulfilled our filtering criteria were validated by Sanger sequencing (Supplementary Text; Supplementary Table 2). Exons and exon-intron boundaries with low coverage (<10 reads) in the WES analysis were also Sanger sequenced to exclude false-negative calls.

Both DNA strands were directly sequenced using the Big Dye Terminator chemistry ver.3.1 (Applied Biosystems) on an ABI3130/ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Subsequent analysis was performed with SeqScape (ver.2.6).

Copy number analysis

Multiplex Ligation-dependent Probe Amplification (MLPA) was performed to analyze copy dosage of *SNCA*, *PARK2*, *PARK7*, *PINK1* and *APP*. The P051-D1 Parkinson and P170-C2 APP MLPA kits (MRC Holland) were used according to the manufacturer's protocol. Subsequent analysis was performed on an ABI3130/ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The MLPA data were analyzed using GeneMarker Ver.2.4.0 (SoftGenetics, State College, PA, USA).

Table 1: Demographic, clinical and pathological features.

	Total (n=20)	Group 1 (n=10)	Group 2 (n=10)
Probable DLB	20 (100%)	10 (100%)	10 (100%)
Sex, male	15 (75%)	8 (80%)	7 (70%)
Age of onset, years	66.5 (8.6)	64.4 (8.5)	68.6 (8.6)
First symptom			
Parkinsonism	9 (45%)	2 (20%)	7 (70%)
Cognition	10 (50%)	8 (80%)	2 (20%)
Hallucinations	1 (5%)	0 (0%)	1 (10%)
Parkinsonism during disease course	18 (90%)	8 (80%)	10 (100%)
Disease duration[a], years (n=2;10)	5.8 (2.9)	5.5 (4.9)	5.8 (2.8)
Deceased	12 (60%)	2 (20%)	10 (100%)
Family history			
DLB	3 (15%)	0 (0%)	3 (30%)
PD/PDD	3 (15%)	3 (30%)	0 (0%)
AD/dementia	9 (45%)	2 (20%)	7 (70%)
Combination	5 (25%)	5 (50%)	0 (0%)
Autopsied	11 (55%)	1 (10%)	10 (100%)
Braak α-synuclein stage			
5	2 (18% [b])	1 (100% [b])	1 (10%)
6	9 (82% [b])	0 (0% [b])	9 (90%)
Braak neurofibrillary tangle stage			
1	2 (18% [b])	0 (0% [b])	2 (20%)
2	3 (27% [b])	0 (0% [b])	3 (30%)
3	5 (45% [b])	1 (100% [b])	4 (40%)
4	1 (9% [b])	0 (0% [b])	1 (10%)
CERAD			
O	2 (18% [b])	0 (0% [b])	2 (20%)
A	2 (18% [b])	0 (0% [b])	2 (20%)
B	4 (36% [b])	1 (100% [b])	3 (30%)
C	3 (27% [b])	0 (0% [b])	3 (30%)
Thal phase			
0	1 (9% [b])	0 (0% [b])	1 (10%)
1	1 (9% [b])	1 (100% [b])	0 (0%)
2	1 (9% [b])	0 (0% [b])	1 (10%)
3	5 (45% [b])	0 (0% [b])	5 (50%)
4	3 (27% [b])	0 (0% [b])	3 (30%)

Data are presented as mean (SD) or n (%). [a] first symptom until death. [b] in percentage of patients who were autopsied. CERAD: Consortium to Establish a Registry for Alzheimer's Disease, DLB: dementia with Lewy bodies, PD: Parkinson's disease, PDD: Parkinson's disease dementia, AD: Alzheimer's disease.

C9orf72 repeat expansion analysis

A previously described repeat-primed PCR assay[20] was used to screen for the presence of a pathogenic chromosome 9p21 GGGGCC hexanucleotide repeat expansion. Fragment length analysis was performed on an ABI3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Subsequent data were analyzed using GeneMarker Ver.2.4.0 (SoftGenetics, State College, PA, USA). A pathogenic *C9orf72* expansion was defined as more than 30 repeats.[20] Positive controls were included in this analysis.

Table 2: Genetic variants.

Position	dbSNP 142 accession number	Nucleotide change	Amino acid change	Variant type	Allele frequency GoNL (alleles)	Allele frequency ExAC-NFE (alleles)	Allele frequency GnomAD (alleles)	Allele frequency HEX (alleles)	Functional predictions: pathogenic (total)	Group 1 Clinically diagnosed and positive family history (n=10)	Group 2 Pathologically diagnosed and positive family history (n=10)
PD genes											
GBA											
Exon 6	rs147138516	c.535G>C	p.D140H (p.D179H)	missense	0.10% (1)	0.01% (7)	0.01% (20)	.	4 (10)	2 (family 1 and 2)	1
Exon 10	rs76763715	c.1226A>G	p.N370S (p.N409S)	missense	.	0.36% (242)	0.23% (623)	0.31% (3)	7 (10)	0	1
Exon 11	rs80356771	c.1504C>T	p.R463C (p.R502C)	missense	.	0.01% (4)	0.01% (37)	0.15% (1)	9 (10)	1 (family 3)	0
Exon 9	rs2230288	c.1093G>A	p.E326K (p.E365K)	missense	2.30% (23)	1.20% (798)	1.01% (3025)	0.94% (9)	3 (9)	2[a] (family 1 and 2)	2[a]
Exon 9	rs75548401	c.1223C>T	p.T369M (p.T408M)	missense	1.10% (11)	0.98% (627)	0.62% (1702)	0.92% (6)	3 (9)	0	1
LRRK2											
Exon 40	rs201012950	c.5870G>T	p.R1957L	missense	.	0.00% (2)	0.01% (22)	.	5 (10)	1 (family 4)	0
PARK2											
Exon 3	rs55774500	c.245C>A	p.A82E	missense	0.40% (4)	0.71% (471)	0.35% (978)	0.10% (1)	1 (9)	1 (family 5)	0
AD genes											
ABCA7											
Exon 5	novel	c.403G>A	p.A135T	missense	.	.	.	.	4 (9)	1 (family 6)	0
SORL1											
Exon 13	rs545522170	c.1729T>C	p.S577P	missense	0.10% (1)	0.00% (3)	0.00% (11)	.	5 (10)	0	1

[a] Both the p.D140H and p.E326K were observed in two patients from group 1 and one patient from group 2. PD: Parkinson's disease, AD: Alzheimers's disease, GoNL: Genome of the Netherlands, ExAC-NFE: Exome Aggregation Consortium-Non Finnish Europeans, GnomAD: Genome Aggregation Database (non-Finnish Europeans), HEX: Healthy Exomes > 60 years, Alzforum. Transcript references: NM_001005742 (*GBA*); NM_198578 (*LRRK2*), NM_004562 (*PARK2*), NM_019112 (*ABCA7*), NM_003105 (*SORL1*).

APOE ε risk allele analysis

Genotypes for allelic variants rs7412 and rs429358 were determined using Taqman Allelic Discrimination (Supplementary Text). Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using sequence detection system 2.3 software (Applied Biosystems Inc.).

Statistical analysis

Differences in demographic and clinical features between genetic variant carriers and non-carriers were analysed with the independent Student's t-test, χ^2-test or Fisher's Exact Test where appropriate. Statistical analyses were performed in IBM SPSS Statistics 21.0 for Windows (SPSS Inc., IL, USA). *P* values of <0.05 were considered statistically significant.

Results

Patient features

Table 1 shows the demographic, clinical and pathological features of the patients. The mean age at disease onset of the total group (75% male) was 66.5 ± 8.6 years. The mean disease duration was 5.8 ± 2.9 years. Fifteen percent of the patients had relatives with DLB, 15% relatives with PD or PD dementia (PDD), 45% relatives with dementia including AD, and 25% relatives with a combination of the previously mentioned diseases. All patients that were autopsied (n=11) had a Braak α-synuclein stage[14] of ≥5 and a Braak neurofibrillary tangle stage[15] of ≤4. CERAD[21] was B or C in 64% of the patients and Thal phase[21] was ≥3 in 73% of the patients.

Genes associated with PD

Seven heterozygous variants in three genes associated with PD (*GBA*, *LRRK2* and *PARK2*) were identified in 45% (9/20) of patients (Table 2; Figure 1).

Glucocerebrosidase

GBA heterozygous variants were observed in seven of the 20 patients (35%). Two patients carried a single rare variant (p.N370S or p.R463C), three patients carried a rare variant (p.D140H) in combination with a more common variant (p.E326K), and two patients carried a single, more common variant (p.E326K or p.T369M). The observed rare variants cause Gaucher disease (in homozygous or compound heterozygous state) and act as confirmed PD risk factors (in single heterozygous state). The more common variants do not cause Gaucher disease (in homozygous or compound heterozygous state), but still act as a mild risk factors for PD (in single heterozygous state)[22, 23] (Table 2).

Co-segregation analysis was possible for three DLB patients from group 1. In family 1, the proband (II-1) carried both the p.D140H and p.E326K variant, whereas his half-brother with DLB (II-3) carried the p.E326K variant only. In family 2, both the proband (III-2) and

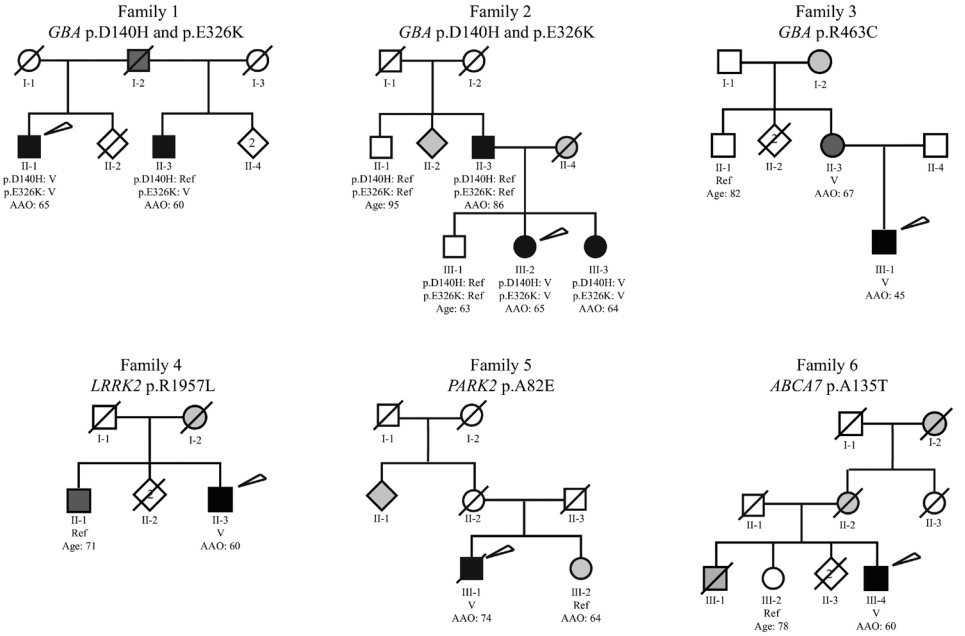

Figure 1: Pedigrees of patients with genetic variants identified.
A circle represents a female patient; a square represents a male patient; a diamond represents a patient with unknown sex; black symbols indicate patients affected by DLB; dark grey symbols indicate patients with PD or PDD and light grey symbols indicate patients with AD or unspecified dementia. V: variant, Ref: reference (wild type genotype), AAO: age at onset, DLB: dementia with Lewy bodies, PD: Parkinson's disease, PDD: Parkinson's disease dementia, AD: Alzheimer's disease.

her sister with DLB (III-3) carried these two variants, but not the father with DLB (II-3). In family 3, the proband (III-1) and his mother with PDD (II-3) carried the p.R463C variant, which was absent in his unaffected uncle (II-1) (Figure 1).

Other genes associated with PD

In two patients we detected single rare variants with unknown significance in *LRRK2* (p.R1957L) and in *PARK2* (p.A82E).

The proband of family 4 (II-3) carried the *LRRK2* p.R1957L variant, which was absent in his brother with PD (II-1) (Figure 1). The MAF of this variant is 0.01% in the GnomAD (Genome Aggregation Database (non-Finnish Europeans)), whereas it is absent in GoNL. It is predicted to be possibly pathogenic by M-CAP and by four out of nine other *in-silico* programs (Table 2; Supplementary Table 3).

The proband of family 5 (III-1) carried a heterozygous *PARK2* p.A82E variant, which was absent in his sister with dementia (III-2) (Figure 1). The MAF of this variant is 0.35% in the GnomAD and is predicted to be benign in nearly all *in-silico* programs (Table 2; Supplementary Table 3).

No dosage abnormalities in *SNCA*, *PARK2*, *PARK7* or *PINK1* were found by MLPA

analysis. This analysis was not possible in one patient from the second group, because of low DNA quality.

Genes associated with AD

Two heterozygous variants in two genes associated with AD (*ABCA7* and *SORL1*) were identified in 10% (2/20) of patients (Table 2; Figure 1).

The proband of family 6 (III-4) carried the *ABCA7* p.A135T variant, which was absent in his unaffected sister (III-2) (Figure 1). This variant is not present in public databases, and is predicted to be possibly pathogenic by four out of nine *in-silico* programs.

The p.S577P variant in *SORL1* is found in one patient from group 2. This variant has a MAF of 0.004% in the GnomAD and is predicted to be pathogenic by half of the *in-silico* programs.

The allele frequency for *APOE* ε2, ε3 and ε4 were 0%, 63% and 38%, respectively (Table 3). Furthermore, no dosage abnormalities in *APP* and no pathogenic *C9orf72* repeat expansions were found. The MLPA analysis was not possible in one patient from the second group, because of low DNA quality. Supplementary Table 4 shows an overview of the pathological findings in the genetic variant carriers.

Table 3: *APOE* ε allele frequencies.

***APOE* allele**	**Total (n=20)**	**Group 1 (n=10)**	**Group 2 (n=10)**
ε2	0 (0%)	0 (0%)	0 (0%)
ε3	25 (63%)	15 (75%)	10 (50%)
ε4	15 (38%)	5 (25%)	10 (50%)

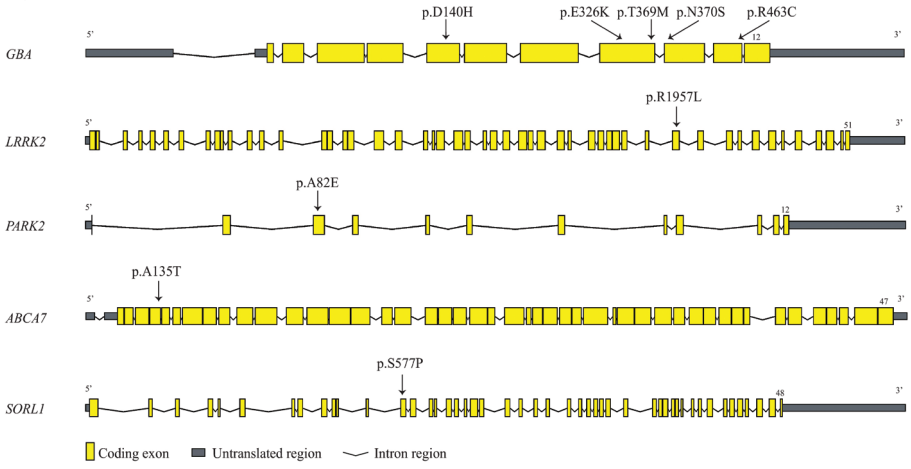

Figure 2: Gene structures.
Locations of the variants found in this study are indicated. Genes are displayed with 5'to 3'orientation and the number of exons is displayed. Transcript references: NM_001005742 (*GBA*), NM_198578 (*LRRK2*), NM_004562 (*PARK2*), NM_019112 (*ABCA7*) and NM_003105 (*SORL1*).

Table 4: Demographic and clinical features in *GBA* variant carriers and non-carriers and *APOE* ε4 allele carriers versus non-carriers.

	***GBA* variant carriers (*GBA*+) (n=7)**	**Non *GBA* variant carriers (*GBA*-) (n=13)**	***p* value**	***APOE* ε4 allele carriers (*APOE* ε4+) (n=13)**	**Non *APOE* ε4 allele carriers (*APOE* ε4-) (n=7)**	***p* value**
Sex, male	5 (71%)	10 (77%)	1.0	10 (77%)	5 (71%)	1.0
Age of onset, years	61.4 (9.6)	69.2 (6.9)	0.05*	68.8 (8.1)	62.3 (8.3)	0.11
First symptom			0.77			1.0
Parkinsonism	4 (57%)	5 (38%)		6 (46%)	3 (43%)	
Cognition	3 (43%)	7 (54%)		6 (46%)	4 (57%)	
Hallucination	0 (0%)	1 (8%)		1 (8%)	0 (0%)	
Parkinsonism during disease course	7 (100%)	11 (85%)	0.52	11 (85%)	7 (100%)	0.52
Disease duration[a], years (n=4;8|12;2)	8.5 (2.1)	4.4 (2.3)	0.01*	4.9 (2.3)	10.0 (1.4)	0.02*
Family history[b] (n=4;8|8;3)			0.15			0.006*
PD/PDD	2 (67%)	1 (13%)		0 (0%)	3 (100%)	
AD/dementia	1 (33%)	7 (87%)		8 (100%)	0 (0%)	

Data are presented as mean (SD) or n (%). [a] first symptom until death. [b] only first-degree relatives, a family history of both PD/PDD and AD/dementia were not taken into account.* Statistically different between groups. PD: Parkinson's disease, PDD: Parkinson's disease dementia, AD: Alzheimer's disease.

Genotype-phenotype correlations

Demographic and clinical features in *GBA* variant carriers (*GBA*+) and non-carriers (*GBA*-), and *APOE* ε4 allele carriers (*APOE* ε4+) and non-carriers (*APOE* ε4-) are displayed in Table 4. Given the small number of patients, we consider the following results as exploratory. In the *GBA*+ group, age of onset was earlier and disease duration longer than in the *GBA*- group (61.4 ± 9.6 years vs 69.2 ± 6.9 years, *p* value 0.05; 8.5 ± 2.1 years vs 4.4 ± 2.3 years, *p* value 0.01).

When stratifying on the presence of at least one *APOE* ε4 allele, disease duration was significantly shorter in the *APOE* ε4+ group than in the *APOE* ε4- group (4.9 ± 2.3 years vs 10.0 ± 1.4 years, *p* value 0.02). Furthermore, age of onset was significantly earlier in the *GBA*+ group compared to the *APOE* ε4+ group (58.5 ± 8.2 (n=4) vs 69.8 ± 7.2 (n=10), *p* value 0.03; patients with both a *GBA* variant and the *APOE* ε4 allele were excluded).

Interestingly, the *APOE* ε4+ group had significantly more often a family history of AD or dementia than with PD or PDD in contrast to the *APOE* ε4- group (family history AD/dementia; PD/PDD: 100%; 0% vs 0%; 100%, *p* value 0.006). When comparing the type of family history between the *GBA*+ group and the *APOE* ε4+ group, a family history of PD or PDD was more often found in *GBA*+ patients and a family history of AD or dementia in *APOE* ε4+ patients (family history PD/PDD; AD/dementia: 100%; 0% (n=2) vs 0%; 100% (n=7), *p* value 0.03; patients with both a *GBA* variant and the *APOE* ε4 allele were excluded).

Supplementary Figure 1 gives a graphical overview of the first-degree relatives with DLB (in black), PD or PDD (in grey), and AD or dementia (in white) referred among the *GBA*+ (Figure 1a) and *APOE* ε4+ patients (Figure 1b).

Discussion

Rare and common *GBA* variants and the *APOE* ε4 allele were frequently observed in this cohort of familial DLB patients. Furthermore, rare variants with unknown significance were found in *LRRK2*, *PARK2*, *ABCA7* and *SORL1*. This study provides further evidence that genes associated with PD and AD are also involved in the etio-pathogenesis of DLB, and suggests that genetic risk factors also influence disease course.

GBA variants have been associated with PD in several studies[10], but they are also increasingly recognized as risk factors for DLB[7]. We found *GBA* variants in 35% of our cohort of familial patients. Twenty-five percent of our patients carried a rare PD-associated variant in *GBA*. *GBA* variant frequencies in DLB range between studies due to differences in population, diagnostic criteria and genetic analysis methods.[13] The largest multicenter study published so far, reported a rare variant frequency of 7.5% in 721 clinically or pathologically diagnosed DLB patients and 0.97% in 1962 controls.[7] The higher frequency of these rare *GBA* variants in our study could represent a higher load of genetic factors in our patients who were selected on the basis of a positive family history, and the proportion of pathologically confirmed patients with a low Braak neurofibrillary tangle stage (<4), which has been associated with higher *GBA* variant frequencies.[24] The *GBA* variants observed in our study have all been previously associated with PD and DLB.[7,10,22,24-26] PD and DLB-associated variants in *GBA* can be divided into mild and severe[27], depending on the severity of the phenotype associated in Gaucher disease patients who carry these variants in homozygous state. A recent, large study of 1000 Ashkenazi-Jews with PD showed odds ratios for PD as different as 2.2 for mild variants (e.g. p.D140H and p.N370S) and 10.3 for severe variants (e.g. p.R463C). Furthermore, age of PD onset was significantly earlier in carriers of severe variants than those who carried mild variants.[27] In our study, we found one severe variant (p.R463C) and two mild variants (p.D140H and p.N370S). The combination of the p.D140H and E326K variant was found in three of our patients. Interestingly, the p.D140H and E326K variant combination has, together with the p.R463C variant, been associated with an increased cognitive decline in PD patients.[28] Further studies are necessary to determine if these variants are more frequently associated with DLB than PD.

The *APOE* ε4 allele is the most frequent, known risk factor for AD[11,12], and it has repeatedly been associated with DLB[4,8,9]. In our cohort, the mean *APOE* ε4 allele frequency (38%) is comparable to that previously reported in DLB (approximately 30%).[13,29-31]

We found several rare variants of unknown significance in *LRRK2*, *PARK2*, *ABCA7* and *SORL1*. The *LRRK2* p.R1957L variant is of interest, because it is extremely rare in public databases (MAF: <0.01%), is predicted to be pathogenic by half of the *in-silico* programs,

and is located in the kinase domain like the G2019S mutation, which is the most common genetic cause of PD.[32]

Pathogenic repeat expansions in *C9orf72* have been found in clinically and pathologically diagnosed AD patients[33], but not in pathologically confirmed DLB patients[34,35]. Our results are in line with the above-mentioned studies, as no pathogenic *C9orf72* repeat expansions were identified.

Given the small number of patients, exploratory analyses were performed concerning genotype-phenotype correlations of *GBA* variants and the *APOE* ε4 allele. These results must be interpreted with caution. Our analysis showed that the age of onset was earlier in the *GBA*+ group versus *GBA*- group, which has been reported before in previous studies.[7,36,37] Disease duration was longer in the *GBA*+ group versus *GBA*- group. Previous studies reported a similar disease duration between these groups.[36,37] Likewise, as in previous studies[5,8] disease duration was shorter in the *APOE* ε4+ group compared to the *APOE* ε4- group, which may be explained by a higher burden of AD concomitant pathology in *APOE* ε4+ patients.[9,38,39] Furthermore, we showed that *GBA*+ patients had more often a family history of PD or PDD than AD or dementia, and that *APOE* ε4+ patients had more often a family history of AD or dementia than PD or PDD. Based on these results, we speculate that, when looking at the PD-DLB-AD spectrum, DLB *GBA*+ patients are pathophysiological more related to the PD spectrum, and DLB *APOE* ε4+ patients to AD.

Our data support a genetic overlap between DLB, PD and AD, and they suggest that these diseases share common molecular mechanisms. This study confirms that the same genetic variant (e.g. *GBA* variants or the *APOE* ε4 allele) can be associated with different phenotypes in the DLB-PD or DLB-AD spectrum; this suggests that other genetic or non-genetic factors are important for the final resulting clinical and pathological phenotypes. Interestingly, studies also suggest[4,28,40] that specific variants within the same gene (for example the *GBA* p.D140H and E326K variant combination) may be more often associated with a specific phenotype within this spectrum. We also described new variants in DLB patients located in genes known to be involved in PD and AD, but whether they play a role in the disease development in our patients remains unknown.

This study has limitations. The total number of patients is low; clinical data were collected retrospectively in half of them; and postmortem examination was not performed in all. Particularly, the results of our genotype-phenotype correlations should be interpreted with caution, and further studies are necessary to support our findings.

Conclusion

This study confirms that *GBA* variants and the *APOE* ε4 allele play important roles in familial DLB. These findings also support the notion that genetic overlaps exist between DLB, PD and AD. Future stratification based on *GBA* and *APOE* variants may help to predict disease course and select patients for clinical trials of disease-modifying drugs.

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Supplementary Information

PCR protocol Sanger sequencing

Our standard PCR mix contained 2 µl PCR buffer, 1.6 µl dNTPs 2.5 mM, 1 µl forward primer 10 µM, 1 µl reverse primer 10 µM, 0.1 µl Fast-start taq or Platinum taq, 2 µl DNA (12.5 ng/µl) and 12.3 µl H_2O per reaction. An adjusted PCR mix was used to amplify fragment 1 of *GBA* and contained 10 µl TAKARA GCII buffer, 1.6 µl dNTPs 2.5 mM, 1 µl forward primer 10 µM, 1 µl reverse primer 10 µM, 0.1 µl TAKARA LA taq, 2 µl DNA (12.5 ng/µl) and 4.4 µl H_2O per reaction. PCR cycling consisted of initial denaturation for 5.5 minutes at 96°C, 31 or 32 cycles with denaturation for 30 seconds at 96°C, annealing for 30 seconds at 60°C and extension for 1.5 minutes at 72°C, and a final extension for 5.5 minutes at 72°C.

PCR protocol APOE ε risk allele analysis

Reactions were performed in a 384-wells format in a total volume of 2 µl containing 2 ng DNA, 1x genotyping assay (Thermo Fisher Scientific) and 1x genotyping master mix (Thermo Fisher Scientific). PCR cycling consisted of initial denaturation for 10 minutes at 95°C, and 40 cycles with denaturation of 15 seconds at 96°C and annealing and extension for 60 seconds at 60°C.

Supplementary Table 1: Genes with a well-established involvement in Parkinson's disease or Alzheimer's disease.

	Chromosome	Start	End	Mode of inheritance
PD genes				
GBA	1	155204239	155214653	autosomal dominant / risk factor
LRRK2	12	40618813	40763087	autosomal dominant
MAPT	17	43971702	44105700	autosomal dominant
SNCA	4	90645250	90759447	autosomal dominant
VPS35	16	46693589	46723144	autosomal dominant
RAB39B	X	154487526	154493852	X-linked
DJ1	1	8021714	8045342	autosomal recessive
DNAJC6	1	65720133	65881552	autosomal recessive
PARK2	6	161768590	163148834	autosomal recessive
PINK1	1	20959948	20978004	autosomal recessive
AD genes				
ABCA7	19	1040102	1065571	autosomal dominant / risk factor
APP	21	27252861	27543446	autosomal dominant
PSEN1	14	73603143	73690399	autosomal dominant
PSEN2	1	227057885	227083804	autosomal dominant
SORL1	11	121322912	121504471	autosomal dominant / risk factor
APOE	19	45409039	45412650	risk factor

Coordinates based on human reference genome (hg19; NCBI). PD: Parkinson's disease, AD: Alzheimer's disease.

Supplementary Table 2: Primers for Sanger sequencing.

Primer name	Sequence	Use
GBA_fragment1_Fa	cctaaagttgtcacccatac	PCR
GBA_fragment1_Ra	agcagacctaccctacagttt	PCR + sequencing
GBA_fragment3_Fa	tgtgtgcaaggtccaggatcag	PCR + sequencing
GBA_fragment3_Ra	accacctagaggggaaagtg	PCR
GBA_exon6_F	cccaggagcccaagttccc	sequencing
GBA_exon9_R	ctggacaggaagggcttctg	sequencing
GBA_exon10_F	ctgacctacccacagctgc	sequencing
GBA_exon10_R	tgatgggactgtcgacaaag	sequencing
GBA_exon11_F	gagagccagggcagagcctc	sequencing
GBA_exon11_R	tgagtcacccaaaccattgc	sequencing
LRRK2_exon40_F	gaagaaatggaaagtttgctatgatcc	PCR + sequencing
LRRK2_exon40_R	tcagggaaatggtagttttcatcc	PCR + sequencing
PARK2_exon3_F	tgtaactgctgtgggcaaagg	PCR + sequencing
PARK2_exon3_R	caaagtactccacctacagtgatgtctcc	PCR + sequencing
ABCA7_exon5_F	CAACTTCAACGACTCCCTgtgagc	PCR + sequencing
ABCA7_exon5_R	GGAGACTGCTTGGTTGGTTGAGG	PCR + sequencing
SORL1_exon13_F	cctttgccttagagactttcactgc	PCR + sequencing
SORL1_exon13_R	tcaattacctccctatgctttttgc	PCR + sequencing

[a] As *GBA* has a pseudogene which is very similar, two different sets of primers were used. Large fragments, specific for *GBA*, were amplified using PCR, after which other primers were used to be able to sequence the smaller regions of interest.

Supplementary Table 3: *In-silico* pathogenicity predictions of genetic variants.

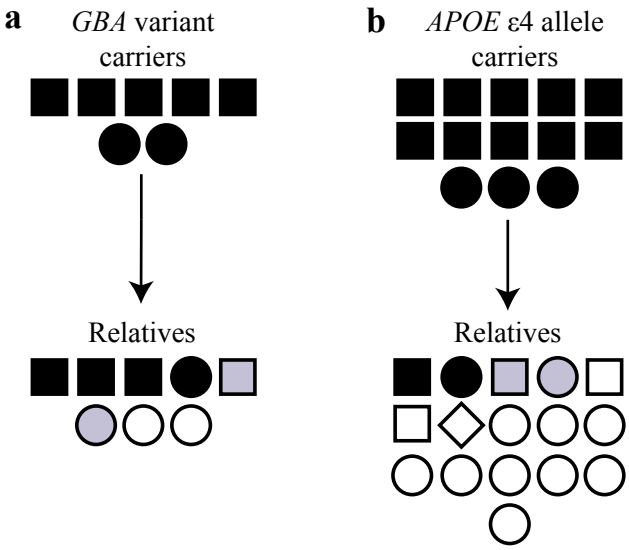
							Variant-effect predictions software (scores)									
Gene	**Chromo-some**	**Genomic position**	**Reference**	**Alternative**	**Nucleotide change**	**Amino acid change**	**GERP**	**SIFT**	**Polyphen2 HDIV**	**Polyphen2 HVAR**	**LRT**	**Mutation Assessor**	**FATHMM**	**Radial SVM**	**LR**	**CADD phred**	**M-CAP**
GBA																	
	1	155208361	C	G	c.535G>C	p.D140H (p.D179H)	2.62	**T** (0.16)	**P** (0.712)	**P** (0.653)	**N** (0.007)	**L** (1.88)	**D** (-5.8)	**D** (0.903)	**D** (0.925)	11.51	**PP** (0.404)
	1	155205634	T	C	c.1226A>G	p.N370S (p.N409S)	3.53	**D** (0.02)	**P** (0.607)	**B** (0.292)	**D** (0)	**L** (1.57)	**D** (-5.77)	**D** (1.036)	**D** (0.903)	18.4	**PP** (0.746)
	1	155204987	G	A	c.1504C>T	p.R463C (p.R502C)	2.11	**D** (0.02)	**D** (0.999)	**D** (0.932)	**N** (0.003)	**M** (2.34)	**D** (-5.84)	**D** (1.058)	**D** (0.968)	19.35	**PP** (0.917)
	1	155206167	C	T	c.1093G>A	p.E326K (p.E365K)	3.67	**T** (0.43)	**B** (0.03)	**B** (0.043)	**N** (0.075)	**L** (1.01)	**D** (-5.77)	**D** (0.675)	**D** (0.85)	11.06	**NA**
	1	155206037	G	A	c.1223C>T	p.T369M (p.T408M)	3.57	**T** (0.12)	**B** (0.275)	**B** (0.113)	**N** (0.006)	**L** (1.5)	**D** (-5.71)	**D** (0.902)	**D** (0.879)	11.4	**NA**
LRRK2																	
	12	40728881	G	T	c.5870G>T	p.R1957L	4.85	**T** (0.26)	**P** (0.834)	**P** (0.635)	**N** (0.003)	**L** (0.975)	**D** (-3.23)	**D** (0.574)	**D** (0.725)	21.1	**PP** (0.123)
PARK2																	
	6	162683724	G	T	c.245C>A	p.A82E	-0.77	**T** (0.81)	**B** (0.026)	B (0.007)	**N** (0.652)	**N** (0.695)	**D** (-2.83)	**T** (-0.805)	**T** (0.265)	5.08	**NA**
ABCA7																	
	19	1042163	G	A	c.403G>A	p.A135T	1.08	**T** (0.71)	**B** (0.035)	**B** (0.01)	**NA**	**N** (0.75)	**D** (-2.13)	**D** (0.075)	**D** (0.78)	8.46	**PP** (0.038)
SORL1																	
	11	121414300	T	C	c.1729T>C	p.S577P	5.8	**T** (0.09)	**D** (0.999)	**D** (0.996)	**D** (0)	**L** (1575)	**T** (1.04)	**T** (-0.747)	**T** (0.222)	28	**PP** (0.044)

M-CAP does not provide pathogenicity scores, when the mean allele frequency is not < 1% in all databases. GERP: Genomic Evolutionary Rate Profiling, SIFT: Sorting Intolerant From Tolerant, PolyPhen2 HDIV: Polymorphism Phenotyping version 2 human diversity, PolyPhen2 HVA: Polymorphism Phenotyping version 2 human variation, LRT: Likelihood Ratio Test, FATHMM: Functional Analysis Through Hidden Markov Models; SVM: Support Vector Machine, LR: Logistic Regression, CADD: Combined Annotation Dependent Depletion, M-CAP: Mendelian Clinically Applicable Pathogenicity, T: tolerated, D: damaging/disease causing, B: benign, N: polymorphism/neutral, P: polymorphism automatic, L: low, M: medium, PP: probably pathogenic, LB: likely benign, NA: not available. Transcript references: NM_001005742 (*GBA*), NM_198578 (*LRRK2*), NM_004562 (*PARK2*), NM_019112 (*ABCA7*), NM_003105 (*SORL1*).

Supplementary Table 4: Pathological findings in genetic variant carriers.

	Case	Sex	Age at death	Genetic variant	*APOE* ε genotype	Braak α-synuclein stage	Braak neurofibrillary tangle stage	CERAD	Thal phase
Group 1	1	M	76	*PARK2* p.A82E	34	5	3	B	1
Group 2	1	F	61	*GBA* p.D140H (p.D179H) *GBA* p.326K (p.E365K)	34	6	1	B	3
	2	M	73	*GBA* p.326K (p.E365K)	34	6	3	C	3
	3	M	67	*GBA* p.N370S (p.N409S)	33	6	2	O	0
	4	M	79	*SORL1* p.S577P	34	6	1	A	2
	5	M	84	*GBA* p.T369M (p.T408M)	34	6	2	O	3

CERAD: Consortium to Establish a Registry for Alzheimer's Disease.



Supplementary Figure 1: Graphical representation of total amount of 1st degree relatives with PD, PDD or DLB versus AD or dementia in *GBA* variant carriers (a) and *APOE* ε4 allele carriers (b). A circle represents a female patient; a square represents a male patient; a diamond represents a patient with unknown sex; black indicates patients with DLB, grey indicates patients with PD or PDD; white indicates patients with AD or unspecified dementia. DLB: dementia with Lewy bodies, PD: Parkinson's disease, PDD: Parkinson's disease dementia, AD: Alzheimer's disease.

3.2

Chapter 3.2

Neuropathological and genetic characteristics of a post-mortem series of cases with dementia with Lewy bodies clinically suspected of Creutzfeldt-Jakob's disease

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Abstract

Introduction

The disease course of dementia with Lewy bodies (DLB) can be rapidly progressive, clinically resembling Creutzfeldt-Jakob's disease (CJD). To better understand factors contributing to this rapidly progressive disease course, we describe load and distribution of neuropathology, and the presence of possible disease-associated genetic defects in a post-mortem series of DLB cases clinically suspected of CJD.

Methods

We included pathologically confirmed DLB cases with a disease duration of 3.5 years or less from the Dutch Surveillance Center for Prion Diseases, collected between 1998 and 2014. Lewy body disease (LBD) and Alzheimer's disease (AD)-related pathology were staged and semi-quantitatively scored in selected brain regions. Whole exome sequencing analysis of known disease-associated genes, copy number analysis, *APOE* ε genotyping and *C9orf72* repeat expansion analysis were performed to identify defects in genes with a well-established involvement in Parkinson's disease or AD.

Results

Diffuse LBD was present in nine cases, transitional LBD in six cases and brainstem-predominant LBD in one case. Neocortical alpha-synuclein load was significantly higher in cases with intermediate-to-high than in cases with low-to-none AD-related pathology (p=0.007). We found two *GBA* variants (p.D140H and p.E326K) in one patient and two heterozygous rare variants of unknown significance in *SORL1* in two patients.

Conclusion

A high load of neocortical alpha-synuclein pathology was present in most, but not all DLB cases. Additional burden from presence of concomitant pathologies, synergistic effects and specific genetic defects in the known disease-associated genes may have contributed to the rapid disease progression.

Introduction

Dementia with Lewy bodies (DLB) is a neurodegenerative dementia characterized by parkinsonism, fluctuating cognitive symptoms, visual hallucinations, REM-sleep behavior disorders and neuroleptic sensitivity.[1] Clinical symptoms of DLB are gradually progressive, with a median disease duration of seven to eight years.[2] However, DLB may occasionally present with a rapid deterioration leading to death within three years from the start of the first symptoms.[3-9] These rapidly progressive DLB cases frequently show additional focal neurological signs such as myoclonus, pyramidal and cerebellar signs, and akinetic mutism.[3-9] As these neurological signs are core symptoms of Creutzfeldt-Jakob's disease (CJD), a rapidly progressive prion disease[10], the clinical differential diagnosis often includes CJD. In autopsy-series of clinically possible and probable CJD cases, DLB was the neuropathological diagnosis in 2-8% of cases.[3-5] To date, it is unknown whether these cases should be regarded as a distinct entity within the Lewy body disease (LBD) spectrum. Studying the neuropathological and genetic correlates of this clinical phenotype may aid in a better understanding of factors contributing to the rapidly progressive disease course in DLB.

The main neuropathological lesions in DLB are alpha-synuclein immunoreactive Lewy bodies (LBs) and Lewy neurites (LNs). In most DLB cases, LB pathology is present in brainstem and limbic (transitional LBD), or in brainstem, limbic and neocortical areas (diffuse LBD).[11] Additionally, Alzheimer's disease (AD)-related changes including amyloid-β plaques, neurofibrillary pathology and neuritic plaques are often present.[12] Neuropathological correlates of disease progression and survival in DLB have previously been assessed in prospectively[13] and retrospectively studied DLB[14] or combined Parkinson's disease (PD) dementia and DLB[15] cohorts. These studies revealed that patients with diffuse LBD had a shorter disease duration[13-15] and showed a more rapid cognitive decline[14] than patients with transitional LBD. Both a higher alpha-synuclein load and amyloid-β load, and to a lesser extent also neurofibrillary pathology load, were associated with a shorter disease duration.[13]

To better understand neuropathological and genetic factors contributing to a rapidly progressive disease course in DLB, we describe here the load and distribution of alpha-synuclein pathology and concomitant pathology, and the presence of possible disease-associated genetic defects in a post-mortem series of DLB cases suspected of CJD from the Dutch Surveillance Centre for Prion Diseases.

Methods

Patient selection

All cases clinically suspected of CJD with LBD at autopsy collected in the period from 1998 to 2014 by the Dutch Surveillance Center for Prion Diseases at the University Medical Center in Utrecht were included. The Dutch Surveillance Center obtained permission for

brain autopsy and written informed consent for use of clinical, pathological and genetic data for research purposes from the patients during life or from their next of kin after death. The medical ethics committee (MEC) of the University Medical Center Utrecht approved all procedures of autopsy. We retrieved information from medical records on symptoms and signs of DLB and sporadic CJD as described by the McKeith criteria and the criteria from the World Health Organization (WHO).[10,11] Symptoms were only regarded as present or not present when explicitly stated in the clinical information. An experienced neurologist (AL) classified all patients to the clinical criteria for DLB[11] and WHO criteria for CJD[10].

Inclusion criteria for this study were: 1) presence of LBD at autopsy, 2) negative screen for prion protein at autopsy, 3) disease duration of 3.5 years or less from the start of first symptoms, 4) no other major neurological or systemic diseases that provided sufficient explanation for a rapid deterioration, and 5) presence of sufficient clinical data.

Neuropathological assessment

Post-mortem examination was performed within 4 to 8 hours post-mortem. A total of 25 tissue blocks were taken from the following regions: frontal, parietal, temporal and occipital cortices, hippocampus, striatum, thalamus, mesencephalon, pons, medulla oblongata and cerebellum.

After decontamination using 98% formic acid for five minutes, brain tissue blocks were formalin-fixed, paraffin-embedded and cut into sections of 10 μm thickness. Routine histological stainings were performed with haematoxylin-eosin, Gallyas silver staining and combined Luxol fast blue-periodic acid-Schiff. All regions were examined for the presence of prion protein with the use of monoclonal antibody 3F4 (1:400, Signet labs, United States). Immunohistochemistry was performed using primary antibodies against alpha-synuclein (clone KM51; 1:500; Monosan, the Netherlands), hyperphosphorylated tau (clone AT8; 1:1000; Innogenetics, Belgium), and amyloid-β (clone 6f/3d; 1:100; Dako, United States). For staging and semi-quantitative scores of alpha-synuclein pathology, Brain Net Europe (BNE) consensus guidelines[16] and modified McKeith criteria[17] were used. LBs and LNs were scored in brainstem regions, LNs in the CA2 regions and LBs in other limbic and neocortical regions. For pathological staging of neurofibrillary tangles, amyloid-β plaques and neuritic plaques, National Institute on Aging - Alzheimer Association (NIA-AA) criteria were used.[17] Mean cerebral load of alpha-synuclein, neurofibrillary and amyloid-β pathology was calculated based on semi-quantitative load of pathology in selected regions used for pathological staging (Supplementary Methods). Presence of age-related astrogliopathy (ARTAG)[18], argyrophilic grain disease[19], atherosclerosis, ischemic or hemorrhagic lesions and small vessel disease[17] was reported, and cerebral amyloid angiopathy (CAA)[20] was classified. Spongiform changes were assessed based on presence of vacuoles in the entorhinal and temporo-occipital cortex. Neuropathological assessment was performed by an experienced neuropathologist (AR), and semi-quantitative load of pathology was scored according to consensus criteria[16,17,21] by the same assessor (HG) in all cases.

Genetic analysis

Fresh-frozen temporal cortex was available for eleven cases (Table 2). Genomic DNA was isolated from this tissue with the AllPrep DNA/RNA/miRNA Universal Kit of Qiagen. The Nimblegen SeqCap EZ Exome v.2.0 44Mb kit (Roche Nimblegen, Inc., Madison, WI) was used on a HiSeq2000 sequencer (paired-end 2x100) for the whole exome sequencing (WES). We selected non-synonymous, stop-gain or stop-loss variants in exons and variants near splice sites with a mean allele frequency of <1% in the ExAC-NFE (Exome Aggregation Consortium-Non Finnish Europeans) database and the GoNL (Genome of the Netherlands) database in genes with a well-established involvement in Parkinson's disease (PD) (*SNCA*, *Parkin*, *PINK1*, *DJ1*, *LRRK2*, *GBA*, *VPS35*, *DNAJC6*, *RAB39B*), AD (*APP*, *PSEN1*, *PSEN2*, *SORL1*, *TREM2*, *APOE*, *ABCA7*) and frontotemporal dementia (*MAPT*, *GRN*). All variants in *GBA* were selected regardless the mean allele frequency reported in public databases. Subsequently, Sanger sequencing was performed to validate selected variants and to exclude false-negative results in exons and intron-exon boundaries with low coverage (<10 reads).

Furthermore, copy dosage analysis of *SNCA*, *Parkin*, *PARK7*, *PINK1* and *APP* was done using Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland), *APOE* ε genotyping using Taqman Allelic Discrimination and *C9orf72* repeat expansions analysis using a repeat-primed PCR assay. Further details on genetic analyses techniques are provided in the Supplementary Methods.

Statistical analyses

Mean and standard deviation were calculated for normally distributed continuous data (age at onset, age at death), median and interquartile range was used for not normally distributed data (disease duration) and ordinal data (load of different types of pathology). Correlation between cerebral alpha-synuclein, neurofibrillary and amyloid-β load was assessed with Spearman's rank correlations, and the cerebral alpha-synuclein load in a group of cases with low-to-none versus a moderate-to-high level of AD-related pathology was compared using the Mann-Whitney U test. Data were considered significant if $p<0.05$. All statistical analyses were done using IBM SPSS version 22.

Results

Neuropathological diagnoses in all cases suspected of CJD

Of 512 consecutive autopsy cases between 1998 and 2014, autopsy revealed a prion disease in 296 cases (58%), including 259 cases with sporadic CJD. Neurodegenerative diseases were the most frequent alternative diagnoses (48%), followed by immune diseases (17%), malignant diseases (10%), toxic metabolic disorders (10%), vascular diseases (9%) and other causes (6%) (Supplementary Table 1). LBD was found in 26 cases (12%), of whom sixteen cases fulfilled inclusion criteria (Supplementary Methods).

Clinical diagnosis and demographics

All sixteen cases were diagnosed during life with rapidly progressive dementia. CJD was considered the most likely clinical diagnosis or was included in the differential diagnosis in all cases by the treating neurologists, and DLB was explicitly listed in the differential diagnosis in eight cases. In retrospect, thirteen cases fulfilled clinical criteria for probable DLB and three cases for possible DLB.[11] Clinical criteria categorized one case as probable CJD, eight cases as possible CJD, and seven cases as no CJD.[10] Median disease duration was 12 months (interquartile range 6.5 to 24 months), with a mean age at onset of 77 years and a mean age at death of 78 years (SD 7.3; range 62 to 87 years). Basic demographic features, clinical symptoms, ancillary investigations and clinical diagnoses of CJD and DLB are listed in Table 1.

Distribution and load of alpha-synuclein pathology

Mild to moderate atrophy was present in 50% of cases (Table 2). Moderate to severe neuronal loss in the substantia nigra was present in all cases. Brainstem-type LBD was present in one case (Figure 1, 5a-c), transitional LBD in six cases and diffuse LBD in nine cases. Eleven cases showed Braak alpha-synuclein stage 6 (Table 2). A large proportion of the cases showed a moderate to severe load of alpha-synuclein pathology in the substantia nigra (81%), temporo-occipital cortex (75%), temporal cortex (56%), and frontal cortex (38%, Figure 2).

Presence of concomitant AD-related pathology, vascular pathology and spongiform changes

All cases showed neurofibrillary pathology, with nine cases reaching Braak neurofibrillary stage 3 or higher. One case (case 8) showed ARTAG, none of the cases showed argyrophilic grain disease. Diffuse and/or classical amyloid-β positive plaques were present in thirteen cases and neuritic plaques were present in the neocortex of nine cases (Table 2). The level of AD-related pathology was none in two cases, low in six cases, intermediate in six cases and high in two cases (case 1 and 11; Figure 1, 2d-e, Table 2).

Case 16 showed severe capillary CAA, Thal stage 3 (Figure 1, 3e). Additionally, CAA type 2 was present in six cases. Signs of small vessel disease were present in three cases, and severe atherosclerosis of large vessels combined with multiple cortical and subcortical infarctions were present in two cases (case 13 and 15). Mild to severe spongiform cortical changes were present in twelve cases (75%) (Table 2).

Table 1: Basic demographic features, clinical symptoms, ancillary investigations and diagnoses of dementia with Lewy bodies and Creutzfeldt-Jakob's disease in sixteen cases with dementia with Lewy bodies clinically suspected of Creutzfeldt-Jakob's disease.

Sex	9 M / 7 F
Disease duration, months	12 (4–42)
Age at onset, years	77 (7.3)
Age at death, years	78 (7.3)
Presence of clinical symptoms (yes / no / NA)	
- Dementia	16 (100%)/ 0 / 0
- Parkinsonism	14 (88%)/ 2 / 0
- Fluctuating cognition	11 (69%)/ 1 / 4
- Visual hallucinations	12 (75%)/ 0 / 4
- Delusions	9 (56%)/ 0 / 7
- Neuroleptic sensitivity	9 (56%)/ 1 / 6
- History of depression	5 (31%)/ 0 / 11
- Autonomic dysfunction	3 (19%)/ 0 / 13
- Myoclonus	10 (63%)/ 1 / 5
- Pyramidal symptoms	6 (38%)/ 5 / 5
- Cerebellar symptoms	4 (25%) / 8 / 4
- Akinetic mutism	3 (19%)/ 4 / 9
Ancillary investigations	
- PSWCs on EEG	Present in 0 of 11 cases with a reported EEG (non-periodic bi- or triphasic complexes in 3 cases)
- Hyperintensities on MR-DWI	Present in 0 of 7 cases with a reported MRI
- 14-3-3 protein in CSF	2 negative; 1 inconclusive; 2 positive of 5 cases with a reported 14-3-3 CSF test
Clinical diagnosis of DLB[11]	0 no; 3 possible; 13 probable
Clinical diagnosis of CJD[10]	7 no; 8 possible; 1 probable

Data are presented as mean (SD), median (range) or n (%). PSWCs: periodic sharp wave complexes, EEG: electroencephalogram, MR-DWI: magnetic resonance diffusion weighted imaging, CSF: cerebrospinal fluid, NA: not available.

Table 2: Staging of Lewy body disease and Alzheimer's disease related pathology, presence of vascular pathology and spongiform changes, genetic variants in disease-associated genes and *APOE* ε genotype in sixteen cases with dementia with Lewy bodies clinically suspected of Creutzfeldt-Jakob's disease.

Case number	Sex	Age at death	Clinical criteria for DLB	Clinical criteria for CJD	Atrophy	Athero-sclerosis	Infarctions	Braak alpha-synu-clein stage	McKeith Lewy body stage	Braak neurofi-brillary stage	Thal phase for amy-loid-beta	CERAD age-re-lated score	Level of AD-related pathology	Small vessel disease	Cerebral amyloid angiopa-thy	Spon-giform changes	Genetic variants in disease-as-sociated genes	*APOE* ε genotype
1	M	74	Probable DLB	No CJD	no	no	no	6	diffuse	5	4	C	high	no	type 2 stage 2	moderate	ND	ND
2	M	79	Probable DLB	Possible CJD	moderate (global, wide ventricles)	no	no	5	transiti-onal	3	1	O	low	yes	no	mild	*SORL1* (p.R1799Q)	2/3
3	F	87	Probable DLB	Possible CJD	no	no	no	6	diffuse	2	3	O	low	no	type 2 stage 2	severe	-	3/3
4	F	81	Probable DLB	No CJD	no	no	no	6	transiti-onal	2	2	O	low	no	no	moderate	ND	ND
5	M	67	Probable DLB	No CJD	no	mild	no	6	diffuse	3	3	B	intermediate	no	type 2 stage 1	none	ND	ND
6	F	86	Probable DLB	Possible CJD	mild (frontal)	moderate	no	6	diffuse	4	3	B	intermediate	yes	no	mild	-	3/3
7	M	75	Probable DLB	No CJD	no	no	no	6	transiti-onal	2	2	O	low	no	no	mild	*GBA* (p.D140H + p.E326K)	3/3
8	M	71	Possible DLB	Possible CJD	mild (frontotemporal)	moderate	no	6	diffuse	3	3	B	intermediate	no	type 2 stage 1	mild	-	3/3
9	M	85	Probable DLB	Possible CJD	no	moderate	no	5	transiti-onal	1	3	B	low	no	no	none	-	3/3
10	F	76	Possible DLB	No CJD	mild (frontotemporal)	moderate	no	3	brainstem	1	0	O	none	no	no	none	-	2/3
11	M	75	Probable DLB	Possible CJD	mild (frontotemporal)	mild	no	6	diffuse	6	4	C	high	no	no	severe	ND	ND
12	F	80	Probable DLB	Possible CJD	no	mild	no	5	transiti-onal	1	0	O	low	no	no	mild	-	2/4
13	M	84	Probable DLB	No CJD	moderate (frontal)	severe	microscopical infarctions: CA1/CA2 region hippocampus right, caudate nucleus right and left, occipital cortex	6	diffuse	3	4	C	intermediate	yes	type 2 stage 1	severe	*SORL1* (p.D140N)	3/4
14	F	62	Possible DLB	Possible CJD	mild (frontal, amygdala, brainstem)	mild	no	4	transiti-onal	1	0	O	none	no	no	none	-	3/3
15	F	87	Probable DLB	Probable CJD	mild	severe	cerebellar left (2cm), microscopical infarctions in temporal cortex and thalamus	6	diffuse	3	5	C	intermediate	no	type 2 stage 1	mild	-	3/4
16	M	78	Probable DLB	No CJD	no	mild	no	6	diffuse	3	4	B	intermediate	no	type 1	x	x	x

CERAD: Consortium to Establish a Registry for Alzheimer's Disease, ND: not done.

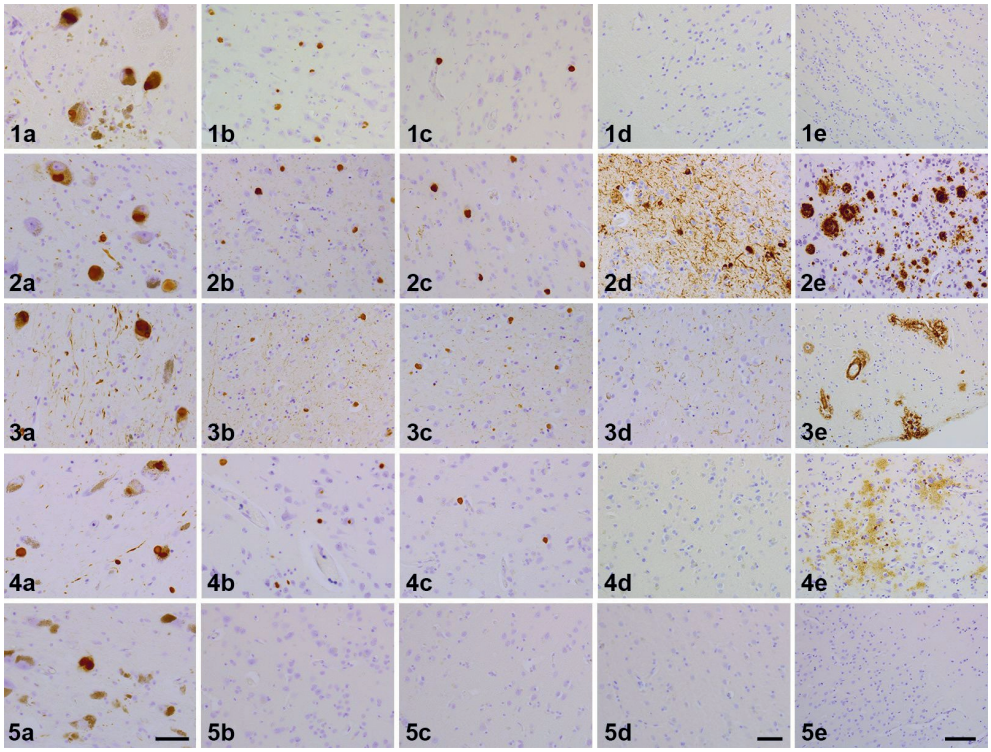

Figure 1: Representative overview of the neuropathological features visualized with immunohistochemistry in sixteen cases with dementia with Lewy bodies clinically suspected of Creutzfeldt-Jakob's disease.
Load of alpha-synuclein pathology was high in limbic and neocortical regions in most cases (**1b-4b, 1c-4c**), but a few cases only showed brainstem-predominant (**5a-c**) or transitional Lewy body disease (LBD). Neurofibrillary and amyloid-β pathology were absent or low in some (**1d-e, 4d-e, 5d-e**), but severe in other cases (**2d-e**). One case showed severe capillary cerebral amyloid angiopathy (**3e**). A case with two *GBA* variants (p.D140H and p.E326K) showed diffuse LBD (**4a-c**) and low levels of Alzheimer's disease (AD)-related pathology (**4d-e**). One case showed nigral degeneration and brainstem-predominant LBD (5a-c) without signs of AD-related pathology (**5d-e**).
Representative microscopy images from case 4 (**1a-e**), 11 (**2a-e**), 16 (**3a-e**), 7 (**4a-e**) and 10 (**5a-e**). Immunohistochemistry against alpha-synuclein (clone KM51) was performed on **a:** substantia nigra, **b:** trans-entorhinal cortex and **c:** temporal cortex. **d:** Immunohistochemistry against hyperphosphorylated tau (clone AT8) on temporal cortex. **e:** Immunohistochemistry against amyloid-β (clone 6f/3d) on temporal (1e, 2e, 4e and 5e) and frontal (3e) cortex. The scale bar in 5a represents 50 µm and applies to 1a-5a, 1b-5b and 1c-5c. The scale bar in 5d represents 50 µm and applies to 1d-5d. The scale bar in 5e represents 100 µm and applies to 1e-5e.

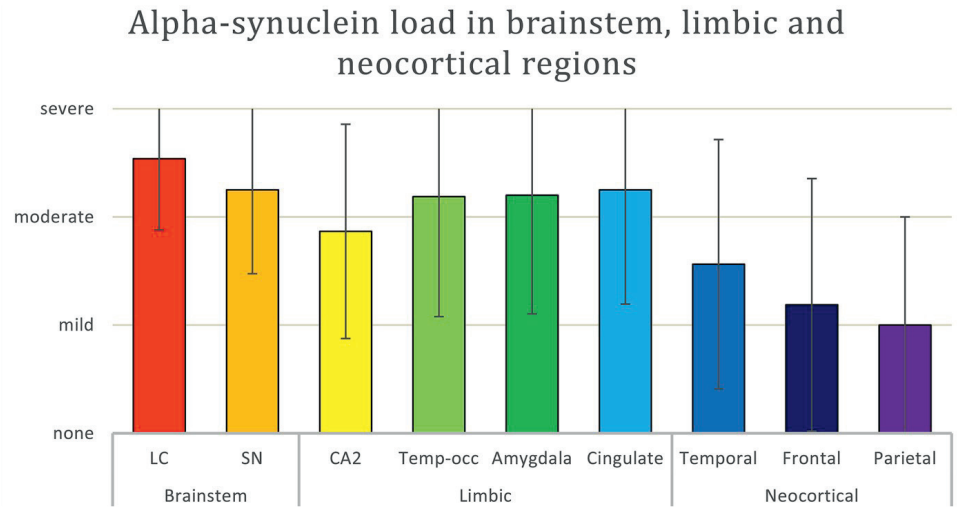

Figure 2: Semi-quantitative load of alpha-synuclein pathology in brainstem, limbic and neocortical brain regions in sixteen cases with dementia with Lewy bodies clinically suspected of Creutzfeldt-Jakob's disease.
Lewy bodies (LBs) and Lewy neurites (LNs) were scored in brainstem regions, LNs in the CA2 region and LBs in other limbic and neocortical regions. Bars represent mean values, error bars represent standard deviations. LC: locus coeruleus, SN: substantia nigra, CA2: cornu ammonis region 2, temp-occ: temporo-occipital cortex, cingulate: posterior cingulate gyrus, temporal: medial temporal gyrus, frontal: frontal gyrus (Brodmann area 10), parietal: inferior parietal lobe.

Correlations between cerebral load of alpha-synuclein, neurofibrillary and amyloid-β pathology

Cases with an intermediate-to-high load of AD-related pathology (n=8) had a significantly higher cerebral load of alpha-synuclein pathology compared to cases with no-to-low level of AD-related pathology (n=8, p=0.007). The load of alpha-synuclein pathology was significantly correlated to both the load of neurofibrillary (Spearman's rho 0.757; p=0.001) and amyloid-β pathology (Spearman's rho 0.707; p=0.002).

Presence of genetic defects and their neuropathological correlates

In three of the eleven cases (45%) analyzed, possible disease-associated variants were detected in genes with a well-established involvement in PD or AD (Supplementary Table 2).

In case 7, a rare variant and a common variant were detected in *GBA* (p.D140H and p.E326K) and confirmed by Sanger sequencing. E326K and E326K+D140H variants have been associated with a more rapid cognitive decline in PD.[22,23] At autopsy, case 7 showed a high load of neocortical alpha-synuclein and a low level of AD-related pathology (Figure 1, 4a-e).

Additionally, the p.R1799Q and p.D140N variants in *SORL1* were found in case 2 and 13 respectively, and confirmed by Sanger sequencing. These variants have an allele frequency

of 0.005% and 0.03% in the GnomAD (Genome Aggregation Database) respectively. The p.D140N variant is predicted to be pathogenic by half of the *in-silico* programs, whilst the p.R1799Q variant is predicted to be likely benign by the majority of programs. The case with the p.D140N variant showed an intermediate level of AD-related pathology, whereas the case with the likely benign p.R1799Q variant showed only a low level of AD-type pathology.

No *SNCA*, *Parkin*, *PARK7*, *PINK1* or *APP* dosage abnormalities and no pathogenic *C9orf72* repeat expansions were found. Three cases carried an *APOE* ε4 allele, of which one had no AD-related pathology and two had an intermediate level of AD-related pathology (Table 2).

Discussion

Until now, few studies have reported the neuropathological characteristics of DLB cases suspected of CJD. Here, we describe neuropathological and genetic features in sixteen of these cases collected by the Dutch Surveillance Center for Prion Diseases. A high load of neocortical alpha-synuclein pathology was present in most, but not all DLB cases, and cases showed a variable load of AD-related pathology. We found two *GBA* variants in one patient and two heterozygous rare variants of unknown significance in *SORL1* in two patients.

In the current study, ten cases retrospectively fulfilled clinical criteria for possible or probable CJD, and all cases retrospectively fulfilled clinical criteria for possible or probable DLB, with a median disease duration of 12 months. The overlap of clinical symptoms in cases with rapidly progressive dementias is well-known, and it has been proven difficult to discriminate rapidly progressive DLB and CJD based on clinical symptoms and signs.[3-5,7]

The neuropathological correlates of rapidly progressive DLB have only been studied in few case-studies and very small case-series. Transitional LBD was present in four cases, and diffuse LBD in 16 out of 20 cases described.[4,7,8,24] The level of AD-related pathology according to the NIA-AA[17] ranged from none to severe.[7-9] Additionally, concomitant pathology included argyrophilic grain disease[7], TDP-43 positive inclusions[8], atherosclerosis[8], infarctions[8], small vessel disease[7-9], CAA[7], and a subarachnoidal hemorrhage[8]. In conclusion, the neuropathology described in these cases was variable, but studies were small with heterogeneous inclusion criteria.

In 56% of DLB cases suspected of CJD in the current study, the neuropathology was characterized by diffuse LBD, with an intermediate to high load of alpha-synuclein pathology in neocortical regions. In comparison, a previous large post-mortem series of 807 DLB cases (mean disease duration 8.8 ± 4.0 years) revealed diffuse LBD in 57% of the cases.[14] However, due to differences in case selection, a direct comparison of different cohorts should be interpreted with caution.[25] Although diffuse LBD has been related to a more rapidly progressive disease course in DLB[13,14] and LBD[15], our results show that the pathological correlate of rapidly progressive DLB can be transitional LBD or even brainstem-predominant LBD.

The load of AD-related pathology varied from none to severe in this study. A negative correlation between load of AD-related pathology and survival in DLB has been shown by neuropathological studies[13,15] and a recent cerebrospinal fluid biomarker study[26]. However, the current study shows that DLB symptoms can also progress rapidly in cases with little concomitant pathology. Also, concomitant vascular pathology was present in several cases, which may have added to disease progression.

Spongiform changes were found in 75% of cases in the current study, which is in line with a previous post-mortem study of 40 DLB cases, where 82% showed spongiform changes.[27] In this study, there was no correlation between spongiform changes and disease duration[27], which is corroborated by the low frequency of severe spongiform changes in the current series. The heterogeneous neuropathology in our cases is in line with previous reports on the presence of neuropathological hallmarks in DLB cases suspected of CJD[7-9], and with the neuropathology of DLB cases in general[13,14].

In this study, a higher alpha-synuclein load was associated with a higher load of AD-related pathology. This is in line with results from previous autopsy studies, that revealed a correlation between alpha-synuclein pathology and AD-related pathology in LBD[15], and DLB[13] cases. Together with evidence from *in vitro* and animal cross-seeding experiments, this suggests synergistic interactions between hyperphosphorylated tau, amyloid-β and alpha-synuclein aggregates.[28]

The relation between genetic defects and a rapidly progressive phenotype in DLB has been understudied. In a few rapidly progressive DLB cases, a genetic screening has been performed, without finding evidence for presence of genetic defects.[8,24]

Interestingly, in one diffuse LBD case with a low level of AD-related pathology, a combination of two genetic variants was found in *GBA* (p.D140H and p.E326K). This combination of variants has been described before in Gaucher disease[23] and PD[22]. PD carriers of the p.E326K variant and the D140H+E326K complex allele were shown to have a faster cognitive decline than non-*GBA* associated PD cases.[22] As *GBA* variants have been shown to be associated with more severe motor and cognitive dysfunction in DLB[29], these variants may have contributed to the rapid cognitive decline.

Furthermore, two other heterozygous variants in the *SORL1* gene (p.D140N and p.R1799Q) were observed in two different cases. According to recent criteria for *SORL1* variants in AD, both variants are categorized as 'likely benign'.[30] However, as these criteria have been adopted for AD, the role of these variants in DLB disease progression is still uncertain.

Limitations of the current study are the small sample size, limited availability of tissue for genetic analysis, inclusion of cases based on clinical referrals, and the retrospective nature of the clinical data. Additionally, rapid clinical deterioration in DLB can be induced by a hypersensitive reaction to neuroleptic treatment, which may be misinterpreted as signs of CJD.[6] This iatrogenic cause of disease progression may play a role in some cases in this study. Studies in larger, prospectively followed cohorts of patients are needed to draw more

generalizable conclusions on the neuropathological or genetic substrates of rapid disease progression in DLB, especially regarding the clinical and pathological heterogeneity in this group of patients. However, this may be difficult to realize in clinical practice, as DLB cases clinically suspected of CJD are very rare.

In short, a high load and neocortical distribution of alpha-synuclein pathology is present in some, but not all DLB cases in this post-mortem series. This suggests that a different set of factors contribute to the rapidly progressive disease course in cases with no or a low load of neocortical alpha-synuclein pathology. Additional burden from presence of concomitant pathologies, synergistic effects and specific genetic defects may have contributed to the disease progression in some cases. Understanding which factors contribute to a rapid disease progression in DLB could aid in the search for biomarkers that enable clinicians and researchers to select patients for therapeutic strategies.

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Supplementary Information

Case selection

Of 26 cases with LBD without presence of prion protein, collected from 1998 to 2014, 16 were included in the study whereas the remaining 10 cases did not meet the inclusion criteria. A disease duration of four years or longer was present in five cases, other neurological diseases were apparent in three cases (one case with an intracerebral B-cel lymphoma, one case with a large recent infarction, and one case with microcephaly), and sepsis with multiple organ failure or bronchopneumonia coincided with the neurological decline in two cases. Thus, sixteen cases were included in the current study.

Calculation of mean load of neurofibrillary, amyloid-β and alpha-synuclein pathology

Alpha-synuclein pathology was calculated as the mean of temporo-occipital, cingulate, temporal, frontal and parietal scores. Neocortical load of neurofibrillary pathology was calculated as the mean of the entorhinal, temporo-occipital, temporal, occipital-peristriatal and occipital-striatal scores, and neocortical amyloid-β load was calculated as the mean of entorhinal, temporo-occipital, cingulate, temporal, frontal, parietal and occipital scores.

DNA quality assessement

All samples were snap frozen in liquid nitrogen at autopsy and stored at -80°C until use. Degradation of DNA was assessed using DNA electrophoresis on agarose gels, which did not reveal DNA degradation in any of the samples. Quantification of DNA purity by Nanodrop® showed 260/280 ratios above 1.8 for all samples.

Whole exome sequencing

The Burrows-Wheeler alignment tool was used to align reads to the human reference genome (hg19). Picard (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK) were used to process aligned reads following standard procedures.[1] We determined single nucleotide variations using GATKs HaplotypeCaller, and used ANNOVAR for functional annotation.[2]

Sanger sequencing

Direct sequencing of both strands was done using the Big Dye Terminator chemistry version 3.1 (Applied Biosystems), and fragments were run on an ABI3130 or ABI3730 sequencer. SeqScape (version 2.6) was used for analysis.

Multiplex Ligation-dependent Probe Amplification

The P051-D1 Parkinson and P170-C2 APP MLPA kits (MRC Holland) were used according to the manufacturer's protocol. An ABI3130 or ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and GeneMarker Ver.2.4.0 (SoftGenetics, State College, PA, USA) were used for further analysis.

APOE ε genotyping

APOE ε genotyping (rs7412 and rs429358) was done using Taqman Allelic Discrimination. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using sequence detection system 2.3 software (Applied Biosystems Inc.).

C9orf72 repeat expansions analysis

To detect pathogenic *C9orf72* repeat expansions, a previously described repeat-primed PCR assay[3] was used. An ABI3130 or ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and GeneMarker Ver.2.4.0 (SoftGenetics, State College, PA, USA) were used for further analysis. A pathogenic *C9orf72* expansion was defined as more than 30 repeats.[3]

Supplementary Table 1: Neuropathological diagnosis in 512 consecutive autopsy cases in period 1998-2014 at the Dutch Surveillance Center for Prion Diseases.

Prion disease	**296**		
		Sporadic CJD	259
		Sporadic CJD	252
		Sporadic CJD + AD	7
		Other prion diseases	37
Non-prion disease	**216**		
		Neurodegenerative diseases	104
		AD	46
		LBD	26
		Mixed AD + vascular pathology	14
		PSP	4
		FTD-tau or FTD-TDP	8
		ALS	2
		Multiple sclerosis	4
		Immune diseases	37
		Limbic encephalitis	27
		Viral infections	4
		Neurocoeliac disease	4
		HIV encephalopathy	2
		Malignant diseases	22
		Myeloproliferative disease	11
		Metastases	5
		Intravascular lymphoma	3
		Astrocytoma or glioblastoma	3
		Toxic metabolic disorders	21
		Vascular diseases	20
		Thrombotic or vascular lesions	18
		Non-CAA angiopathy	2
		Other	12
Total	**512**		

CJD: Creutzfeldt-Jakob's disease, AD: Alzheimer's disease, LBD: Lewy body disease, PSP: progressive supranuclear palsy, FTD: frontotemporal dementia, ALS: amyotrophic lateral sclerosis, HIV: human immunodeficiency virus, CAA: cerebral amyloid angiopathy.

Supplementary Table 2: Variants in disease-associated genes in dementia with Lewy bodies cases clinically suspected of Creutzfeldt-Jakob's disease.

Case nr	Gene	Position	db SNP 142 accession number	Nucleotide change	Amino acid change	Mean allele frequency GoNL (%)	Mean allele frequency ExAC-NFE (%)	Mean allele frequency GnomAD (%)	Functional predic-tions: pathogenic (total)	Pathogenicity in AD according to criteria by Holstege et al.[4]	Pathogenic in literature
7	*GBA*	Exon 6	rs147138516	c.535G>C	p.D140H (p.D179H)	0.1	0.01	0.01	4 (10)	-	Yes[5]
7	*GBA*	Exon 9	rs2230288	c.1093G>A	p.E326K (p.E365K)	2.30	1.20	1.01	3 (9)	-	Risk factor[5]
2	*SORL1*	Exon 40	rs530863434	c.5396G>A	p.R1799Q	-	0.01	0.005	1 (10)	Likely benign	Not described
13	*SORL1*	Exon 3	rs140888526	c.418G>A	p.D140N	-	0.05	0.03	5 (10)	Likely benign	Not likely[6]

ExAC-NFE: Exome Aggregation Consortium - Non Finnish Europeans, GoNL: Genome of the Netherlands, GnomAD: Genome Aggregation Database (non-Finnish Europeans). Transcript references: NM_001005742 (*GBA*), NM_198578 (*LRRK2*) and NM_004562 (*Parkin*).

Supplementary References

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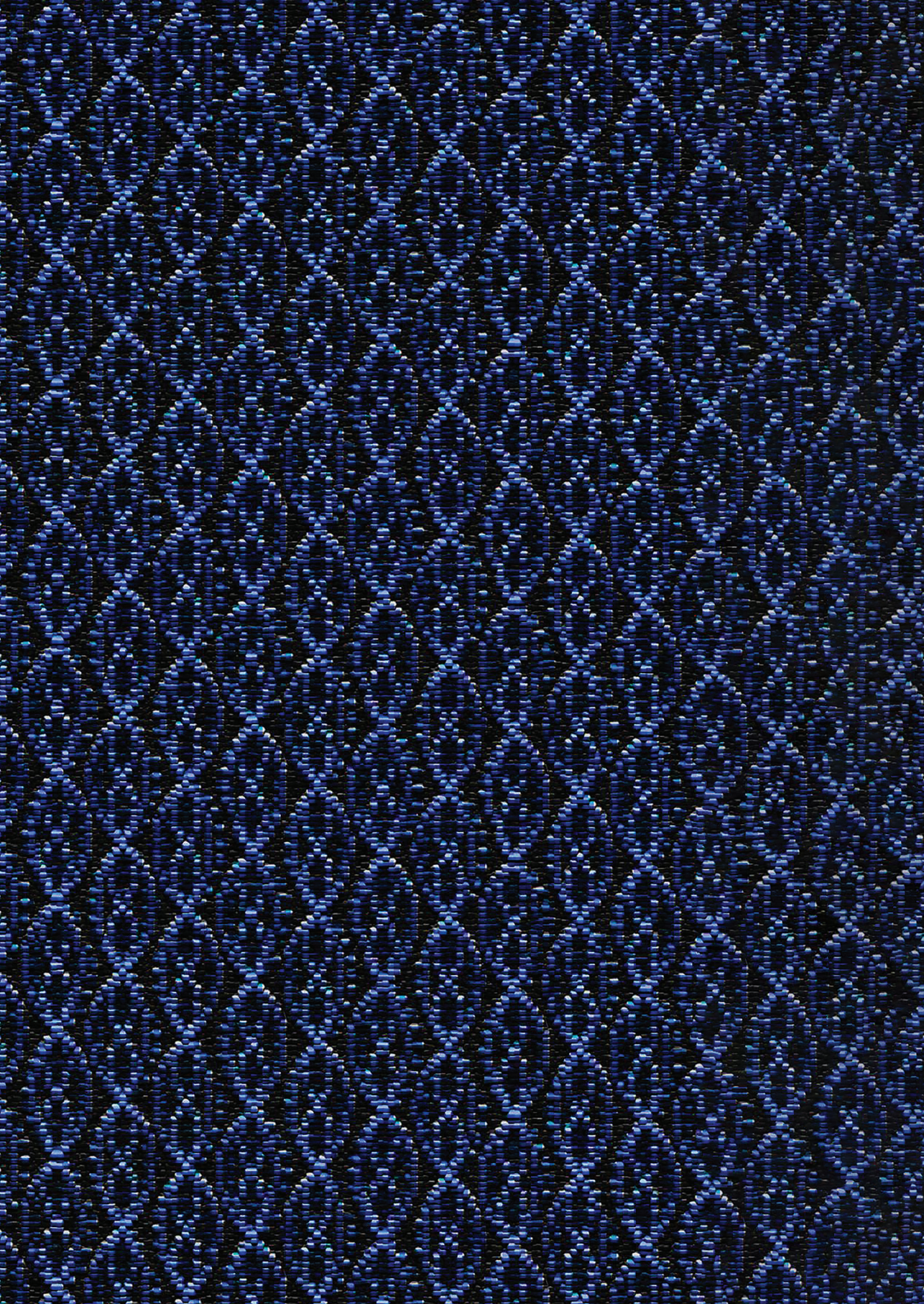

Part 4

LRP10 variants associated with dementia with Lewy bodies and related disorders

4.1

Chapter 4.1

LRP10 variants in Parkinson's disease and dementia with Lewy bodies in the South-West of the Netherlands

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Abstract

Objective

To analyse *LRP10* variants, recently associated with the development of Parkinson's disease (PD), Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB), in a series of patients and controls from the South-West of the Netherlands (Walcheren).

Methods

A series of 130 patients with PD, PDD or DLB were clinically examined, and a structured questionnaire used to collect information about family history of PD and dementia. The entire *LRP10* coding region was sequenced by Sanger methods in all patients, and haplotype analysis was performed for one recurrent *LRP10* variant. The fragments containing possibly pathogenic *LRP10* variants were sequenced in 62 unaffected control subjects from the same region. Other known PD-associated genes were analyzed by exome sequencing and gene dosage in the carriers of *LRP10* variants.

Results

Four patients were carriers of a rare heterozygous, possibly pathogenic *LRP10* variant: p.Arg151Cys, p.Arg263His, and p.Tyr307Asn. None of these variants was detected among the controls, nor were additional mutations identified in known PD-associated genes in the four *LRP10* variant carriers. The previously reported p.Tyr307Asn variant was identified in two patients (with PD and PDD), who are connected genealogically within six generations, and in one of their relatives with cognitive decline. Haplotype analysis suggests a common founder for the p.Tyr307Asn variant carriers analyzed.

Discussion

We report three possibly pathogenic *LRP10* variants in patients with PD and PDD from a local Dutch population. The identification of additional patients carrying the p.Tyr307Asn variant provides some further evidence that this variant is pathogenic for PD and PDD.

Introduction

Parkinson's disease (PD), Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB) are common neurodegenerative diseases, which share clinical, pathological and genetic features.[1,2] These disorders occur in approximately 1-2% of the population above 60 years old[3,4], and their common hallmark is Lewy pathology, observed primarily in the brainstem in PD, and more diffusely throughout the brain in PDD and DLB[2,5].

Although PD, PDD and DLB manifest mostly as sporadic diseases, during the last decades mounting evidence showed that genetic factors play an important role in the disease etiopathogenesis.[6-8] Recently, genetic defects in the *low-density lipoprotein receptor related protein 10* gene (*LRP10*) have been reported in familial PD, PDD and DLB.[9]

The aim of this study was to screen *LRP10* in a series of patients and unaffected subjects from an isolated region in the South-West of the Netherlands.

Methods

Participants

Between 2007 and 2010 we ascertained a series of 130 patients with PD (n=71), PDD (n=55), or DLB (n=4), as well as 62 unrelated and unaffected subjects originating from a region in the South-West of the Netherlands (Walcheren). This area had maintained the features of an island until 1870, and for centuries its population remained geographically isolated from the surrounding areas. All patients were neurologically examined by a neurologist (AR). Structured questionnaires were used to collect information about family history of PD and dementia and The Mini Mental State Examination (MMSE) was administered as screening tool for the cognitive status. Clinical follow-up was available until July 2018. The diagnosis of PD required the exclusion of secondary causes of parkinsonism, and the presence of at least two of the following signs: bradykinesia, rigidity, or rest tremor; or, presence of one of these signs together with improvement with dopaminergic medications. Diagnosis of DLB or PDD was made according to the criteria described by McKeith and co-workers[10] and by Emre and co-workers[11], respectively. Unaffected spouses of patients or of relatives were recruited as controls. Blood samples were collected from patients, available relatives and controls for DNA isolation. The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam, the Netherlands (MEC-2005-206) and written informed consent was obtained by all participating subjects.

Genetic Studies

The entire open reading frame and the intron-exon boundaries of *LRP10* were sequenced in all patients using a reported Sanger protocol[9] with minor modifications (Supplementary Information). Variants were considered as possibly pathogenic if they were: (1) present in

heterozygous state (as expected for variants acting in a dominant fashion); (2) rare, defined as with a minor allele frequency (MAF) <0.001 by the Genome Aggregation Database (GnomAD v2.1.1; https://gnomad.broadinstitute.org/); (3) exonic and non-synonymous, or predicted to affect splicing; and (4) predicted as pathogenic by at least five of 11 *in-silico* programs (Supplementary Information). The *LRP10* fragments containing possibly pathogenic variants identified in the patients were sequenced in the controls.

Whole exome sequencing (WES) and multiple ligation probe amplification (MLPA, P051-Parkinson mix 1, MRC Holland) were also performed in the patients who carried possibly pathogenic *LRP10* variants to rule out mutations in other known genes associated with PD or parkinsonism (Supplementary Table 1).

Haplotype analysis of a 6-Mb genomic region flanking *LRP10* was performed in the carriers of one recurrent *LRP10* variant (c.919T>A, p.Tyr307Asn), by typing short tandem repeat (STR) markers. DNA of the Italian PDD patient previously reported by us with the same variant[9], was also included in this analysis (Supplementary Figure 1).

LRP10 protein conservation analysis was performed using the T-Coffee multiple sequence alignment program (https://www.ebi.ac.uk/Tools/msa/tcoffee/) (Supplementary Figure 2). Details on the methodologies are reported in the Supplementary Information.

Results

The demographic and clinical characteristics of the 130 index patients are reported in Supplementary Table 2. The index patients had a mean age at disease onset of 62.4 ± 9.6 years and the 62 unrelated and unaffected participants had a mean age of 63.5 ± 12.3 years at recruitment.

Four index patients carried *LRP10* variants that fulfill our criteria for being considered as possibly pathogenic: c.451C>T/p.Arg151Cys (GnomAD MAF 0.000056, 16 alleles) in one patient; c.788G>A/p.Arg263His (GnomAD MAF 0.000007, 2 alleles) in another patient; and c.919T>A/p.Tyr307Asn (GnomAD MAF 0.000059, 15 alleles) in two other patients (Table 1). None of these variants were detected among the 62 unaffected subjects. Additional variants detected in patients but not fulfilling our criteria to be considered as possibly pathogenic are reported in Supplementary Table 3. Our WES and MLPA analyses in the four patients carrying *LRP10* possibly pathogenic variants detected no pathogenic variants in any of the other known genes associated with PD or parkinsonism (WES reached an average depth >190x, with 99.1% of the target region covered >20x).

Two patients, whose genealogy can be traced back to a common ancestor within six generations, carry the *LRP10* p.Tyr307Asn heterozygous variant. The diagnosis of PD in the first patient (Family 1, II-2) was established based on rigidity, bradykinesia, reduced arm swing and reduced facial expression, after he presented with rest tremor of the left hand at 63 years of age. The patient had multiple depressive episodes. He did not report PD or dementia among his first-degree relatives (Figure 1). DNA was available from two of his

Table 1: Details of the possibly pathogenic *LRP10* variants identified in this study and previous studies.

Genomic position	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP accession number	MAF GnomAD (alleles)	Functional predictions: pathogenic (total)	Study	Patients	Diagnosis	AAO (years)	AAD (years)	First symptom
14:23345076	c.919T>A	p.Tyr307Asn	5	missense	rs139650807	0.005% (15)	11/11	Current	Patient II-2 (Family 1)	PD	63	-	Rest tremor
								Current	Patient II-2 (Family 1)	PDD	69	79	Motor difficulties
								Current	Patient II-3 (Family 1)	Cognitive decline*	78	-	NA
								Quadri et al., 2018[9]	Patient II-1 (Family 5)	PDD	71	-	Rest tremor
								Tesson et al., 2018[12]	Patient I-9 (FPD-083)	PD	73	NA	Rest tremor
								Tesson et al., 2018[12]	Patient II-1 (FPD-083)	PD	45	NA	Rest tremor
14: 23344945	c.788G>A	p.Arg263His	5	missense	rs372858291	0.0007% (2)	6/11	Current	Patient III-1 (Family 3)	PD	68	-	Rest tremor and bradykinesia
14:23344608	c.451C>T	p.Arg151Cys	5	missense	rs774043484	0.006% (16)	9/11	Current	Patient II-2 (Family 4)	PDD	66	74	Rest tremor and hypokinesia

GRCh37: genome reference consortium human 37; *LRP10* variant nomenclature is assigned based on reference sequence NM_014045.4. MAF: minor allele frequency, GnomAD: Genome Aggregation Database, AAO: age at onset, AAD: age at death, *: progressive cognitive decline of neurodegenerative nature, NA: not available, PD: Parkinson's disease, PDD: Parkinson's disease dementia.

offspring, asymptomatic at the age of 33 and 47 years, respectively; both also carried the *LRP10* p.Tyr307Asn variant in heterozygous state (Figure 1 and Supplementary Figure 1). The second patient carrying the *LRP10* p.Tyr307Asn variant (Family 2, II-2) developed motor difficulties in the right leg at the age of 69. PD was diagnosed one year later based on bradykinesia, reduced facial expression, hypersalivation and orthostasis, in absence of rest tremor. He developed a paresis of the left arm after a small intracerebral hemorrhage in the right basal ganglia at the age of 75, followed by cognitive deterioration and periods of confusion. A dementia was diagnosed one year later and he died at 79 years old. He did not report PD among his first-degree relatives. However, his brother was recently diagnosed with a progressive cognitive decline of neurodegenerative nature, and DNA testing revealed that he also carries the *LRP10* p.Tyr307Asn variant (Figure 1 and Supplementary Figure 1).

A third patient (Family 3, III-1), carrying a *LRP10* p.Arg263His variant, developed a rest tremor of both hands and bradykinesia at 68 years of age. The PD diagnosis was established two years later, when rigidity and reduced facial expression were also present. He has mild memory impairments, concentration and orientation problems at the current age of 78. One of his cousins was also diagnosed with PD (paternal side), the patient's father had memory complaints and his three sisters were affected by dementia (Figure 1). Unfortunately, co-segregation studies could not be performed in the affected relatives due to unavailability of DNA.

A fourth patient (Family 4, II-2), carrying a *LRP10* p.Arg151Cys variant, was diagnosed with PD based on hypokinesia and rest tremor of the left hand at 66 years of age. He developed rigidity, hallucinations and depression several years later, and memory complaints at the age of 70. A diagnosis of dementia was established at 73 years of age, one year before he died. His father suffered from Alzheimer's disease, walking problems and frequent falls which started at the end of his seventies.

As expected from the genealogical links, our haplotype studies showed that the patients in the two Dutch families with the *LRP10* p.Tyr307Asn variant share an extended haplotype of several megabases flanking the *LRP10* gene. The Italian PDD patient previously reported by us with the same variant [9] shares a smaller haplotype of ~1Mb, therefore compatible with a common but more distant ancestor.

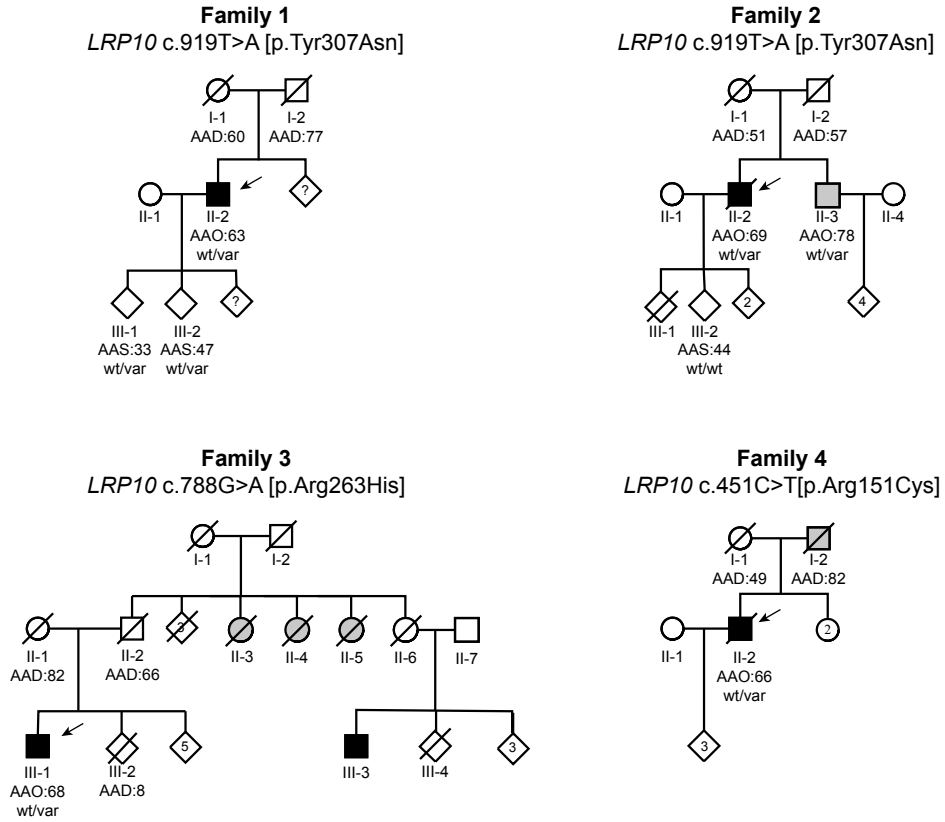

Figure 1: Pedigrees of patients carrying *LRP10* variants.
Circles indicate females, squares indicate males, diamonds indicate sex-disguised individuals; black symbols indicate patients affected by Parkinson's disease or Parkinson's disease dementia; grey symbols indicate patients with Alzheimer's disease, cognitive decline or unspecified type of dementia; diagonal lines indicate deceased individuals; arrows indicate index patients; numbers within a circle, square or diamond indicate the number of individuals; question marks indicate that there is no information about individuals' disease status. AAD: age at death, AAO: age at onset, AAS: age at sampling, wt: wild-type, var: variant.

Discussion

Here, we report three rare, possibly pathogenic *LRP10* variants in a relatively small series of 130 patients from the South-West of the Netherlands. The identified variants were absent in 62 unaffected subjects from the same region, and extremely rare in public databases (Table 1). Moreover, WES and MLPA performed in our four index patients with *LRP10* variants detected no pathogenic variants in any of the other genes previously associated with PD or parkinsonism.

Of interest, the p.Tyr307Asn variant identified here in two Dutch patients with PD and PDD and in one relative with cognitive decline, was initially observed by us in one patient with PDD from Italy [9]. The same variant was subsequently reported in a parent-offspring pair with PD, in a screening of 25 PD/DLB French families[12]; another two relatives affected by PD in that family did not share the p.Tyr307Asn variant, and might represent phenocopies.

Thus, the *LRP10* p.Tyr307Asn variant has been identified at least three times so far, in independent studies targeting patients of European ancestry with familial forms of PD, PDD or DLB, and including a total of 701 unrelated probands: 608 patients of European ancestry in our initial study[9]; 25 French patients studied by Tesson and colleagues[12]; and 68 Dutch patients in the current study (here we consider Family 1 an 2 as part of one extended kindred). The frequency of this variant among patients with familial forms of PD, PDD or DLB (3/701) is significantly higher compared to that present in GnomAD v2.1.1 (13/74109, only considering non-Finnish Europeans and Latino individuals, two-sided Fisher's Exact test, p=0.0048). Furthermore, functional studies showed that this variant leads to decreased stability of the LRP10 protein compared to the wild-type[9]. Taken together, these genetic and functional data support the contention that this variant plays a role in the development of PD and PDD. The penetrance of the p.Tyr307Asn variant as well as of the p.Arg151Cys and p.Arg263His variants might be incomplete. However, an accurate estimation is currently impossible because, although PD occurred sporadically in some carriers, in many of them the parents had died before the age at disease development observed in their offspring, and the patients' offspring are still younger than that age.

Considering together all patients with p.Tyr307Asn reported so far (n=6), the initial diagnosis was PD in five patients and progressive cognitive decline of neurodegenerative nature in one, rest tremor was the presenting sign in four out of the five patients diagnosed with PD, and mean age at onset was 66.5 years (SD:11.6; range:45-78; Table 1). During the course of PD, two patients developed dementia (one Italian patient and the Dutch patient II-2 - Family 2), two had no cognitive impairments (French patients), and one had multiple depressive periods (Dutch patient II-2 - Family 1).

Limited data are available for the p.Arg263His and p.Arg151Cys variants reported here in two patients with PD and PDD, respectively. Besides being very rare, predicted to be pathogenic by the majority of *in-silico* programs used here, and to replace highly conserved

amino acids located within conserved protein stretches (Supplementary Figure 2), no additional affected carriers have been reported so far, and functional data are not available. Therefore pathogenicity cannot be confidently established. Of note, the p.Arg151Cys substitution was previously found by us[9] in one of 645 Dutch patients with abdominal aortic aneurysms. However, the neurological status of this subject is unknown.

In conclusion, we report *LRP10* possibly pathogenic variants in patients with PD, PDD and dementia from a local Dutch population. Although our data cannot conclusively prove pathogenicity, the identification of additional patients with PD, PDD and dementia carrying the *LRP10* p.Tyr307Asn variant provides further evidence that this variant might be pathogenic.

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Supplementary Information

Sanger sequencing of LRP10

Amplification of DNA fragments was performed in a total volume of 20 µl, containing 2.0 µl of 10X FastStart Taq DNA Polymerase buffer, 1.6 µl of 2.5 mM dNTPs, 1.0 µl of 10 µM forward primer, 1.0 µl of 10 µM reverse primer, 0.10 or 0.15 µl of FastStart Taq. DNA Polymerase (Roche, Basel, Switzerland), and 25 ng of genomic DNA. In addition, 4 µl of 1X GC-RICH solution (Roche) was used for exon 1, 5 and 7. We used the same primers that have been previously reported by us.[1] Initial denaturation was performed for 5 minutes at 94˚C, followed by 30 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C, and 90 seconds at 72˚C. The final extension step was done for 5 minutes at 72˚C. Removal of unconsumed dNTPs and primers was performed with 3µl of PCR product, using 5 units of ExoI and 0·5 unit of Fast AP (Thermo Fisher Scientific, Waltham, MA, USA) for 45 minutes at 37˚C and 15 minutes at 80˚C. DNA strands were sequenced directly using the Big Dye Terminator (version 3·1; Thermo Fisher Scientific) according to the manufacturer's protocol. SephadexG50 (GE Healthcare, Little Chalfont, UK) was used to remove dye terminators. Fragments were loaded on an ABI 3730XL Genetic Analyzer (Thermo Fisher Scientific). Sequences were analyzed using the software packages Seqscape v3·0 (Thermo Fisher Scientific) and Sequencing Analysis v6·0 (Thermo Fisher Scientific). For annotating *LRP10* sequence variants transcript NM_0140445.4 was adopted and the Human Genome Variation Society (HGVS) nomenclature was applied to describe the sequence variants.[2]

Whole exome sequencing and copy number analysis of LRP10 variant carriers

Whole exome sequencing (WES) was performed in the possibly pathogenic *LRP10* variant carriers by Nimblegen SeqCap EZ MedExome 47 Mb in combination with Illumina Paired-End Library Preperation and 2x 150 bp Sequencing on an Illumina HiSeq2000 sequencer. Using standard procedures[3], reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler alignment[4] tool and processed using Picard (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK)[5]. GATKs Haplotype Caller was used for variant calling and ANNOVAR for the annotation (version 2018Apr16)[6].

To screen for copy number variants of *SNCA*, *PARK7*, *PINK1*, and *PARK2*, Multiplex Ligation-dependent Probe Amplification (MLPA) was performed. The P051-D1 Parkinson kit (MRC Holland) was used according to the manufacturer's protocol. Analysis was done using an ABI 3730XL Genetic Analyzer (Thermo Fisher Scientific) and Seqscape v3·0 (Thermo Fisher Scientific).

Haplotype analysis

Nine short tandem repeat (STR) markers located within a ~6 Mb genomic region containing the *LRP10* locus were genotyped. PCR reactions were performed in a total volume of 20 µl, containing 30 ng of genomic DNA, 1x PCR buffer (FastStart, Roche), 200 µM dNTPs, 0.5 µM fluorescent forward primer, 0.5 µM reverse primer, and 0.5 unit FastStart Taq Polymerase (Roche). Primers for the amplification of markers D14S261, D14S283 and D14S275 were taken from the available ABI Prism Linkage Mapping Set Version 2.5. Details of the other primers used for STR markers genotyping are available in Supplementary Table 5. PCR was carried out following an initial denaturation at 94˚C for 4 minutes, followed by 30 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C, and 90 seconds at 72˚C. The final extension step was done for 7 minutes at 72˚C. Fluorescent PCR products were mixed with GeneScan 500-LIZ Size Standard (Applied Biosystems), loaded on an ABI 3730XL automated sequencer (Thermo Fisher Scientific), and analyzed using GeneMarker v.2.4.0 software package (SoftGenetics, State College, PA, USA).

Supplementary Table 1: Genes with established or proposed association with PD or parkinsonism.

Chr	Start	End	Gene	Mode of inheritance
1	8021714	8045342	*PARK7*	AR
1	17312453	17338467	*ATP13A2*	AR
1	20959948	20978004	*PINK1*	AR
1	65720133	65881552	*DNAJC6*	AR
2	25013136	25016251	*PTRHD1*	AR
6	161768590	163148834	*PARK2*	AR
15	62144588	62352664	*VPS13C*	AR
21	33997269	34100351	*SYNJ1*	AR
22	32870707	32894818	*FBXO7*	AR
22	38507502	38577857	*PLA2G6*	AR
1	11072462	11085549	*TARDBP*	AD
1	155204239	155214653	*GBA**	AD
3	132136361	132257876	*DNAJC13*	AD
4	90645250	90759447	*SNCA*	AD
7	56169266	56174187	*CHCHD2*	AD
12	40618813	40763087	*LRRK2*	AD
14	23340822	23350789	*LRP10*	AD
16	46693589	46723144	*VPS35*	AD
17	42422491	42430474	*GRN*	AD
17	43971702	44105700	*MAPT*	AD
20	5049129	5093736	*TMEM230*	AD
X	154487526	154493852	*RAB39B*	X-linked R

* Heterozygous variants in *GBA* are known risk factors for PD. GRCh37: genome reference consortium human 37. AR: autosomal recessive, AD: autosomal dominant, PD: Parkinson's disease.

Supplementary Table 2: Demographic and clinical characteristics of the patients included in the study.

Sex, male		81 (62%)
Diagnosis at baseline	PD	130 (100%)
Age at baseline, years		69.9 (8.4)
Age at onset, years		62.4 (9.6)
MMSE at baseline*		26.8 (3.8)
Family history of PD	only 1st-degree	21 (16%)
	only 2nd-degree	13 (10%)
	1st- and 2nd-degree	3 (2%)
	absent	93 (72%)
Family history of dementia	only 1st-degree	31 (23%)
	only 2nd-degree	8 (6%)
	1st and 2nd-degree	4 (3%)
	absent	87 (68%)
Family history of PD and/or dementia	1st- and/or 2nd-degree	68 (52%)
Diagnosis at follow up	PD	71 (55%)
	PDD	55 (42%)
	DLB	4 (3%)
Follow up, years		7.0 (3.2)
N. of deceased patients		70 (54%)
Disease duration**, years		14.0 (8.3)

Data are presented as mean (SD) or n (%).*MMSE data was available in 128 patients; ** Data on disease duration was available in 70 patients. MMSE: Mini Mental State Examination, PD: Parkinson's disease, PDD: Parkinson's disease dementia, DLB: dementia with Lewy bodies.

Supplementary Table 3: Additional *LRP10* variants identified in the study.

N. of patients	Genomic position	Nucleotide change	Amino acid change	Exon / Intron	Coding effect	dbSNP accession number	MAF GnomAD (N. of alleles)	Functional predictions: pathogenic (total)	Splicing predictions: deleterious (total)
1	14:23341969	c.57C>T	p.Asp19=	exon 2	synonymous	rs772482762	0.004% (11)	NA	0/5
1	14:23346279	c.1685G>A	p.Arg562His	exon 7	missense	rs142153001	0.70% (1974)	9/10	n.a.
2	14:23342497	c.80-23G>A	n.a.	intron 2	n.a.	rs145476957	0.69% (1950)	NA	n.a.
1	14:23345195	c.1038 C>T	p.Asp346=	exon 5	synonymous	rs201657631	0.007% (20)	NA	0/2

GRCh37: genome reference consortium human 37; NM_014045.4. MAF: minor allele frequency, GnomAD: Genome Aggregation Database, NA: not available, n.a.: not applicable. Splicing prediction programs for p.Asp19=: SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, and Human Splicing Finder integrated in Alamut Visual version 4.2 (Interactive Biosoftware, Rouen, France). Splicing prediction programs for p.Asp346= : Berkeley Drosophila Genome Project Searches Splice Site prediction by Neural Network http://www.fruitfly.org/seq_tools/splice.html; NatGene2 Server http://www.cbs.dtu.dk/services/NetGene2/.

Supplementary Table 4: *In-silico* pathogenicity predictions for the *LRP10* variants.

				***In-silico* predictions** (scores)										
Genomic position	**Nucleotide change**	**Amino acid change**	**GERP**	**SIFT**	**Polyphen2 HDIV**	**Polyphen2 HVAR**	**LRT**	**Mutation Taster**	**Mutation Assessor**	**FATHMM**	**Meta-SVM**	**Meta-LR**	**CADD phred**	**M-CAP**
14:23345076	c.919T>A	p.Tyr307Asn	5.97	D (0.0)	D (0.999)	D (0.942)	D (0.000)	D (1.000)	M (2.525)	D (-3.32)	D (0.771)	D (0.848)	26.0	D (0.140)
14: 23344945	c.788G>A	p.Arg263His	5.97	D (0.02)	D (1.0)	D (0.927)	D (0.000)	D (0.975)	L (1.7)	T (2.25)	T (-1.146)	T (0.096)	29.1	T (0.008)
14:23344608	c.451C>T	p.Arg151Cys	5.01	D (0.0)	P (0.472)	B (0.037)	D (0.000)	D (1.000)	M (2.285)	D (-3.92)	D (0.545)	D (0.777)	32	D (0.172)
14:23341969	c.57C>T	p.Asp19=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
14:23346279	c.1685G>A	p.Arg562His	5.23	T (0.68)	D (0.999)	D (0.972)	D (0.000)	D (1.000)	M (2.2)	D (-3.37)	D (0.451)	D (0.772)	26.6	NA
14:23342497	C.80-23G>A	n.a.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
14:23345195	c.1038 C>T	p.Asp346=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

GERP: Genomic Evolutionary Rate Profiling, SIFT: Sorting Intolerant From Tolerant, PolyPhen2 HDIV: Polymorphism Phenotyping version 2 human diversity, PolyPhen2 HVAR: Polymorphism Phenotyping version 2 human variation, LRT: Likelihood Ratio Test, FATHMM: Functional Analysis Through Hidden Markov Models, SVM: Support Vector Machine, LR: Logistic Regression, CADD: Combined Annotation Dependent Depletion, M-CAP: Mendelian Clinically Applicable Pathogenicity, T: tolerated, D: damaging/ disease causing, B: benign, N: polymorphism/neutral, P: polymorphism automatic, L: low, M: medium, PP: probably pathogenic, LB: likely benign, NA: not available, n.a.: not applicable. Transcript references: NM_014045.4. Annovar (version 2018Apr16).

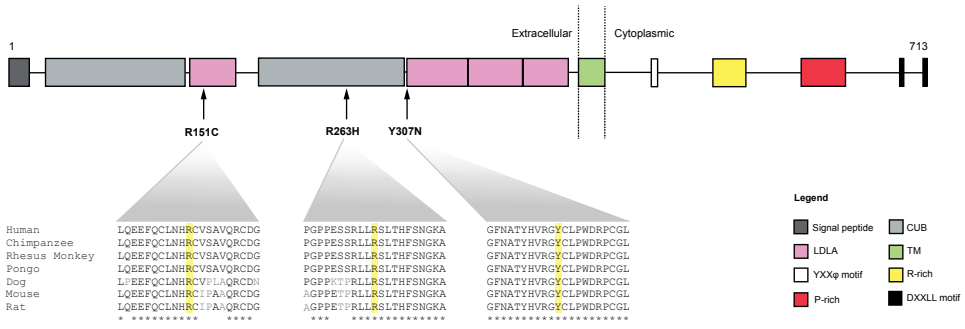
Supplementary Table 5: PCR Primers for markers genotyping.

Marker	Oligo Name	Oligo Sequence
CGR6800	CGR6800-FAM-FWD	5'-TGCCTGGCAAAACACACACAC-3'
	CGR6800-REV	5'-GGCTGAGGCAGGACAATCAC-3'
CGR4118	CGR4118-FAM-FWD	5'-ATTTCCAGCCTCCCTCTAGCC-3'
	CGR4118-REV	5'-GCCCAGTGTCTGGGGAGTAGG-3'
CGR7011	CGR7011-FAM-FWD	5'-GGCCACATTCGACTGTCATAGC-3'
	CGR7011-REV	5'-CTTCCAAGCCGACAGGATGG-3'
D14S1023	D14S1023-FAM-FWD	5'-AAAGGACCTCACAAATTCCTTCTAGC-3'
	D14S1023-REV	5'-TCTTGATAGTCTTAAGGTAGCAACAACAGC-3'
D14S990	D14S990-FAM-FWD	5'-ATATTGGGGGTGGGCTGTGG-3'
	D14S990-REV	5'-GCTGAATAAAGTTGCACTGTGACTGG-3'
D14S972	D14S972-FAM-FWD	5'-GAGGTACAAGAAACTTAGAGAACCTCAAGC-3'
	D14S972-REV	5'-TGTCTACAGATTCAATGCAATACTAACAGG-3'

Primers for the amplification of markers D14S261, D14S283 and D14S275 were according to the ABI Prism Linkage Mapping Set Version 2.5.

	Family 1						Family 2						Italian Patient*		Genomic Position (bp)
	II-2		III-1		III-2		II-2		II-3		III-2				
D14S261	299	297	297	297	297	297	301	297	301	297	297	297	295	303	20,472,545
D14S1023	296	294	296	294	296	294	292	294	292	294	294	294	296	294	21,074,061
D14S283	135	129	129	129	129	129	149	129	149	129	149	141	149	149	22,319,885
CGR6800	294	294	294	294	298	294	294	294	294	294	294	292	294	294	22,826,800
LRP10 p.Tyr307Asn	T	A	T	A	T	A	T	A	T	A	T	T	T	A	22,881,580
CGR4118	390	390	390	390	390	390	390	390	390	390	390	390	392	390	22,884,118
CGR7011	270	270	270	270	290	270	290	270	290	270	290	270	290	270	22,997,011
D14S990	201	207	201	207	195	207	191	207	191	207	191	199	193	199	23,217,423
D14S972	257	255	253	255	253	255	253	251	253	251	253	253	251	253	23,978,736
D14S275	153	149	155	149	153	149	155	151	155	151	155	153	149	155	26,327,814

Supplementary Figure 1: Haplotype analysis.
Haplotype analysis of the *LRP10* region in two Dutch families (genealogically related within six generations) and one Italian patient reported previously with the same *LRP10* c.919T>A, p.Tyr307Asn variant (* Patient II-1, in Quadri et al., 2018); for pedigree position refer to Figure 1 in the manuscript. All *LRP10* p.Tyr307Asn variant carriers share three markers flanking the variant, displayed in red. The individuals from the two Dutch families share a longer haplotype (displayed in orange), in keeping with a more recent common founder. Other markers specific to each family are reported in blue and green. Genomic positions are according to the Genome Reference Consortium human genome build 38 (GRCh38).



Supplementary Figure 2: *LRP10* protein structure and variants conservation analysis.
Representation of LRP10 protein structure and conservation analysis between LRP10 orthologs of p.Arg151, p.Arg263 and p.Tyr307 amino acids. CUB: compement C1R/C1S, urchin EGF, BMP1, LDLA: low-density lipoprotein receptor class A, TM: transmembrane domain, R-rich: Arginine-rich domain, YXXφ: a motife of Tyrosine plus two other amino acids, then an amino acid with a large bulky hydrophobic side chain, P-rich: Roline-rich domain, DXXLL: a motif of an aspartic acid, two other amino acids, then two leucines. We used the following National Centre for Biotechnology Information (NCBI) LRP10 Reference Sequence: Human, Homo Sapiens NP_054764.2; Chimpanzee, Pan troglodytes XP_509843; Rhesus monkey, Macaca mulatta NP_001244860.1; Pongo, Sumatran orangutan NP_001125058; Dog, Canis lupus familiaris XP_537364; Mouse, Mus musculus NP_075369; Rattus norvegicus NP_001032866.

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4.2

Chapter 4.2

Clinical and pathological phenotypes of patients with *LRP10* variants

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Under review

Abstract

Objective

Rare *LRP10* variants have recently been implicated in the etiology of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Here, we searched for *LRP10* variants in a new series of brain donors with dementia and Lewy pathology (LP) at autopsy, or dementia-parkinsonism without LP but with different neurodegenerative pathologies.

Methods

Sanger sequencing of *LRP10* was performed in 233 donors collected by the Netherlands Brain Bank.

Results

Rare, possibly pathogenic heterozygous *LRP10* variants were present in three patients: p.Gly453Ser in a patient with mixed Alzheimer's disease (AD)/Lewy body disease (LBD), p.Arg151Cys in a DLB patient, and p.Gly326Asp in an AD patient without LP. All three patients had a positive family history for dementia or PD.

Conclusion

Rare *LRP10* variants are present in some patients with dementia and different brain pathologies including DLB, mixed AD/LBD and AD. These findings suggest a role for *LRP10* across a broad neurodegenerative spectrum.

Introduction

Genetic factors play an important role in the etiology of both Parkinson's disease (PD) as well as dementia with Lewy bodies (DLB).[1-3] We recently reported rare variants in the *low-density lipoprotein receptor related protein 10* gene (*LRP10*) in familial forms of PD and DLB, including one large family with genome-wide significant linkage evidence, and several smaller families with additional, albeit more limited evidence of segregation of *LRP10* variants with disease.[4] In three probands with *LRP10* variants, autopsy studies showed diffuse-neocortical Lewy pathology (LP) and concomitant Alzheimer's disease (AD) pathology. Since our initial report, additional rare *LRP10* variants have been reported in patients with PD, DLB, or other neurodegenerative diseases by some but not all studies.[5-12]

LRP10 encodes transmembrane protein LRP10, which shuttles between the plasma membrane and the Golgi system and co-localizes with VPS35, a retromer component and the product of another known PD-causing gene.[13-15] LRP10 also interacts with GGA proteins, which are reported to affect α-synuclein trafficking and aggregation.[16,17] Furthermore, LRP10 has been implicated in the metabolism of APOE lipoproteins and direct as well as GGA-mediated regulation of APP trafficking and processing, and may therefore also influence amyloid-β homeostasis.[14,18,19] Taken together, this suggests a broad role for *LRP10* in neurodegeneration.

The aim of this study was to search for *LRP10* variants in a series of 233 brain donors from the Netherlands Brain Bank (NBB), who were clinically diagnosed with dementia, and had LP at autopsy or, if LP-negative, had developed parkinsonism in addition to dementia during their lifetime.

Methods

Donor selection

The entire series of brains available from the NBB (n=3853) from 1989 to 2017 was considered. For all donors, a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained from the donor or the next of kin.

For Group 1, inclusion criteria were: 1) Presence of dementia; 2) Braak Lewy body stage ≥4 according to the BrainNet Europe (BNE) criteria[20]; 3) At least low levels of AD pathology according to National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines[21].

For Group 2, inclusion criteria were: 1) Presence of dementia and parkinsonism; 2) Presence of any type of neurodegenerative disease upon autopsy; 3) Braak Lewy body stage 0 or 1 according to the BNE criteria[20]. Parkinsonism was defined as bradykinesia in combination with rigidity and/or tremor. Donors with possible drug-induced parkinsonism were excluded.

Donors with known disease-causing mutations in genes other than *LRP10* were excluded. Fresh-frozen brain tissue from cerebellum, cortical or subcortical grey matter was available from 126 donors from Group 1 and 107 donors from Group 2.

Clinical and neuropathological assessment

Clinical information was abstracted from the clinical records of the selected donors (Supplementary Methods). For all donors in the study selection, standardized neuropathological examinations had been performed by an experienced neuropathologist (AJMR) (open access: wwww.brainbank.nl). Neuropathological assessment was done according to NIA-AA and BNE guidelines[20, 21], and standardized evaluation strategies for cerebral amyloid angiopathy (CAA)[22], granulovacuolar degeneration[23], limbic-predominant age-related TDP-43 encephalopathy (LATE)[24], and aging-related tau astrogliopathy (ARTAG)[25] (Supplementary Methods).

Genetic analyses

Genomic DNA was isolated from brain tissue using standard methods. We performed Sanger sequencing using the reported protocol of Quadri et al. (2018)[4] with minor modifications (Supplementary Methods), for the entire open reading frame and the exon-intron boundaries of *LRP10*. We considered variants as possibly pathogenic according to the previously used criteria: 1) heterozygous state; 2) minor allele frequency <0.1% in the Genome Aggregation Database (GnomAD v2.1); 3) exonic location and non-synonymous, or predicted to affect splicing; and 4) predicted as pathogenic by at least five of 11 *in-silico* programs.[4,11]

To exclude (possibly) pathogenic variants in other known genes causing parkinsonism or dementia (Supplementary Table 1) we performed whole exome sequencing (WES) and multiple ligation-dependent probe amplification (MLPA, P051-Parkinson mix 1) in the possibly pathogenic *LRP10* variant carriers (Supplementary Methods). *APOE* genotyping was performed using TaqMan® SNP Genotyping Assay (ThermoFisher Scientific).

Results

Clinicopathological characteristics of the study groups

Demographic, clinical and neuropathological information from the 233 selected donors are summarized in Supplementary Table 2 and 3. In Group 1, pathological diagnoses included PD with dementia, DLB, AD with LP, mixed AD and Lewy body disease (AD/LBD), frontotemporal dementia (FTD) with LP and progressive supranuclear palsy (PSP) with LP. Distribution of LP was amygdala-predominant (24%), limbic-transitional (38%), or diffuse-neocortical (38%). In Group 2, pathological diagnoses included AD, FTD, vascular dementia, PSP, auto-immune encephalitis, corticobasal degeneration, CRASH syndrome, multiple sclerosis, multiple system atrophy (MSA), neurodegeneration with brain iron accumulation, neuronal intranuclear inclusion disease and spinocerebellar ataxia. Among donors with a

specified clinical diagnosis, 77.5% was confirmed upon autopsy in Group 1 and 61.3% in Group 2.

Genetic findings

Two donors (Patient 1 and 2) from Group 1 carried each a different, possibly pathogenic *LRP10* variant: c.1357G>A (p.Gly453Ser) and c.451C>T (p.Arg151Cys) (mean allele frequency (MAF) GnomAD 0.004% and 0.006% respectively, predicted as pathogenic in 5/11 and 9/11 *in-silico* programs respectively, Supplementary Table 4 and 5). One donor (Patient 3) from Group 2 carried a possibly pathogenic *LRP10* variant: c.977G>A (p.Gly326Asp) (MAF GnomAD 0.006%, predicted as pathogenic in 6/11 *in-silico* programs, Supplementary Table 4 and 5). Additional *LRP10* variants, which did not fulfill our criteria for possible pathogenicity, are listed in Supplementary Table 5 and 6. Additional WES and MLPA in possibly pathogenic *LRP10* variant carriers showed only one possibly pathogenic variant, namely the *ABCA7* p.Gly1741Arg variant in Patient 1 (Supplementary Table 7). *APOE* genotypes of Patient 1, 2 and 3 were ε3/ε4, ε3/ε3, and ε2/ε4 respectively.

Clinical phenotype of the LRP10 variant carriers

Patient 1 was clinically diagnosed with DLB. Onset symptoms at the age of 64 were parkinsonism, followed by dementia within a year. The patient died at the age of 72. The father of the patient had been diagnosed with PD. Patient 2 was clinically diagnosed with AD. She developed the first signs of dementia at age 80, and died at the age of 90. Her mother had also been diagnosed with dementia. Patient 3 was clinically diagnosed with AD. Onset symptoms at the age of 77 were walking problems and memory disturbances, followed by parkinsonism and dementia. He died at the age of 88. The brother and two aunts of the patient also suffered from dementia. All three patients died in an end-stage of dementia (Table 1 and Supplementary Results).

Pathological characteristics of the LRP10 variant carriers

Macroscopic examination of the brains of the three possibly pathogenic *LRP10* variant carriers showed mild to moderate frontotemporal atrophy in Patient 1 and 3 (Table 1). Microscopy showed LP in brainstem, limbic and neocortical brain areas in Patient 1 and 2 (Figure 1 I-IIA,B), but no LP in Patient 3 (Figure 1 IIIA,B). Nigral degeneration was present in Patient 1 and 2. An intermediate or high level of AD-type pathology was present in Patient 2 and 3, and Patient 1 respectively (Figure 1 I-IIIC-E). All three patients showed ARTAG, as well as CAA, including capillary CAA in Patient 2 and 3, and granulovacuolar degeneration in hippocampus and amygdala (Figure 1 I-IIIF, II-IIIG). Patient 1 showed LATE stage 1 (Figure 1G). The neuropathological diagnoses in the three patients can be summarized as mixed AD/LBD, DLB and AD respectively.

Table 1: Clinical and pathological characteristics of possibly pathogenic *LRP10* variant carriers.

	Patient 1	**Patient 2**	**Patient 3**
LRP10 variant	p.Gly453Ser	p.Arg151Cys	p.Gly326Asp
Sex	F	F	M
Age at death , years	72	90	88
Clinical diagnosis	DLB	AD	AD
Age at onset, years	64	80	77
Disease duration, years	8	10	11
Dementia duration, years	6	6	7
Parkinsonism	yes	not mentioned in clinical records	yes
Family history	father with PD	mother with dementia	brother and two aunts with dementia
Cause of death	pneumonia at end-stage dementia	cachexia and pressure ulcers at end-stage dementia	pneumonia at end-stage dementia
Neuropathological diagnosis	mixed AD/LBD	DLB	AD
PMD, hh:mm	05:35	05:35	05:00
APOE ε4	3/4	3/3	2/4
Brain weight, g	1043	1053	1133
Atrophy	moderate frontotemporal and hippocampal atrophy, severe dilation of temporal horn of lateral ventricle	none	mild frontotemporal atrophy, severe diffuse dilation of ventricles
Pigmentation substantia nigra	pale	pale	normal
Pigmentation locus coeruleus	pale	pale	normal
Thal amyloid-β phase[21]	5	3	5
Braak neurofibrillary stage[21]	6	4	4
CERAD score[21]	C	B	B
AD-level[21]	high	intermediate	intermediate
Braak Lewy body stage[20]	6	6	0
McKeith Lewy body stage[20]	neocortical	neocortical	none
Microvascular lesions	yes	no	yes
Hippocampal sclerosis	no	no	no
Argyrophilic grain disease	no	no	no
ARTAG[25]	yes	yes	yes
CAA type	2	1	1
Thal CAA stage[22]	1	1	1
Granulovacuolar degeneration[23]	yes, stage ≥4	yes, stage ≥4	yes, stage ≥4
Spongiform changes	severe	no	mild
LATE[24]	yes, stage 1	no	no

DLB: dementia with Lewy bodies, AD: Alzheimer's disease, PD: Parkinson's disease, LBD: Lewy body disease, PMD: post-mortem delay, ARTAG: aging-related tau astrogliopathy, CAA: cerebral amyloid angiopathy, LATE: limbic-predominant age-related TDP43 encephalopathy.

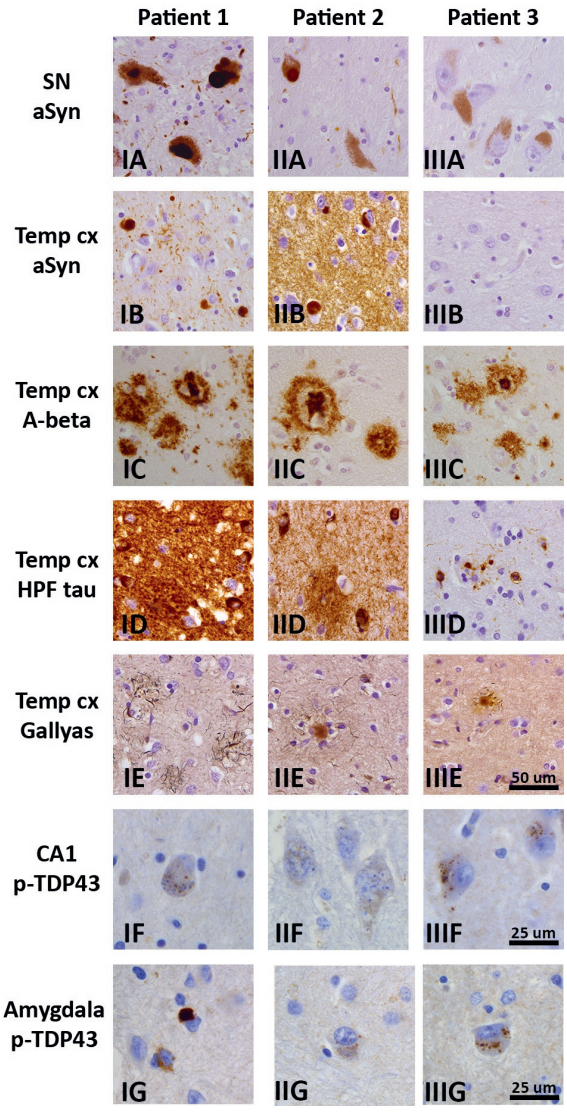

Figure 1: Brain pathology in three patients with possibly pathogenic *LRP10* variants, with representative photomicrographs of selected brain regions for Patient 1 (IA-G), Patient 2 (IIA-G) and Patient 3 (IIIA-G). Immunohistochemistry for α-synuclein (clone KM51) showed neocortical Lewy bodies and Lewy neurites in brainstem, limbic and neocortical brain areas in Patient 1 and 2 (I-IIA,B), but no α-synuclein positivity in Patient 3 (IIIA,B). Diffuse and cored amyloid-β plaques (clone 6F/3D) were present in all three patients (I-IIIC). Immunostaining for hyperphosphorylated tau (clone AT8) showed neurofibrillary tangles, threads and plaques in all three patients, with a moderate or high load in the temporal cortex in Patient 3 (IIID), and Patient 1 and 2 (I-IID) respectively. Gallyas silver stain showed a moderate or high load of neuritic plaques in Patient 2 and 3 (IIE, IIIE), and Patient 1 respectively (IE). Immunostaining for phospho-TDP43 (polyclonal rabbit antibody) showed granulovacuolar degeneration in the hippocampus and amygdala of all three patients (I-IIIF, II-IIIG). Additionally, Patient 1 showed TDP-43-positive neuronal inclusions and threads in the amygdala fitting limbic-predominant age-related TDP-43 encephalopathy (LATE; IG). Scalebar in IIIE represents 50 µm and applies to panels A-E. Scalebars in IIIF and IIIG represent 25 µm and apply to panels I-IIIF-G.

Discussion

We found two possibly pathogenic *LRP10* variants among 126 donors with dementia and LP, and one among 107 donors with dementia and parkinsonism without LP. The first variant, p.Gly453Ser, was carried by a patient with clinically diagnosed DLB and mixed AD/LBD upon autopsy. The second variant, p.Arg151Cys, was carried by a patient clinically diagnosed with AD, and a neuropathological diagnosis of DLB. The third variant, p.Gly326Asp, was carried by a patient with clinically diagnosed AD plus parkinsonism, and pure AD pathology upon autopsy. In the patient with the *LRP10* p.Gly453Ser variant, an additional rare variant (p.Gly1741Arg) in *ABCA7* was found. Rare disease-associated variants in *ABCA7* are considered risk factors for AD and PD[26,27], and therefore, the *ABCA7* p.Gly1741Arg variant may have contributed to the mixed AD/LBD pathology in this patient, together with the *LRP10* p.Gly453Ser variant.

While the three *LRP10* variants are extremely rare in control databases, they have all been reported in independent studies in other patients with neurodegenerative diseases. The p.Gly453Ser variant has previously been found in a patient with clinically diagnosed PD.[5] The p.Arg151Cys variant has previously been identified in a patient with PD with dementia, who had a father with AD and gait problems.[11] This variant was also observed by us in one out of 645 controls who were not neurologically examined.[4] Last, the p.Gly326Asp variant has been previously reported in a pathologically confirmed MSA patient[7], and found by us in a patient with clinically diagnosed PSP (unpublished observations). The lack of pathological examination in some of the previously reported patients complicates the overall interpretation of these findings. We acknowledge that in some of these patients, the *LRP10* variant could have been a chance occurrence, with no roles in the disease development. Nevertheless, these results are also compatible with a *bona-fide* pathogenic role for *LRP10* variants in a broad spectrum of neurodegenerative disorders.

LRRK2 is a paradigmatic example of marked pathological pleomorphism associated with pathogenic variants in the same gene[28], even in different members of the same family.[29] Similarly, *LRP10* variants may be related to a variety of neuropathological lesions, including LP, AD pathology and possibly tauopathies. Especially the association with AD pathology, next to LP pathology, is interesting as all three *LRP10* variant carriers reported in this study, as well as the three patients we previously described[4], had an intermediate or high level of AD pathology. This could possibly be explained by the putative role of *LRP10* in APP trafficking and amyloid-β homeostasis.[14,18]

Of note, all three *LRP10* variant carriers in this study had one or more first- or second-degree relatives with parkinsonism or dementia. Unfortunately, the family of these brain donors could not be contacted. Therefore, a co-segregation analysis of the *LRP10* variants could not be performed, which is a limitation of our study.

In conclusion, we observed possibly pathogenic *LRP10* variants in two patients with Lewy body diseases, and in one with pure AD pathology. Further studies of *LRP10* in large, well-characterized cohorts of patients with different types of neuropathology are warranted.

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Supplementary Information

Clinical information

Clinical information was abstracted form the database from the Netherlands Brain Bank and included information on sex, age at onset, age at death, disease duration from time at onset of first symptoms, presence and timing of parkinsonism and dementia, a history of parkinsonism or dementia in first-degree or second-degree relatives, the clinical diagnosis by the treating physician and cause of death.

Neuropathological assessment

For the possibly pathogenic *LRP10* variant carriers, 17 different brain regions were formalin-fixed and paraffin-embedded, cut into 8 µm sections, and stained with haematoxylin and eosin. For selected regions, histological stainings were performed using Congo red and Gallyas silver stains. Immunohistochemical analysis was performed on selected regions using primary antibodies against α-synuclein (clone KM51; 1:500; Monosan, The Netherlands), hyperphosphorylated tau (clone AT8; 1:1000; Innogenetics, Belgium), amyloid-β (clone 6F/3D; 1:100; Dako, United States) and phospho-TDP43 (rabbit polyclonal; 1:1000 Cosmo Bio, United States). Stages of amyloid-β plaques, neurofibrillary pathology and neuritic plaques were scored according to the NIA-AA guidelines[1], and stages of LP were scored according to BNE criteria[2]. Standardized staging systems for cerebral amyloid angiopathy (CAA)[3], granulovacuolar degeneration[4], and limbic-predominant age-related TDP-43 encephalopathy (LATE)[5] were applied. Presence of aging-related tau astrogliopathy (ARTAG) was evaluated based on tau immunostaining (clone AT8) of sections of the hippocampus at the level of the lateral geniculate nucleus, amygdala and temporal pool.[6]

Sanger sequencing

A total volume of 20 µl, containing 2.0 µl of 10X FastStart Taq DNA Polymerase buffer, 1.6 µl of 2.5 mM dNTPs, 1.0 µl of 10 µM forward primer, 1.0 µl of 10 µM reverse primer, 0.10 or 0.15 µl of FastStart Taq. DNA Polymerase (Roche, Basel, Switzerland), and 25 ng of genomic DNA, was used to amplify the fragments. We added 4 µl of 1X GC-RICH solution (Roche) for exon 1, 5 and 7. An overview of used primers can be found in Supplementary Table 8. We performed the initial denaturation for 5 minutes at 94˚C followed by 30 cycles of 30 seconds at 94˚C, 30 seconds at 60˚C, 90 seconds at 72˚C, with a final extension step for 5 minutes at 72˚C. We used 5 units of ExoI and 0·5 unit of Fast AP (Thermo Fisher Scientific, Waltham, MA, USA) for 45 minutes at 37˚C and 15 minutes at 80˚C to remove unconsumed dNTPs and primers from the PCR product (3µl). The Big Dye Terminator (version 3·1; Thermo Fisher Scientific) was used according to the manufacturer's protocol to sequence both DNA strands directly and sephadexG50 (GE Healthcare, Little Chalfont, UK) was used to remove dye terminators. We used an ABI 3730XL Genetic Analyzer (Thermo

Fisher Scientific) and Seqscape v3·0 (Thermo Fisher Scientific) for the analysis. The LRP10 NM_0140445.4 transcript was used for sequence variants annotation and the human genome variome society (HGVS) recommendations[7] was used for variants nomenclature.

Whole exome sequencing

Whole exome sequencing (WES) was performed with the same protocol as previously reported in Jamra et al. (2017)[8]. An average depth of >84x was reached, with 99% of the target region covered >20x.

Copy number analysis

The P051-D1 Parkinson kit (MRC Holland) was used according to the manufacturer's protocol. An ABI 3730XL Genetic Analyzer (Thermo Fisher Scientific) and Seqscape v3·0 (Thermo Fisher Scientific) were used for analysis.

Clinical information

Patient 1, carrier of the p.Gly453Ser variant, was clinically diagnosed with DLB. Throughout her life, the patient suffered from recurrent depressions, anxiety and panic attacks. At the age of 64, she developed severe constipation and a tremor of both hands, left more than right, progressing into a hypokinetic-rigid syndrome a year later. Her cognition rapidly deteriorated and neuropsychological examination showed deficits in memory, visuoconstruction and orientation. Brain MRI showed mild general atrophy. Visual hallucinations, personality changes and apraxia developed during the course of the disease. The patient died at the age of 72 due to pneumonia in an end-stage of dementia. The father of the patient had been diagnosed with PD.

Patient 2, carrier of the p.Arg151Cys variant, was clinically diagnosed with AD. She died at the age of 90 due to cachexia and decubitus wounds, after a disease duration of 10 years. The dementia was characterized by memory loss, anxiety, visual hallucinations and delusions. Any other neurological symptoms, in particular parkinsonism, were not mentioned in the clinical records. Her mother was also demented, but the clinical diagnosis of the mother is not known.

Patient 3, carrier of the p.Gly326Asp variant, was clinically diagnosed with AD. The patient had a disease duration of 11 years, starting with walking problems and memory disturbances, followed by parkinsonism, aphasia, frontal disinhibited behavior and anxiety. He died at the age of 88 due to pneumonia. The brother and two aunts of the patient also suffered from dementia.

Supplementary Table 1: Genes previously established or nominated as causative in parkinsonism or dementia.

Chr	Start	End	Gene	Mode of inheritance
			PD genes	
1	8021714	8045342	*PARK7*	AR
1	17312453	17338467	*ATP13A2*	AR
1	20959948	20978004	*PINK1*	AR
1	65720133	65881552	*DNAJC6*	AR
2	25013136	25016251	*PTRHD1*	AR
6	161768590	163148834	*PARK2*	AR
15	62144588	62352664	*VPS13C*	AR
21	33997269	34100351	*SYNJ1*	AR
22	32870707	32894818	*FBXO7*	AR
22	38507502	38577857	*PLA2G6*	AR
1	11072462	11085549	*TARDBP*	AD
1	155204239	155214653	*GBA**	AD
3	132136361	132257876	*DNAJC13*	AD
4	90645250	90759447	*SNCA*	AD
7	56169266	56174187	*CHCHD2*	AD
12	40618813	40763087	*LRRK2*	AD
14	23340822	23350789	*LRP10*	AD
16	46693589	46723144	*VPS35*	AD
17	42422491	42430474	*GRN*	AD
17	43971702	44105700	*MAPT*	AD
20	5049129	5093736	*TMEM230*	AD
X	154487526	154493852	*RAB39B*	X-linked R
			FTD genes	
1	11072462	11085549	*TARDBP*	AD
3	87276413	87304698	*CHMP2B*	AD
7	144149034	144533488	*TBK1*	AD
9	35056065	35072739	*VCP*	AD
16	31191431	31206192	*FUS*	AD
17	43971702	44105700	*MAPT*	AD
17	42422491	42430474	*GRN*	AD
X	56590025	56593443	*UBQLN2*	X-linked D
			AD genes	
1	227057885	227083804	*PSEN2*	AD
11	121322912	121504471	*SORL1*	AD
14	73603143	73690399	*PSEN1*	AD
19	1040102	1065571	*ABCA7**	AD
21	27252861	27543446	*APP*	AD
			Perry syndrome gene	
2	74588281	74619214	*DCTN1*	AD
			Niemann-Pick C genes	
14	74942900	74960084	*NPC2*	AR
18	21086148	21166581	*NPC1*	AR

* also risk gene. The Genome Reference Consortium Human Build 37 (hg19) was used. AR: autosomal recessive, AD: autosomal dominant or Alzheimer's disease, PD: Parkinson's disease, FTD: frontotemporal dementia.

Supplementary Table 2: Demographic and clinical characteristics of the two study groups.

	Dementia with Lewy pathology (n=126)	**Dementia with parkinsonism without Lewy pathology (n=107)**
Sex, male	56 (44%)	56 (52%)
Age at death, years	78.1 (9.2)	74.4 (11.2)
Age at onset, years	67.2 (13.5)	66.2 (11.8)
Disease duration, years	9.6 (5.9)	8.1 (5.9)
Dementia duration, years	6.3 (4.3)	5.1 (3.6)
Parkinsonism	72 (56%)	107 (100%)
Familial parkinsonism (n=21;22)	8 (38%)	4 (18%)
Familial dementia (n=68;66)	46 (68%)	36 (55%)
Clinical diagnoses		
AD	48 (38%)	20 (19%)
Corticobasal syndrome	1 (1%)	2 (2%)
DLB	24 (19%)	5 (5%)
Frontotemporal dementia	4 (3%)	26 (24%)
Multiple system atrophy	1 (1%)	2 (2%)
PD with dementia	22 (17%)	7 (7%)
Progressive supranuclear palsy	1 (1%)	13 (12%)
Vascular dementia	9 (7%)	18 (17%)
Asymmetrical cortical degenerative syndrome	1 (1%)	
No definite clinical diagnosis	15 (12%)	14 (13%)

Data are presented as mean (SD) or n (%). AD: Alzheimer's disease, DLB: dementia with Lewy bodies, PD: Parkinson's disease.

Supplementary Table 3: Pathological characteristics of the two study groups.

	Dementia with Lewy pathology (n=126)	Dementia with parkinsonism without Lewy pathology (n=107)
PMD, hours	5.5 (1.4)	5.6 (5.6)
APOE ε4 (n=75;87)		
0	24 (32%)	50 (58%)
1	39 (52%)	25 (29%)
2	12 (16%)	11 (13%)
Thal amyloid-β phase	4 (3-4)	2 (0-4)
Braak neurofibrillary stage	5 (3-6)	3 (1-4)
CERAD score	B (B-C)	A (O-B)
AD-level (n=123;98)		
not	0 (0%)	25 (26%)
low	24 (20%)	34 (35%)
intermediate	49 (40%)	21 (21%)
high	50 (41%)	18 (18%)
Braak Lewy body stage		
typical	109 (87%); 6 (5-6)	107 (100%); 0 (0-0)
atypical	17 (13%)	0 (0%)
McKeith Lewy body stage (n=110;107)		
none	0 (0%)	104 (97%)
brainstem predominant	0 (0%)	3 (3%)
limbic-transitional	42 (38%)	0 (0%)
neocortical-diffuse	42 (38%)	0 (0%)
amygdala predominant	26 (24%)	0 (0%)
Microvascular lesions	45 (36%)	44 (41%)
Hippocampal sclerosis	27 (21%)	17 (16%)
Argyrophilic grain disease	5 (4%)	7 (7%)
CAA		
type 1	51 (40%)	12 (11%)
type 2	57 (45%)	40 (37%)
Pathological diagnoses		
AD		37 (34%)
AD with Lewy pathology	38 (30%)	
Auto-immune encephalitis		1 (1%)
Corticobasal degeneration		1 (1%)
CRASH syndrome		1 (1%)
DLB	38 (30%)	
Frontotemporal dementia	1 (1%)	29 (27%)
Mixed AD/LBD	28 (22%)	
Multiple sclerosis		1 (1%)
Multiple system atrophy		1 (1%)
Neurodegeneration with brain iron accumulation		1 (1%)
Neuronal intranuclear inclusion disease		1 (1%)
PD with dementia	20 (16%)	
Progressive supranuclear palsy	1 (1%)	15 (14%)
Spinocerebellar ataxia		1 (1%)
Vascular dementia		18 (17%)

Data are presented as mean (SD), median (IQR) or n (%). PMD: post mortem delay, AD: Alzheimer's disease, CAA: cerebral amyloid angiopathy, DLB: dementia with Lewy bodies, PD: Parkinson's disease.

Supplementary Table 4: Possibly pathogenic *LRP10* variants.

Genomic position	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP 142 accession number	MAF GnomAD (alleles)	Functional predictions: pathogenic (total)	Study	Patient number	Clinical diagnosis	Age at onset (years)	Age at death (years)	Pathological diagnosis
14:23345514	c.1357G>A	p.Gly453Ser	5	missense	rs146378015	0.004% (10)	5/11	Current	1	DLB	64	72	Mixed AD/ LBD
								Kia et al., 2019[9]	NA	PD	NA	NA	NA
14:23344608	c.451C>T	p.Arg151Cys	5	missense	rs774043484	0.006% (16)	9/11	Current	2	AD	80	90	DLB
								Vergouw et al., 2019[10]	Patient II-2 (family 4)	PD with dementia	66	74	NA
								Quadri et al., 2018[11]	NA	control, but neurological status was not available	NA	NA	NA
14:23345134	c.977G>A	p.Gly326Asp	5	missense	rs547591765	0.006% (14)	6/11	Current	3	AD	77	88	AD
								Unpublished observations	NA	PSP	55	65	NA
								Pihlström et al., 2018[12]	NA	MSA	NA	NA	MSA

The Genome Reference Consortium Human Build 37 (hg19) and transcript NM_014045-4 were used. MAF: minor allele frequency, GnomAD: Genome Aggregation Database, NA: not available, AD: Alzheimer's disease, PSP: progressive supranuclear palsy, MSA: multiple system atrophy, DLB: dementia with Lewy bodies, PD: Parkinson's disease, LBD: Lewy body disease.

Supplementary Table 5: *In-silico* pathogenicity predictions.

				Variant-effect predictions software (scores)										
Genomic position	**Nucleotide change**	**Amino acid change**	**GERP**	**SIFT**	**Polyphen2 HDIV**	**Polyphen2 HVAR**	**LRT**	**Mutation Taster**	**Mutation Assessor**	**FATHMM**	**MetaSVM**	**MetaLR**	**CADD phred**	**M-CAP**
14:23344608	c.451C>T	p.Arg151Cys	5.01	D (0.0)	P (0.472)	B (0.037)	D (0.000)	D (1.000)	M (2.285)	D (-3.92)	D (0.545)	D (0.777)	32	D (0.172)
14:23345134	c.977G>A	p.Gly326Asp	4.14	T (0.368)	B (0.297)	B (0.172)	N (0.009)	D (0.887)	L (1.245)	D (-3.23)	D (0.067)	D (0.644)	15.59	D (0.034)
14:23345514	c.1357G>A	p.Gly453Ser	5.08	D (0.024)	P (0.944)	B (0.113)	N (0.000)	D (1.000)	L (1.175)	D (-3.34)	T (-0.475)	T (0.330)	21.0	D (0.031)
14: 23341951	c.39C>T	p.Gly13=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
14:23344264	c.216G>C	p.Arg72Ser	5.13	T (1.0)	B (0.064)	B (0.047)	D (0.000)	D (0.997)	L (1.385)	T (1.64)	T (-1.076)	T (0.046)	3.153	T (0.014)
14:23344572	c.415A>G	p.Met139Val	2.05	T (0.302)	B (0.002)	B (0.003)	N (0.665)	N (1.000)	N (-0.625)	D (-2.19)	T (-1.010)	T (0.026)	3.321	NA
14:23346279	c.1685G>A	p.Arg562His	5.23	T (0.68)	D (0.999)	D (0.972)	D (0.000)	D (1.000)	M (2.2)	D (-3.37)	D (0.451)	D (0.772)	26.6	NA
14:23346529	c.1935C>T	p.Pro645=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

The Genome Reference Consortium Human Build 37 (hg19), transcript NM_014045-4 and ANNOVAR_V5.0.sh were used. GERP: Genomic Evolutionary Rate Profiling, SIFT: Sorting Intolerant From Tolerant, PolyPhen2 HDIV: Polymorphism Phenotyping version 2 human diversity, PolyPhen2 HVAR: Polymorphism Phenotyping version 2 human variation, LRT: Likelihood Ratio Test, FATHMM: Functional Analysis Through Hidden Markov Models, SVM: Support Vector Machine, LR: Logistic Regression, CADD: Combined Annotation Dependent Depletion, M-CAP: Mendelian Clinically Applicable Pathogenicity, T: tolerated, D: damaging/disease causing, B: benign, N: polymorphism/ neutral, P: polymorphism automatic, L: low, M: medium, NA: not available.

Supplementary Table 6: Other *LRP10* variants which did not fulfill our criteria for possible pathogenicity.

Patient (s)	**Genomic position**	**Nucleotide change**	**Amino acid change**	**Exon**	**Coding effect**	**dbSNP 142 accession number**	**Allele frequency GnomAD (alleles)**	**Functional predictions: pathogenic (total)**
4-6	14:23341951	c.39C>T	p.Gly13=	2	synonymous	rs34294471	2.73% (7725)	NA
7	14:23344264	c.216G>C	p.Arg72Ser	4	missense	rs201675483	0.011% (28)	2/11
8	14:23344572	c.415A>G	p.Met139Val	5	missense	rs28534929	0.70% (1974)	1/10
9-11	14:23346279	c.1685G>A	p.Arg562His	7	missense	rs142153001	0.70% (1974)	9/10
12	14:23346529	c.1935C>T	p.Pro645=	7	synonymous	-	0.002% (6)	NA

The Genome Reference Consortium Human Build 37 (hg19) and transcript NM_014045-4 were used. Only variants in exons or at the exon-intron boundary (-10/+10) are displayed. MAF: minor allele frequency, GnomAD: Genome Aggregation Database, NA: not available.

Supplementary Table 7: Possibly pathogenic variant in other known genes causing parkinsonism or dementia in possibly pathogenic *LRP10* carriers.

										Variant-effect predictions software (scores)										
Patient	**Gene**	**Genomic position**	**Nucleotide change**	**Amino acid change**	**Exon**	**Coding effect**	**dbSNP 142 accession number**	**MAF GnomAD (alleles)**	**GERP**	**SIFT**	**Polyphen2 HDIV**	**Polyphen2 HVAR**	**LRT**	**Mutation Taster**	**Mutation Assessor**	**FATHMM**	**MetaSVM**	**MetaLR**	**CADD phred**	**M-CAP**
1	*ABCA7*	19:1058688	c.5221G>A	p.Gly1741Arg	38	missense	rs1311222336	0.0004 (1)	4.23	D (0)	P (1)	P (0.999)	NA	D (1)	M (3.3)	D (-2.42)	D (0.965)	D (0.86)	D (31)	D (0.214)

The Genome Reference Consortium Human Build 37 (hg19), transcript NM_019112 and ANNOVAR_V5.0.sh were used. GERP: Genomic Evolutionary Rate Profiling, SIFT: Sorting Intolerant From Tolerant, PolyPhen2 HDIV: Polymorphism Phenotyping version 2 human diversity, PolyPhen2 HVAR: Polymorphism Phenotyping version 2 human variation, LRT: Likelihood Ratio Test, FATHMM: Functional Analysis Through Hidden Markov Models, SVM: Support Vector Machine, LR: Logistic Regression, CADD: Combined Annotation Dependent Depletion, M-CAP: Mendelian Clinically Applicable Pathogenicity, T: tolerated, D: damaging/disease causing, B: benign, N: polymorphism/neutral, P: polymorphism automatic, L: low, M: medium, NA: not available.

Supplementary Table 8: PCR primers for *LRP10* genomic region.

***LRP10* genomic DNA NM_014045.4**		
Primer name	**Primer sequence 5'>3'**	**Amplicon size (bp)**
LRP10-ex1-fwd	CAAAGTTTGGCCCGAAGAGG	522
LRP10-ex1-rev	gggcaggcaggatagagtgc	
LRP10-ex2-fwd	cggatggctcccttgagttg	287
LRP10-ex2-rev	cacaccgagcctcagcttcc	
LRP10-ex3-fwd	cctccttgcagagcccagac	325
LRP10-ex3-rev	gaagtgatccctgaagacttccaatg	
LRP10-ex4-fwd	gcaccaggggaggagaaagc	367
LRP10-ex4-rev	gcagaggcaccatggagagg	
LRP10-ex5A-fwd	GCCAAGgtaggctggacagg	860
LRP10-ex5B-rev	TGGTAGTTGCAGCGGTCAGC	
LRP10-ex5B-fwd	GTCCCCTCCCTGCCTTGC	1020
LRP10-ex5C-rev	tcaggatctggacctgtcccttac	
LRP10-ex6-fwd	gggaaagccatggcacagc	342
LRP10-ex6-rev	ggccaaaggctgaatgaagg	
LRP10-ex7A-fwd	cctcctggtcccagttctgc	813
LRP10-ex7B-rev	CCCACCAAGTCCCTGAAATCC	

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4.3

Chapter 4.3

LRP10 variants in progressive supranuclear palsy

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Abstract

The aim of this study was to explore whether variants in *LRP10*, recently associated with Parkinson's disease and dementia with Lewy bodies, are observed in two large cohorts (discovery and validation cohort) of patients with progressive supranuclear palsy (PSP). A total of 950 PSP patients were enrolled: 246 PSP patients (n=85 possible (35%), n=128 probable (52%), n=33 definite (13%)) in the discovery cohort, and 704 patients with definite PSP in the validation cohort. Sanger sequencing of all *LRP10* exons and exon-intron boundaries was performed in the discovery cohort, and whole exome sequencing was performed in the validation cohort. Two patients from the discovery cohort and eight patients from the validation cohort carried a rare, heterozygous, possibly pathogenic *LRP10* variant (p.Gly326Asp, p.Asp389Asn, and p.Arg158His, p.Cys220Tyr, p.Thr278Ala, p.Gly306Asp, p.Glu486Asp, p.Arg554*, p.Arg661Cys). In conclusion, possibly pathogenic *LRP10* variants occur in a small fraction of PSP patients and may be overrepresented in these patients compared to controls. This suggests that possibly pathogenic *LRP10* variants may play a role in the development of PSP.

Introduction

Progressive supranuclear palsy (PSP) is an adult-onset, progressive neurodegenerative disorder clinically characterized by parkinsonism, vertical supranuclear gaze palsy, and postural instability with falls. Other PSP features include frontal lobe and bulbar dysfunction, and cognitive decline.[1,2] The clinical presentation of PSP is heterogeneous, and ten different clinical phenotypes have been described in patients with PSP neuropathology.[3] PSP brain pathology includes neurofibrillary tangles, neutrophil threads, tufted astrocytes, neuronal loss and gliosis in multiple subcortical areas and other regions.[4]

PSP is usually considered a sporadic tauopathy of unknown etiology.[5] However, rare familial forms have been reported.[6,7] Mutations in the *microtubule-associated protein tau* (*MAPT*) gene have been reported as the likely disease cause in a few pathologically confirmed PSP patients.[8,9] Furthermore, genome-wide association studies have shown associations between PSP and the *MAPT*, *syntaxin-6* (*STX6*), *myelin-associated oligodendrocyte basic protein* (*MOBP*), and *eukaryotic translation initiation factor 2-alpha kinase* (*EIF2AK*) genes, modulating the risk of developing PSP.[10,11] Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene have also been implicated in a small number of PSP cases[12,13] and are the most common genetic cause of Parkinson's disease (PD)[14]. Interestingly, pleomorphic neuropathology has been observed in PD patients with *LRRK2* mutations, ranging from typical alpha-synuclein-positive pathology (seen in most cases), to PSP-like pathology.[15] Indeed in the original Japanese family, which nominated the linkage region for *LRRK2*, the affected members presented with tauopathy.[16] Because of these genetic and pathological overlaps between PD and PSP, we hypothesized that variants in the *low-density lipoprotein receptor related protein 10* (*LRP10*) gene, recently associated with PD and dementia with Lewy bodies (DLB)[17,18], might also be implicated in PSP. *LRP10* is a surface protein, which function is largely elusive. However, some studies have suggested a role of LRP10 in ligand trafficking between the trans-Golgi network, endosomes, and the plasma membrane. Furthermore, LRP10 has been linked to the metabolism of amyloid-β and α-synuclein.[19,20,21]

The aim of this study is to explore whether possibly pathogenic variants in *LRP10* are observed in a large Dutch cohort of PSP patients (discovery cohort) using Sanger sequencing. Additionally, we try to validate our findings in a large cohort of PSP patients (validation cohort) from the USA and Europe using whole exome sequencing (WES).

Methods

Subjects

PSP patients from two large cohorts were enrolled in this study. PSP was diagnosed according to the criteria of the National Institute for Neurological Disorders and Stroke / Society for PSP (NINDS-SPSP)[1]. The discovery cohort consisted of 246 PSP patients enrolled from a large Dutch cohort[22], consecutively collected between 2003 and 2012 (Table 1). Patients were ascertained at the outpatient clinic of the Erasmus Medical Center Rotterdam, at home or at nursing homes. At inclusion, information about patient's medical and family history and current medical status was collected. Furthermore, neurological examination was performed and a blood sample was collected. The validation cohort consisted of 704 neuropathologically confirmed PSP patients enrolled form a large cohort of patients from the USA and Europe[23] (Table 1). These patients were identified from brain banks, research hospitals and neuropathologists. The study was approved by the relevant Institutional Ethical Authorities and all participants or legal representatives signed informed consent.

Table 1: Demographic and clinical characteristics.

		n=246	**n=704**
Sex, male		127 (52%)	377 (54%)
Diagnosis (NINDS-SPSP)	possible PSP	85 (35%)	0 (0%)
	probable PSP	128 (52%)	0 (0%)
	definite PSP	33 (13%)	704 (100%)
Age at onset, years (n=246;476)		65.8 (7.5)	68.1 (8.4)
Family history of neurodegenerative diseases (n=244;0)	1st-degree	71 (29%)	NA
	2nd-degree	21 (9%)	NA
	no	152 (62%)	NA
Deceased (n=244;704)		242 (98%)	704 (100%)
Age at death, years (n=241;698)		73.7 (7.3)	75.3 (8.2)

Data are presented as n (%) or mean (SD). NINDS-SPSP: National Institute for Neurological Disorders and Stroke / Society for PSP, PSP: progressive supranuclear palsy, NA: not available.

Genetic analyses

Genetic analyses in the two cohorts

Genomic DNA was isolated from blood in the discovery cohort and from brain tissue in the validation cohort using standard methods. Sanger sequencing was performed for the entire open reading frame and exon-intron boundaries of *LRP10* in the discovery cohort (protocol reported by Vergouw et al., 2019[18]). WES was performed in the validation cohort (Supplementary Information). Possibly pathogenic *LRP10* variants identified by WES in the validation cohort were validated by Sanger sequencing (Supplementary Information).

We considered variants as possibly pathogenic according to the following criteria: (1) heterozygous state; (2) rarity, defined as a frequency <0.1% in the Genome Aggregation Database (GnomAD v2.1); (3) exonic location and non-synonymous, or predicted to affect splicing; and (4) predicted as pathogenic by at least five of 11 *in-silico* programs (Supplementary Information).

Additional genetic analyses in possibly pathogenic LRP10 variant carriers in the discovery cohort

WES, multiple ligation-dependent probe amplification (MLPA, P051-Parkinson mix 1), and *C9orf72* repeat expansion analysis were performed in patients who carried possibly pathogenic *LRP10* variants to exclude possibly pathogenic variants in other known genes causing parkinsonism or dementia (Supplementary Table 1). The presence of possibly pathogenic variants in known genes causing parkinsonism or dementia in possibly pathogenic *LRP10* variant carriers decreases the chance of the *LRP10* variant to be truly pathogenic. WES and MLPA were performed as reported previously by Vergouw et al., 2019[18]. Details of the methods of the *C9orf72* repeat expansion analysis can be found in the Supplementary Information.

Results

Demographic and clinical characteristics of the two cohorts

The discovery cohort consisted of 85 (35%) patients with possible PSP, 128 (52%) with probable PSP (52%), and 33 (13%) with definite PSP. The mean disease onset age in this cohort was 65.8 ± 7.5 years and 52% of patients were male; 29% of patients had at least one first-degree relative and 9% had at least one second-degree relative with a neurodegenerative disease. The validation cohort consisted of 704 definite PSP patients. The mean disease onset age in this cohort was 68.1 ± 8.4 years (data only available in n=476) and 54% of patients were male (Table 1).

Genetic findings

Two possibly pathogenic *LRP10* variants were detected in the discovery cohort, each in single patients (p.Gly326Asp and p.Asp389Asn). In the validation cohort, seven possibly pathogenic *LRP10* variants were detected in eight patients (p.Arg158His, p.Cys220Tyr, p.Thr278Ala, p.Gly306Asp, p.Glu486Asp, p.Arg554*, and p.Arg661Cys; see Table 2 and Supplementary Table 2 for specifications). Supplementary Figure 1a shows the *LRP10* gene structure with the location of the identified variants and Supplementary Figure 1b shows the LRP10 protein structure with the location of the amino acid changes. Other variants in *LRP10* which did not fulfill the criteria for possible pathogenicity, as described in section 2.2.1., are depicted in Supplementary Table 3. Additional WES analysis (average depth of >170x with 99% of the target region covered >20x) in the possibly pathogenic *LRP10* variant carriers

Table 2: Possibly pathogenic *LRP10* variants.

	Genetic information									Clinical information			
	Genomic position	**Nucleotide change**	**Amino acid change**	**Exon**	**Coding effect**	**dbSNP 142 accession number**	**Allele frequency GnomAD (alleles)**	**Functional predictions: pathogenic (total)**	**Splicing predictions: deleterious (total)**	**Patient**	**NINDS-SPSP criteria**	**Age at onset (years)**	**Age at death (years)**
Discovery cohort													
	14:23345322	c.1165G>A	p.Asp389Asn	5	missense	rs754181235	0.01% (37)	6/11	n.a.	1	Probable PSP	61	66
	14:23345134	c.977G>A	p.Gly326Asp	5	missense	rs547591765	0.006% (14)	6/11	n.a.	2	Possible PSP	55	65
Validation cohort													
	14:23344630	c.473G>A	p.Arg158His	5	missense	rs764424911	0.005% (13)	6/11	n.a.	1	Definite PSP	68	75
	14:23344816	c.659G>A	p.Cys220Tyr	5	missense	rs867533372	.	10/11	n.a.	2	Definite PSP	70	74
	14:23344989	c.832A>G	p.Thr278Ala	5	missense	.	.	5/11	n.a.	3	Definite PSP	74	87
	14:23345074	c.917G>A	p.Gly306Asp	5	missense	rs375748692	0.007% (21)	9/11	n.a.	4	Definite PSP	NA	66
										5	Definite PSP	74	80
	14:23345931[1]	c.1458G>C	p.Glu486Asp	6	missense	rs142130715	0.01% (32)	11/11	0/4	6	Definite PSP	78	83
	14:23346254	c.1660C>T	p.Arg554*	7	stopgain	rs201213246	0.01% (28)	n.a.	n.a.	7	Definite PSP	74	84
	14:23346575	c.1981C>T	p.Arg661Cys	7	missense	rs771796662	0.004% (10)	8/11	n.a.	8	Definite PSP	NA	84

The Genome Reference Consortium Human Build 37 (hg19) and NM_014045-4 LRP10 transcript were used. * This variant was not validated by Sanger sequencing, because no additional DNA was available. GnomAD: Genome Aggregation Database, NINDS-SPSP: National Institute for Neurological Disorders and Stroke / Society for progressive supranuclear palsy, n.a.: not applicable, NA: not available. Splicing prediction programs: SSF, MaxEnt, NNSPLICE, Gene Splicer.

from the discovery cohort revealed a heterozygous *VPS13C* variant (p.Gln2546*, absent in GnomAD v2.1) in one patient (Supplementary Table 4). No other mutations in genes causing parkinsonism or dementia were found.

Clinical information of possibly pathogenic LRP10 variant carriers

An overview of the clinical information of the possibly pathogenic *LRP10* variant carriers is shown in Table 2. Patient 1 from the discovery cohort (*LRP10* p.Asp389Asn variant) experienced falls from the age of 61, followed by swallowing problems. At the age of 63, a mild downward vertical supranuclear gaze palsy, dysarthric speech, reduced arm swing, palatal tremor and impaired balance, but no clear ataxia were observed. He had a favorable response to levodopa. At neuropsychological examination deficits were observed in attention, concentration and executive functioning. Furthermore, mild memory and naming problems were seen. Brain MRI showed mild parieto-occipital and cerebellar atrophy and hypertrophy of the olivary nuclei. The patient died at the age of 66. Family history was negative for parkinsonism, dementia or motor neuron disease. Brain autopsy was not performed. This patient was diagnosed with probable PSP during life according to the NINDS-SPSP criteria[1] and can retrospectively be classified as probable PSP with Richardson's syndrome (PSP-RS) according to the MDS criteria[23]. Patient 2 from the discovery cohort (*LRP10* p.Gly326Asp variant) experienced tremor of the right leg from the age of 55, followed by falls, rigidity, swallowing, speech and memory problems from the age of 60. At the age of 64, vertical supranuclear gaze palsy, bradykinesia, intermittent rest tremor of arms and legs and balance problems were observed. She had a favorable response to levodopa. Neuropsychological examination showed severe deficits, especially with frontal subcortical and language problems. Brain MRI was unremarkable. The patient died at the age of 65. Family history was negative for parkinsonism, dementia or motor neuron disease. Brain autopsy was not performed. This patients was diagnosed with possible PSP during life according to the NINDS-SPSP criteria[1] and can retrospectively be classified as probable PSP with predominant parkinsonism (PSP-P) according to the MDS criteria[23].

Discussion

In this study we explored the presence of *LRP10* variants in two cohorts with a total of 950 PSP patients (discovery cohort n=246, validation cohort n=704). The PSP diagnosis was pathologically confirmed in 78% of these patients. Two possibly pathogenic *LRP10* variants (p.Gly326Asp and p.Asp389Asn) were identified in two patients from the discovery cohort and seven possibly pathogenic *LRP10* variants (p.Arg158His, p.Cys220Tyr, p.Thr278Ala, p.Gly306Asp, p.Glu486Asp, p.Arg554*, and p.Arg661Cys) were identified in eight patients from the validation cohort. These variants are very rare, are predicted to be pathogenic by ≥5 *in-silico* programs and are mostly located in *LRP10* exon 5, where other probably pathogenic variants were previously found[17]. Interestingly, the frequency of possibly pathogenic *LRP10*

variants is significantly higher in the validation cohort (8/1408 alleles=0.6%) compared to a previous published control cohort of patients with abdominal aneurysms[17] (1/1248 alleles=0.08%; Fishers's Exact test *p* value 0.04). In addition, the p.Gly306Asp variant has been identified previously in two out of 2835 PD patients and one out of 5343 controls[24], the p.Gly326Asp variant in one out of 264 patients with multiple system atrophy and in no controls[25], and the p.Glu486Asp variant in three out of 2835 PD patients and one out of 111 DLB patients compared to none in 5343 and 233 controls, respectively[24]. The p.Arg158His, p.Arg554* and p.Arg661Cys variants have previously been identified in single controls[24-26] (Supplementary Table 5).

Both patients from the discovery cohort displayed uncommon PSP clinical features. Patient 1 had a palatal tremor, inferior olivary hypertrophy and cerebellar atrophy. Inferior olivary hypertrophy is observed in 1.5% of pathologically confirmed PSP patients[27], but associated palatal tremor is very rare in PSP[27,28]. The syndrome of progressive ataxia and palatal tremor[29] may retrospectively also be considered in patient 1, yet the clinical phenotype is most consistent with PSP. An uncommon feature in patient 2 was the presence of an isolated tremor of the right leg in the first 5 years of the disease. Unfortunately, autopsy studies were not performed in these patients, and therefore the diagnosis could not be verified at pathological level. The absence of a family history of PSP or other neurodegenerative disorders in these two patients would be compatible with an incomplete penetrance or a de novo occurrence of the *LRP10* variants.

Of note, a *VPS13C* variant (p.Gln2546*) was observed in one *LRP10* variant carrier. Mutations in *VPS13C* are associated with autosomal recessive forms of early-onset parkinsonism.[30] In our patient the variant was found in heterozygous state and is therefore most likely an incidental finding.

Strengths of this study are the large sample size of the two PSP cohorts, the validation of our findings in an independent cohort, and the high percentage of neuropathologically confirmed PSP patients. Limitations are the lack of screening for *LRP10* genomic deletions of multiplications (not detectable by Sanger methods).

In conclusion, this is the first study of *LRP10* in two large PSP cohorts. We showed that rare, possibly pathogenic *LRP10* variants occur in a small, but substantial fraction of PSP patients. Furthermore, possibly pathogenic *LRP10* variants may be overrepresented in PSP patients compared to controls and may therefore play a role in disease pathogenesis. Further studies are warranted to replicate our findings and to study which molecular mechanisms underlie the possible association between *LRP10* and PSP.

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Supplementary Information

Whole exome sequencing in validation cohort

Whole exome sequencing (WES) was performed using the Nimblegen's VCRome v2.1 (36Mb) capture kit on an Illumina HiSeq sequencer. Sequencing data were analyzed using the in-house DNA Resequencing analysis workflow (DRAW)[1]. Reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler alignment tool (0.7.5)[2] and processed using Picard (http://picard.sourceforge.net/) and the Genome Analysis Toolkit (GATK)[3]. Additional sequencing was done for samples where coverage did not reach 20x for more than 80% of the targeted region and 10x for more than 90% of the targeted regions. Data from multiple sequencing experiments from the same individual were merged using SAMtools[4]. Variant calling was performed by GATKs Haplotype Caller and annotated using SNPEff[5]. An average depth of 33x was reached.

Sanger sequencing in validation cohort

Genomic DNA (~50ng) was amplified using a SimpliAmp Thermal Cycler (Applied Biosystems) in a 20 µl reaction volume with HotStarTaq Master Mix (Qiagen) in the presence of 2 µM primers (IDT). The PCR conditions used were: 95°C 15min followed by 30 cycles of 95°C 20sec, 55°C 30sec, 72°C 2min with a final extension of 72°C 7min. The amplified PCR products were prepared for Sanger sequencing by adding ExoSAP-IT (USB) and incubating at 37°C for 45min followed by 80°C for 15min. The PCR products were then Sanger sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Part No. 4336917 Applied Biosystems). The sequencing reaction contained BigDye® Terminator v3.1 Ready Reaction Mix, 5X Sequencing Buffer, 5M Betaine solution (Part No. B0300 Sigma) and 0.64uM sequencing primer (IDT) in a total volume of 5ul. The sequencing reaction was performed in a SimpliAmp Thermal Cycler (Applied Biosystems) using the following program: 96°C 1min followed by 25 cycles of 96°C 10sec, 50°C 5sec, 60°C 1min15sec. The products were cleaned using XTerminator and SAM Solution (Applied Biosystems) with 30 min of shaking at 1800 rpm followed by centrifugation at 1000 rpm for 2min. The sequencing products were analyzed on a 3130XL Genetic Analyzer (Applied Biosystems) and the sequencing traces were analyzed using Sequencer 5.4 (Gene Code). Information on primers can be given by the Authors on request.

C9orf72 repeat expansion analysis

C9orf72 repeat expansion analysis was performed to screen for the presence of a pathogenic chromosome 9p21 GGGGCC hexanucleotide repeat expansion. A previously described repeat-primed PCR assay[6] was used. An ABI3730XL Genetic Analyzer (Thermo Fisher Scientific) and GeneMarker Ver.2.4.0 (SoftGenetics, State College, PA, USA) were used for analysis. A pathogenic *C9orf72* expansion was defined as more than 30 repeats.[6]

Supplementary Table 1: Known genes causing parkinsonism or dementia.

Chr	Start	End	Gene	Mode of inheritance
			PD genes	
1	8021714	8045342	*PARK7*	AR
1	17312453	17338467	*ATP13A2*	AR
1	20959948	20978004	*PINK1*	AR
1	65720133	65881552	*DNAJC6*	AR
2	25013136	25016251	*PTRHD1*	AR
6	161768590	163148834	*PARK2*	AR
15	62144588	62352664	*VPS13C*	AR
21	33997269	34100351	*SYNJ1*	AR
22	32870707	32894818	*FBXO7*	AR
22	38507502	38577857	*PLA2G6*	AR
1	11072462	11085549	*TARDBP*	AD
1	155204239	155214653	*GBA**	AD
3	132136361	132257876	*DNAJC13*	AD
4	90645250	90759447	*SNCA*	AD
7	56169266	56174187	*CHCHD2*	AD
12	40618813	40763087	*LRRK2*	AD
14	23340822	23350789	*LRP10*	AD
16	46693589	46723144	*VPS35*	AD
17	42422491	42430474	*GRN*	AD
17	43971702	44105700	*MAPT*	AD
20	5049129	5093736	*TMEM230*	AD
X	154487526	154493852	*RAB39B*	X-linked R
			FTD genes	
1	11072462	11085549	*TARDBP*	AD
3	87276413	87304698	*CHMP2B*	AD
7	144149034	144533488	*TBK1*	AD
9	35056065	35072739	*VCP*	AD
16	31191431	31206192	*FUS*	AD
17	43971702	44105700	*MAPT*	AD
17	42422491	42430474	*GRN*	AD
X	56590025	56593443	*UBQLN2*	X-linked D
1	227057885	227083804	**AD genes**	AD
11	121322912	121504471	*PSEN2*	AD
14	73603143	73690399	*SORL1*	AD
19	1040102	1065571	*PSEN1*	AD
21	27252861	27543446	*ABCA7**	AD
			APP	
			Perry syndrome gene	
2	74588281	74619214	*DCTN1*	AD
			Niemann-Pick C genes	AR
14	74942900	74960084	*NPC2*	AR
18	21086148	21166581	*NPC1*	

* also risk gene. The Genome Reference Consortium Human Build 37 (hg19) was used. AR: autosomal recessive, AD: autosomal dominant or Alzheimer's disease, PD: Parkinson's disease, FTD: frontotemporal dementia.

Supplementary Table 2: *In-silico* pathogenicity predictions.

					Variant-effect predictions software (scores)										
	Genomic position	**Nucleotide change**	**Amino acid change**	**GERP**	**SIFT**	**Polyphen2 HDIV**	**Polyphen2 HVAR**	**LRT**	**Mutation Taster**	**Mutation Assessor**	**FATHMM**	**MetaSVM**	**MetaLR**	**CADD phred**	**M-CAP**
Discovery cohort	14:23345134	c.977G>A	p.Gly326Asp	4.14	T (0.368)	B (0.297)	B (0.172)	N (0.009)	D (0.887)	L (1.245)	D (-3.23)	D (0.067)	D (0.644)	15.59	D (0.034)
	14:23345322	c.1165G>A	p.Asp389Asn	4.9	T (0.584)	B (0.022)	B (0.007)	D (0.000)	D (1.000)	L (1.165)	D (-2.67)	T (-0.226)	D (0.535)	17.57	D (0.030)
	14: 23341951	c.39C>T	p.Gly13=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23345195	c.1038 C>T	p.Asp346=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23346279	c.1685G>A	p.Arg562His	5.23	T (0.68)	D (0.999)	D (0.972)	D (0.000)	D (1.000)	M (2.2)	D (-3.37)	D (0.451)	D (0.772)	26.6	NA
	14:23346630	c.2036A>C	p.His679Pro	3.84	T (0.143)	B (0.072)	B (0.037)	N (0.226)	D (1.000)	L (0.975)	D (-3.05)	T (-0.202)	D (0.561)	9.054	D (0.093)
Validation cohort	14:23344630	c.473G>A	p.Arg158His	5.03	T (0.064)	(0.021)	B (0.007)	N (0.000)	D (1.000)	L (1.4)	D (-3.85)	D (0.359)	D (0.768)	22.3	D (0.058)
	14:23344816	c.659G>A	p.Cys220Tyr	5.73	D (0.0)	D (1.0)	D (0.999)	D (0.000)	D (1)	M (2.635)	T (-0.2)	D (0.127)	D (0.520)	25.6	D (0.093)
	14:23344989	c.832A>G	p.Thr278Ala	5.97	T (0.292)	P (0.911)	P (0.621)	D (0.000)	D (0.995)	L (0.975)	T (1.6)	T (-1.087)	T (0.067)	22.7	T (0.006)
	14:23345074	c.917G>A	p.Gly306Asp	5.97	T (0.055)	D (0.959)	P (0.6)	D (0.000)	D (0.997)	L (1.205)	D (-3.35)	D (0.601)	D (0.752)	23.5	D (0.048)
	14:23345931	c.1458G>C	p.Glu486Asp	5.91	D (0.036)	D (0.999)	D (0.963)	D (0.000)	D (1.000)	M (1.95)	D (-3.44)	D (0.841)	D (0.861)	28.1	D (0.039)
	14:23346254	c.1660C>T	p.Arg554*	5.23	NA	NA	NA	D (0.000)	D (1)	NA	NA	NA	NA	42	NA
	14:23346575	c.1981C>T	p.Arg661Cys	4.97	T (0.081)	D (0.999)	P (0.761)	D (0.000)	D (1.000)	L (1.1)	D (-3.18)	D (0.730)	D (0.805)	33	D (0.267)
	14:23341951	c.39C>T	p.Gly13=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23344572	c.415A>G	p.Met139Val	2.05	T (0.302)	B (0.002)	B (0.003)	N (0.665)	N (1.000)	N (-0.625)	D (-2.19)	T (-1.010)	T (0.026)	3.321	NA
	14:23344848	c.691C>T	p.Arg231Trp	3.79	D (0.005)	D (1.0)	D (0.981)	D (0.000)	D (0.936)	M (2.085)	T (2.14)	T (-1.068)	T (0.032)	33	NA
	14:23345048	c.891C>T	p.Gly297=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23345129	c.972C>T	p.Gly324=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23345133	c.976G>A	p.Gly326Ser	4.07	T (0.584)	B (0.006)	B (0.019)	N (0.009)	D (0.861)	N (0.205)	D (-3.16)	T (-0.950)	T (0.115)	16.85	T (0.007)
	14:23346025	c.1552G>A	p.Asp518Asn	5.24	D (0.011)	D (0.993)	P (0.702)	D (0.000)	D (0.987)	L (1.39)	D (-3.11)	D (0.428)	D (0.645)	25.2	NA
	14:23346279	c.1685G>A	p.Arg562His	5.23	T (0.68)	D (0.999)	D(0.972)	D (0.000)	D (1.000)	M (2.2)	D (-3.37)	D (0.451)	D (0.772)	26.6	NA
	14:23346517	c.1923G>A	p.Leu641=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	14:23346682	c.2088G>C	p.Leu696=	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

GERP: Genomic Evolutionary Rate Profiling, SIFT: Sorting Intolerant From Tolerant, PolyPhen2 HDIV: Polymorphism Phenotyping version 2 human diversity, PolyPhen2 HVAR: Polymorphism Phenotyping version 2 human variation, LRT: Likelihood Ratio Test, FATHMM: Functional Analysis Through Hidden Markov Models, SVM: Support Vector Machine, LR: Logistic Regression, CADD: Combined Annotation Dependent Depletion, M-CAP: Mendelian Clinically Applicable Pathogenicity, T: tolerated, D: damaging/disease causing, B: benign, N: polymorphism/neutral, P: polymorphism automatic, L: low, M: medium, NA: not available. Transcript references: NM_014045-4. ANNOVAR (version 2018Apr16).

Supplementary Table 3: Other *LRP10* variants which did not fulfill our criteria for possible pathogenicity.

	Patient(s)	Genomic position	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP 142 accession number	Allele frequency GnomAD (alleles)	Functional predictions: pathogenic (total)	Splicing predictions: deleterious (total)
Discovery cohort	3-7	14:23341951	c.39C>T	p.Gly13=	2	synonymous	rs34294471	2.7% (7725)	NA	n.a.
	8	14:23345195	c.1038 C>T	p.Asp346=	5	synonymous	rs201657631	0.007% (20)	NA	n.a.
	9,10	14:23346279	c.1685G>A	p.Arg562His	7	missense	rs142153001	0.7% (1974)	9/10	n.a.
	11	14:23346630	c.2036A>C	p.His679Pro	7	missense	rs149685154	0.02% (52)	4/10	n.a.
Validation cohort	9-18	14:23341951	c.39C>T	p.Gly13=	2	synonymous	rs34294471	2.7% (7725)	NA	n.a.
	19, 20	14:23344572	c.415A>G	p.Met139Val	5	missense	rs28534929	0.7% (1974)	1/11	0/4
	21	14:23344848	c.691C>T	p.Arg231Trp	5	missense	rs35043211	0.2% (663)	7/11	n.a.
	22, 23	14:23345048	c.891C>T	p.Gly297=	5	synonymous	rs768801096	0.03% (94)	NA	n.a.
	24, 25	14:23345129	c.972C>T	p.Gly324=	5	synonymous	rs750833995	0.002% (5)	NA	n.a.
	26, 27	14:23345133	c.976G>A	p.Gly326Ser	5	missense	rs138170865	0.2% (450)	3/11	n.a.
	28	14:23346025	c.1552G>A	p.Asp518Asn	6	missense	rs74357167	0.4% (1053)	9/10	0/4
	29-40	14:23346279	c.1685G>A	p.Arg562His	7	missense	rs142153001	0.7% (1974)	9/10	n.a.
	41	14:23346517	c.1923G>A	p.Leu641=	7	synonymous	rs371430508	0.02% (60)	NA	n.a.
	42	14:23346682	c.2088G>C	p.Leu696=	7	synonymous	rs148391884	0.002% (6)	NA	1/4

Only variants in exons or at the exon-intron boundary (-10/+10) are displayed. The Genome Reference Consortium Human Build 37 (hg19) and NM_014045-4 LRP10 transcript were used. GnomAD: Genome Aggregation Database, NA.: not available, n.a.: not applicable. Splicing prediction programs: SSF, MaxEnt, NNSPLICE, Gene Splicer.

Supplementary Table S4: Possibly pathogenic variants in known genes causing parkinsonism or dementia.

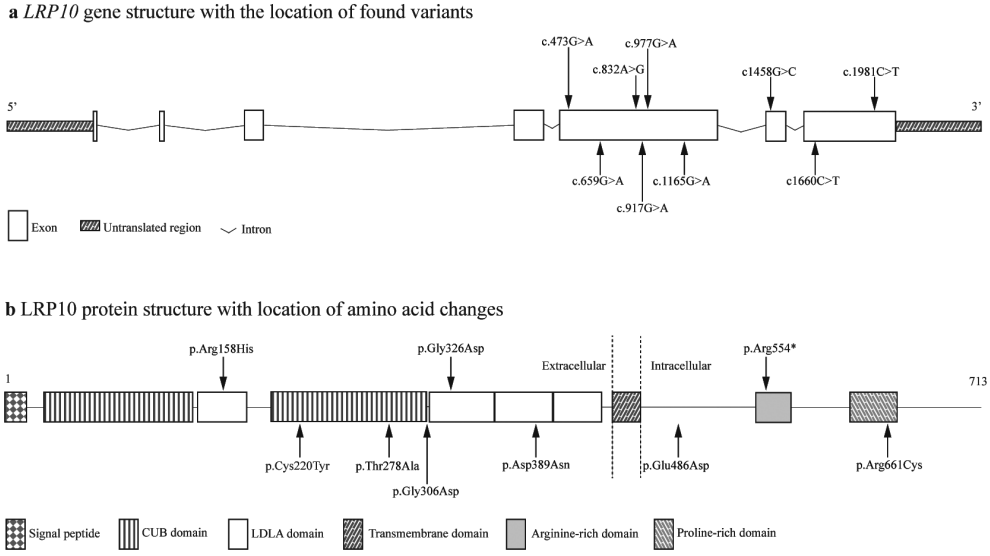
Patient	Gene	Genomic position	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP 142 accession number	Allele frequency GnomAD (alleles)	Functional predictions: pathogenic (total)
1	*VPS13C*	15:62211490	c.7636C>T	p.Gln2546*	58	stopgain	.	.	n.a.

Only variants in exons or at the exon-intron boundary (-10/+10) are displayed. The Genome Reference Consortium Human Build 37 (hg19) and NM_020821 VPS13C transcript were used; GnomAD: Genome Aggregation Database, n.a..: not applicable.

Supplementary Table 5: Overview of identified variants in current and previous studies.

	Genomic position	Nucleotide change	Amino acid change	Study	Present in number of patients (diagnosis)	Present in number of controls
Discovery cohort	14:23345322	c.1165G>A	p.Asp389Asn	Current	1/950 (PSP)	0/645
	14:23345134	c.977G>A	p.Gly326Asp	Current	1/950 (PSP)	0/645
				Pihlström et al., 2018[7]	1/264 (MSA)	0/462
Validation cohort	14:23344630	c.473G>A	p.Arg158His	Current	1/950 (PSP)	0/645
				Kia et al., 2018[8]	0/2835 (PD)	1/5343
	14:23344816	c.659G>A	p.Cys220Tyr	Current	1/950 (PSP)	0/645
	14:23344989	c.832A>G	p.Thr278Ala	Current	1/950 (PSP)	0/645
	14:23345074	c.917G>A	p.Gly306Asp	Current	2/950 (PSP)	0/645
				Kia et al., 2018[8]	2/2835 (PD)	1/5343
	14:23345931	c.1458G>C	p.Glu486Asp	Current	1/950 (PSP)	0/645
				Kia et al., 2018[8]	3/2835 (PD) and 1/111 (DLB)	0/5343 and 0/233
	14:23346254	c.1660C>T	p.Arg554*	Current	1/950 (PSP)	0/645
				Guerreiro et al., 2018[9]	0/1040 (DLB)	1/1422
	14:23346575	c.1981C>T	p.Arg661Cys	Current	1/950 (PSP)	0/645
				Pihlström et al., 2018[7]	0/264 (MSA)	1/462
				Kia et al., 2018[8]	0/2835 (PD)	1/5343

PSP: progressive supranuclear palsy, MSA: multiple system atrophy, PD: Parkinson's disease, DLB: dementia with Lewy bodies.



Supplementary Figure 1:
(a) *LRP10* gene structure with the location of found variants. (b) LRP10 protein structure with the location of amino acid changes.

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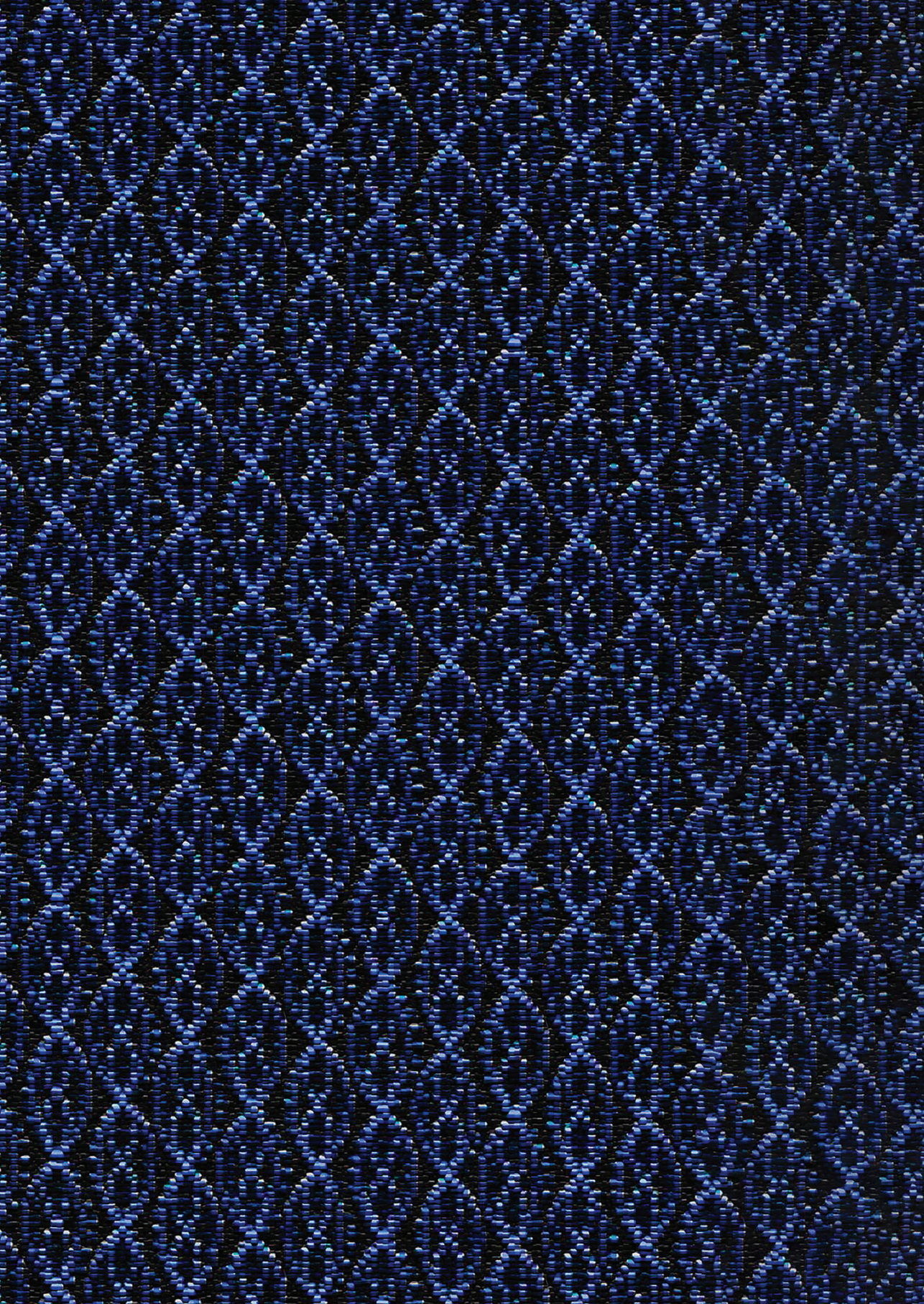

Part 5

Multimodal approach to identify novel genes associated with dementia with Lewy bodies

5.1

Chapter 5.1

Identification of novel cerebrospinal fluid biomarker candidates for dementia with Lewy bodies: a proteomic approach

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Under review

Abstract

Objective

Diagnosis of dementia with Lewy bodies (DLB) is challenging, largely due to a lack of diagnostic tools. Cerebrospinal fluid (CSF) biomarkers have been proven useful in Alzheimer's disease (AD) diagnosis. Here, we aimed to identify novel CSF biomarkers for DLB using a high-throughput proteomic approach.

Methods

We applied liquid chromatography/tandem mass spectrometry with label-free quantification to identify biomarker candidates to individual CSF samples from a well-characterized cohort comprising patients with DLB (n=20) and controls (n=20). Validation was performed using (1) the identical proteomic workflow in an independent cohort (n=30), (2) proteomic data from patients with related neurodegenerative diseases (n=149) and (3) orthogonal techniques in an extended cohort consisting of DLB patients and controls (n=76). Additionally, we utilized random forest analysis to identify the subset of candidate markers that best distinguished DLB from all other groups.

Results

In total, we identified 1995 proteins. In the discovery cohort, 69 proteins were differentially expressed in DLB compared to controls (p<0.05). Independent cohort replication confirmed VGF, SCG2, NPTX2, NPTXR, PDYN and PCSK1N as candidate biomarkers for DLB. The downregulation of the candidate biomarkers was somewhat more pronounced in DLB in comparison with related neurodegenerative diseases. Using random forest analysis, we identified a panel of VGF, SCG2 and PDYN to best differentiate between DLB and other clinical groups (accuracy: 0.82 (95% CI: 0.75-0.89)). Moreover, we confirmed the decrease of VGF and NPTX2 in DLB by ELISA and SRM methods. Low CSF levels of all biomarker candidates, except PCSK1N, were associated with more pronounced cognitive decline (0.37< r <0.56, all p<0.01).

Conclusion

We identified and validated six novel CSF biomarkers for DLB. These biomarkers, particularly when used as a panel, show promise to improve diagnostic accuracy and strengthen the importance of synaptic dysfunction in the pathophysiology of DLB.

Background

Dementia with Lewy Bodies (DLB) is a common cause of dementia in the elderly, accounting for up to 20% of dementia cases.[1] Clinical hallmarks of DLB are cognitive decline accompanied by parkinsonism, visual hallucinations, fluctuating cognition and rapid eye movement (REM)-sleep behavior disorders (RBD).[2] Diagnosis of DLB during life is based on clinical diagnostic consensus criteria[2], but a definite diagnosis of DLB requires post-mortem defined presence of Lewy bodies and Lewy neurites diffusely distributed throughout the brain.[2,3] Diagnosing DLB during life is challenging due to highly variable clinical manifestation and overlap in signs, symptoms and pathology with both Alzheimer's disease (AD) and Parkinson's disease (PD). There is thus a strong need for biomarkers supporting accurate and timely diagnosis of DLB.

Cerebrospinal fluid (CSF) is the best matrix to identify novel biomarkers for central nervous system disorders, due to its direct contact with the brain parenchyma and mirroring biochemical alterations occurring within the brain.[4,5] CSF biomarkers have been proven useful in AD, where a typical CSF profile of decreased levels of amyloid-$\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) combined with increased levels of total and phosphorylated tau (t-tau, p-tau) protein levels supports the diagnosis of AD.[6] So far, no such diagnostic biomarkers are available for DLB. CSF biomarkers for α-synuclein seem promising[7-9], but are still not sensitive and specific enough to function as single diagnostic biomarkers.

Mass spectrometry-based proteomics has emerged as an useful approach for unbiased candidate biomarker discovery in biofluids.[10,11] So far, only few proteomic studies have been performed for DLB, albeit in small and clinically heterogeneous cohorts, and results have not yet been validated.[12-14]

Here, we aimed to identify novel candidate proteins in CSF of DLB patients in a relatively large, well-characterized discovery cohort (20 DLB patients and 20 controls) using a state-of-the-art mass spectrometry workflow. We next thoroughly validated the results by (1) the same proteomic workflow in an independent cohort (n=30), (2) comparison of identified biomarkers values in related neurodegenerative diseases (n=149) and (3) enzyme-linked immunosorbent assays (ELISA) and selected reaction monitoring (SRM) for the most represented candidate biomarkers in an extended cohort (n=76).

Methods

Patient selection

DLB patients and controls enrolled in the current study were selected from the Amsterdam Dementia Cohort and the Erasmus Medical Center. All subjects underwent extensive clinical examination including physical and neurological examination, neuropsychological assessment, electroencephalogram, structural brain imaging and laboratory tests.[15] Additional diagnostic tests, such as ^{123}I[FP-CIT] single photon emission computed tomography

(DaT-SPECT) were performed by indication. Diagnoses were made by consensus in a multidisciplinary meeting according to standard diagnostic criteria. Probable DLB was diagnosed according to the 2005 clinical consensus criteria.[16] All patients also fulfilled novel consensus criteria.[2] Controls were individuals who presented at the memory clinic with cognitive complaints, but no abnormalities on clinical or cognitive testing were observed and criteria for mild cognitive impairment, dementia or other medical conditions associated with cognitive complaints were not met. Furthermore, all controls had normal AD biomarker levels in CSF[17], and preserved normal cognitive function on neuropsychological testing for at least two years after first presentation at the memory clinic. The study was performed according to the ethical principles of the Declaration of Helsinki and was approved by the local ethics committees. Written informed consent was obtained from all subjects.

Phase 1: Discovery

Cohort 1

For the biomarker discovery phase, 20 DLB patients and age- and sex-matched controls were selected from the Amsterdam Dementia Cohort according to the criteria described above. In addition, DLB patients in cohort 1 fulfilled the following additional inclusion criteria: (1) DaT-SPECT scan showing presynaptic dopaminergic deficits and (2) normal AD biomarker levels in the CSF[17].

Phase 2: Proteomics Validation

Cohort 2

A second cohort consisted of an independent set of 17 DLB patients and 13 age- and sex-matched controls selected from the Amsterdam Dementia Cohort (n=27) and the Erasmus Medical Center (n=3) was used for validation using an identical proteomics workflow. The DLB patients in cohort 2 had less stringent inclusion criteria, namely DLB patients were not selected on the basis of normal CSF AD biomarker levels and a DaT-SPECT scan was not required.

Phase 3: Validation of candidate biomarkers

Cohort 3A

For the validation of the identified candidate biomarkers in related neurodegenerative diseases, we analyzed proteomic data previously generated in 20 patients with AD and 21 patients with frontotemporal dementia (FTD) as part of a parallel study (PRODIA Memorabel Project). In addition, proteomic data from 109 PD patients were provided by the Fox Investigation for New Discovery of Biomarkers ("BioFIND") database (http://biofind.loni.usc.edu/).[18]

Cohort 3B

A subset of the identified candidate biomarkers was validated by orthogonal analytical techniques in CSF samples from DLB patients and controls. Cohort 3B consisted of

additional 48 DLB patients and 28 controls selected from the Amsterdam Dementia Cohort. DLB patients in cohort 3B fulfilled similar inclusion criteria as DLB patients in cohort 2.

CSF sample collection and storage

In line with international biobanking guidelines[19], CSF was obtained by lumbar puncture between the L3/L4, L4/L5 or L5/S1 intervertebral space using a 25-gauge needle and collected in 10 mL polypropylene tubes (Starstedt, Nümbrecht, Germany). Part of the CSF was used for basic CSF analysis, and levels of $A\beta_{1-42}$, total tau and p-tau were measured with commercially ELISA (Innotest®, Fujirebio, Gent, Belgium). The remaining CSF was centrifuged at 1800g at 4°C for 10 min, aliquoted in polypropylene tubes of 0.5 mL and stored at -80°C[19] until further analysis.

Biomarker discovery analysis and validation

The workflow for mass-spectrometry biomarker discovery analysis and validation is summarized in Figure 1.

CSF sample preparation and gel electrophoresis

CSF samples were coded and analyzed in a blinded fashion. The depletion of the top-14 high abundant proteins, i.e. albumin, IgG, antitrypsin, IgA, transerrin, haptoglobulin, fibrinogen, α2-macroglobulin, α1-aid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AI, complement C3 and transthyretin, was performed as previously reported[20]. Depleted CSF was further concentrated using 3kDA filters (Millipore, Billericam, CA, USA) prior to loading the whole depleted CSF fraction on 1-D gradient gels from Invitrogen (Carsbad, CA, USA; NuPAGE 4-12% Bis-Tris gel, 1.5mm x 10 wells). SDS-PAGE gels were stained overnight with Coomassie brilliant blue R250 (Pierce, Rockford, IL, USA). To minimize inter-run variability, each gel contained four patients and four controls in an alternating order.

NanoLC-MS/MS analysis

Before Nano liquid chromatography (LC) - tandem mass spectrometry (MS/MS) analysis, separated proteins were in-gel digested as previously described.[21] Peptides were separated by an Ultimate 3000 nanoLC system (Dionex LC-Packings, Amsterdam, the Netherlands) equipped with a 20 cm x 75 µm ID fused silica column custom packed with 3 µm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 µL/min on a 10 mm x 100 µm ID 5 µm 120 Å ReproSil Pur C18 aqua at 2% buffer B (buffer A: 0.05% FC in MilliQ; buffer B: 80% ACN + 0.05% FC in MilliQ). Peptides were separated at 300 nl/min in a 10-40% buffer B gradient in 60 minutes. Eluting peptides were ionized at a potential of +2 kVA and injected in a QExactive mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at resolution 70.000 (at m/z 200) in the Orbitrap using an AGC target value of $3x10^6$ charges. The top 10 peptide signals (charge-states 2^+ and higher) were submitted to MS/MS in the

higher-energy collision cell (4 amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired at resolution 17.500 (at m/z 200) in the Orbitrap using an AGC target value of $2x10^5$ charges and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 seconds.

Protein identification and quantification

MS/MS spectra were searched against the Swissprot human 2018 reference proteome using MaxQuant 1.6.0.16.[22] Enzyme specificity was set to trypsin and up to two missed cleavages were allowed. Cysteine carboxamidomethylation was set as fixed modification and methionine oxidation and N-terminal acetylation as variable modifications. Peptide precursor ions and fragment ions were searched with maximum mass deviation of 4.5 ppm and 20 ppm, respectively. All identifications were filtered at a false discovery rate (FDR) of 1% using the decoy database strategy. Protein abundance was quantified by MS-signal intensity of the area under the chromatographic peak of the peptide precursor ion.

Statistical analysis

To identify differentially expressed proteins among the DLB and control groups raw intensities were processed using the label-free quantification (LFQ) algorithm in MaxQuant and MaxLFQ intensities were obtained.[23] Missing values were imputed from a normal distribution centered at the minimal intensity and a variance equal to the average variance across all proteins. Hierarchical clustering was performed on log10 normalized expression using the Euclidean distance and complete linkage for both sample clustering and protein clustering. Heatmaps were generated to visualize the normalized to zero mean unit variance (z-scores) for individual proteins. Differential expression analysis was performed with the limma package available from the Bioconductor package[24].

Proteomic analysis in CSF samples of AD, FTD and PD patients

We obtained proteomic data from CSF samples from AD and FTD patients that were generated in previous, published[25] and yet unpublished studies from our group. The proteomic analyses were performed using a similar workflow as the proteomic workflow described above. As part of a parallel study (PRODIA Memorabel Project), the generated raw proteomic data of AD, FTD and DLB patients included in cohort 1 and cohort 2 were reanalyzed against the same reference database (Swissprot human 2018 reference proteome using MaxQuant 1.6.0.16). In addition, we obtained CSF proteomic data provided by the BioFind database. CSF samples from PD patients were analyzed using state-of-the-art DEEP SEQ mass spectrometry technology.[26] The PD data were searched against a human protein database (uniprot.org) with Mascot.

Orthogonal methods for validation of candidate biomarkers

For Neurosecretory protein VGF (VGF) and Neuronal pentraxin 2 (NPTX2), we performed

additional validation experiments using orthogonal techniques (i.e., ELISA and SRM) in cohort 3. For VGF, CSF levels of the $VGF_{373-417}$ peptide were measured by quantitative competitive ELISA[27-29] and by SRM (see detailed description below). CSF levels of NPTX2 were detected using a quantitative sandwich ELISA, as previously described.[30]

ELISA analysis of VGF

The human $VGF_{373-417}$ ELISA was carried out as described[27,28], on the basis of the corresponding rat $VGF_{375-420}$ assay[29]. A synthetic peptide corresponding to human $VGF_{375-382}$ (conjugated with keyhole limpet haemocyanina via an additional C-terminal cysteine), was used for rabbit immunization. Briefly, plates were coated with the corresponding synthetic peptide in carbonate/bicarbonate buffer (pH 9.6), blocked, treated with PBS containing normal donkey serum (90 ml/L), aprotinin (20 nmol/L) and EDTA (1 g/L), and incubated with the primary antibody (in the same medium), including serial dilutions of standard peptide, or samples. For the standard curve, a range of concentrations (50 nmol/L to 50 fmol/L) of either $VGF_{373-382}$ or $VGF_{373-417}$ (GGEE-45) synthetic peptide was used. The latter was identified as a natural peptide in human CSF[31], hence was used as "full length" reference. Upon the relevant washings, plates were incubated with biotinylated secondary antibodies (Jackson, West Grove, PA, USA), streptavidin-peroxidase conjugate (Biospa, Milan, Italy), next tetramethylbenzidine (X-tra Kem-En-Tec, Taastrup, Denmark). Upon stopping with HCl (1 mol/L), optical density was measured at 450 nm using a multilabel plate reader (Chameleon: Hidex, Turku, Finland). Using the reference peptide, a 50% inhibition of signal was obtained at 10 pmol/L, while recovery of peptide added to human CSF was >80%. The intra- and inter-assay coefficients of variation (CV) were 4% and 10%, respectively. Serial sample dilutions showed a profile parallel to the standard curve (deviation:<10%). When data were tested vs. duration of sample storage (at –80°C, 1 to >10 years), no correlation was revealed. To gain some insight as to the specificity of the assay for N-terminally cleaved peptides, vs. the same sequence within N-terminally extended forms (possibly including the VGF precursor), a synthetic peptide containing an additional N-terminal Arg residue was tested in the assay (corresponding to Arg_{372} in the di-basic site: human VGF Arg_{371}-Arg_{372} immediately preceding the natural peptide $VGF_{373-417}$ and implicated in its N-terminal cleavage). The data showed a <0.5% cross-reactivity for this peptide, hence indicating a high specificity of the assay for the N-terminally cleaved peptide.

SRM analysis of VGF

For SRM analysis of VGF in CSF samples, 200µL of CSF was spiked with TEAB buffer and a quantitative protein epitope signature tag (QPrEST, kindly provided by Atlas Antibodies AB, #QPrEST20926) of VGF as internal standard. Samples were reduced and alkylated with 1mM TCEP and 1mM CAA at 95°C for 10min. Proteins were digested for 16h at 37°C by adding 1.2µg trypsin/LysC (Promega). Digestion was stopped by addition of 800µL 1.25% TFA and peptides were transferred to strong cation exchange STAGE-Tips32

by centrifugation. Peptides were washed with 0.2% TFA followed by 75mM ammonium acetate/20% acetonitrile/0.5% formic acid and eluted with 125mM ammonium acetate/20% acetonitrile/0.5% formic acid. After vacuum drying, peptides were dissolved in 30µL 6% acetonitrile/0.1% TFA and analyzed by LC-SRM. Analysis of VGF was performed with a QTRAP6500 mass spectrometer (AB Sciex), Eksigent MicroLC200 and Agilent 1260 HPLC pump. Peptides were loaded on a C18 PepMap100, 5µm, 0.3x5 mm trap column (Thermo Fisher Scientific). Separation was performed on an Eksigent HALO Fused-core C18, 2.7µm, 0.5x100 mm column at 40°C with mobile phase A: 4% DMSO/0.1% formic acid, and mobile phase B: 4% DMSO/96% acetonitrile/0.1% formic acid and a linear gradient from 1%-30%B within 9.85min. The following transitions of the proteotypic VGF peptide AQEEAEAEER (aa586-595) were measured: 581.3-962.4 (y8), 581.3-833.4 (y7), 581.3-704.3 (y6) (light peptide); 586.3-972.4 (y8), 586.3-843.4 (y7), 586.3-714.3 (y6) (heavy peptide). For relative quantification, the light-to-heavy peptide ratio (mean of the three transitions) was calculated using Skyline v4.2. CSF QC samples were included in each run. Intra-assay CVs were 5.1-7.9%.

Statistical analysis

All statistical analyses were performed in R v.3.5.1 'Feather Spray'. Demographics were compared using Student's t-test, Wilcoxon signed-rank test or Fisher's Exact Test. Correlations between identified CSF biomarkers and age, sex and MMSE were assessed with Spearman partial correlation, adjusted for cohort. For the validation of the identified candidate biomarker levels in related neurodegenerative diseases (cohort 3A), all protein levels were first normalized according to the mean and standard deviation values of their corresponding control group. The obtained z-scores were compared using general linear models corrected for age. In addition, we performed random forest analyses[33] with the R package randomForest using automated parameter optimization with the caret package to identify a subset of candidate markers that best distinguished DLB from all other groups. We used Monte Carlo sampling with replacement to sample test groups to generate a random forest classifier with the minimum number of predictors, and used the left out data to test the resulting classifier. This procedure was repeated for 1000 iterations. Diagnostic groups differed in sample size, and to avoid class imbalance effects on classifier performance, we down-sampled the larger group to the same size as the smaller group for training. Classification performance on the test data was determined with accuracy, sensitivity and specificity using the R package 'caret'. Finally, general linear models were performed to compare CSF levels of VGF and NPTX2 between DLB patients and controls in cohort 3B. A FDR-value <0.05 was considered statistically significant.

Results

Patient characteristics

Table 1 displays the demographics and CSF characteristics of DLB patients and controls included in cohort 1 & 2. The diagnostic groups had similar age and sex distributions. DLB patients had lower MMSE scores compared to controls. Per inclusion criteria, all DLB patients in cohort 1 and all controls had normal AD biomarker levels in CSF, whereas almost half of the DLB patients in cohort 2 had a CSF AD profile.

Table 1: Demographics and CSF characteristics of DLB patients and controls.

	Cohort 1 (n=40)		**Cohort 2 (n=30)**		**Cohort 3B (n=76)#**	
	DLB (n=20)	**Controls (n=20)**	**DLB (n=17)**	**Controls (n=13)**	**DLB (n=48)**	**Controls (n=28)**
Age, years	65.3 (5.8)	65.1 (5.4)	66.9 (7.5)	65.6 (8.5)	67.8 (6.3)*	64.1 (5.8)
Sex, male	17 (85%)	17 (85%)	13 (76%)	9 (69%)	42 (88%)	24 (86%)
Symptom duration, years	3 [2-4]	NA	2 [2-4]	n.a.	2 [1-4]	n.a.
CSF AD biomarkers						
$A\beta_{1-42,}$ (pg/ml)	846 [637-1011]	820 [691-1039]	611 [478-942]*	959 [932-1054]	660 [536-871]**	856 [691-1027]
Abnormal	0 (0%)	0 (0%)	8 (47%)	0 (0%)	13 (27%)	0 (0%)
total tau, (pg/ml)	238 [200-286]	209 [167-266]	317 [268-599]**	226 [194-253]	299 [224-370]***	190 [156-257]
Abnormal	0 (0%)	0 (0%)	8 (47%)	0 (0%)	11 (23%)	0 (0%)
p-tau, (pg/ml)	37 [29-47]	42 [31-47]	46 [41-71]	41 [37-49]	47 [34-61]*	38 [28-46]
Abnormal	0 (0%)	0 (0%)	7 (41%)	0 (0%)	18 (37%)	0 (0%)
APOE ε4 carrier	9 (45%)	8 (40%)	10 (71%)**	2 (17%)	25 (55%)	10 (38%)

Data are presented as mean (SD), median (IQR) or n (%). CSF cutoff values were set on $A\beta_{1-42}$ <550 pg/ml, total tau >375 pg/ml, p-tau >52 pg/ml.[17] Differences between DLB patients and controls were assessed with Student's t-test, Wilcoxon signed-rank test or Fisher's Exact Test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.#NPTX2 ELISA: n=76 (DLB: n=48, controls: n=28); VGF ELISA: n=66 (DLB: n=44, controls: n=22); VGF SRM: n=65 (DLB: n=44, controls: n=21). $A\beta_{1-42}$: amyloid-β_{1-42}, AD: Alzheimer's disease, CSF: cerebrospinal fluid, DLB: dementia with Lewy bodies, MMSE: Mini-Mental State Examination (score range 0-30), n.a.: not applicable, p-tau: tau phosphorylated at threonine 181.

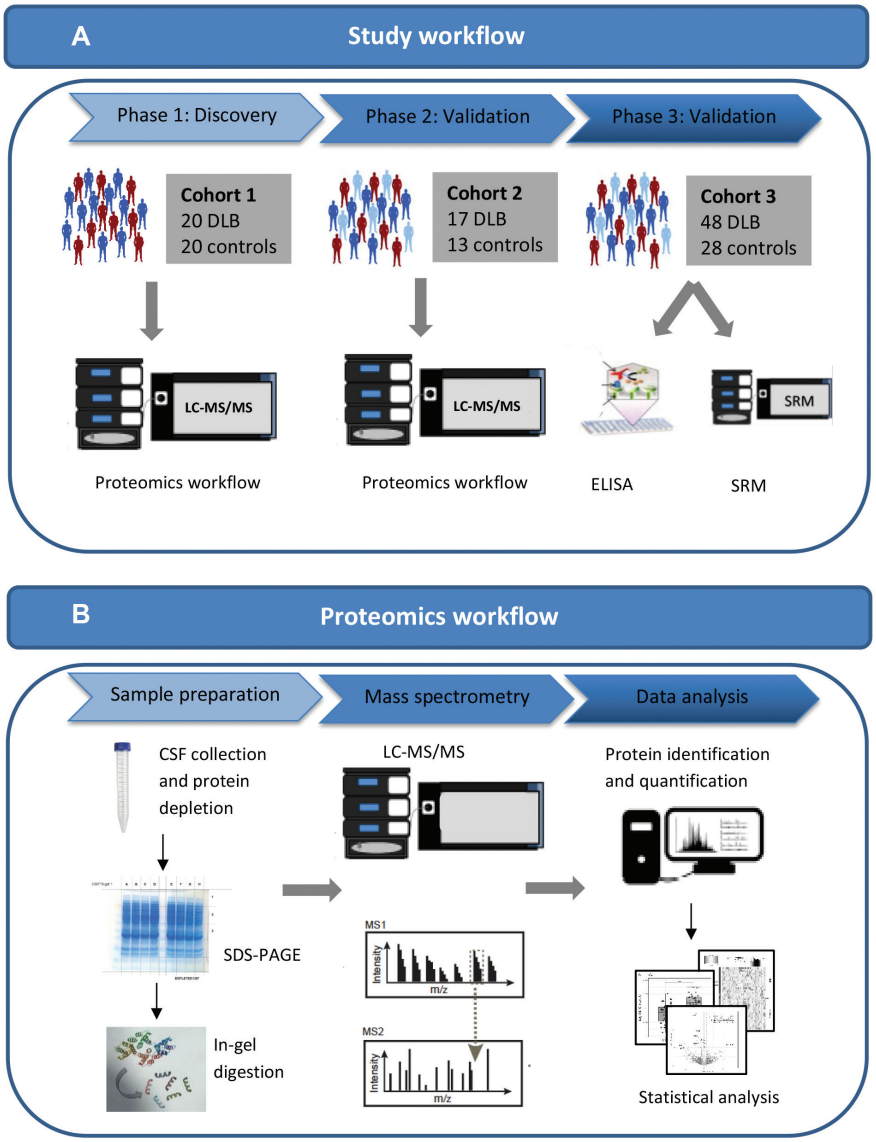

Figure 1: Graphical summary of the workflow used to identify novel CSF biomarkers for DLB. (A) Graphical summary of study workflow. In short, CSF samples from cohort 1 were evaluated using a high-throughput proteomic workflow. The CSF proteome from DLB patients was compared with that of cognitively normal individuals. Validation was performed in an independent validation cohort (cohort 2) using an identical proteomic workflow. Proteins that were significantly altered in abundance in both cohort 1 and cohort 2 were indicated as candidate biomarkers. Levels of the identified candidate biomarkers in DLB patients were compared with the levels of the identified candidate biomarkers as quantified with mass spectrometry in related neurodegenerative diseases (cohort 3A). For a subset of the candidate biomarkers validation was performed using orthogonal methods (ELISA and SRM) in cohort 3B. (B) Graphical summary of the proteomic workflow. We applied an in-depth proteomic workflow, including abundant protein depletion, protein fractionation prior to NanoLC-MS/MS analysis and label-free protein quantification on CSF samples from DLB patients and controls in cohort 1 and 2.

Phase 1: CSF biomarker discovery

In total, 1995 proteins were identified in the discovery cohort (cohort 1). A total of 69 unique proteins showed significantly different abundances (p<0.05). Forty-six proteins were downregulated and 23 proteins were upregulated in DLB (Supplementary Table 1). Figure 2A shows the heatmap and cluster analysis of differentially expressed proteins. Hierarchical cluster analysis including the differentially expressed proteins revealed almost complete separate clustering of DLB patients and controls (87.5% were clustered correctly). The dendrogram illustrates the two distinct clusters: 15 DLB patients were assigned to cluster 1. Interestingly, the 5 DLB patients in cluster 2 clustered together in a subgroup (cluster 2A), while all 20 controls clustered together in subgroup 2B. The level of significance and the magnitude of changes of the quantitative data are visualized in a volcano plot (Figure 2B).

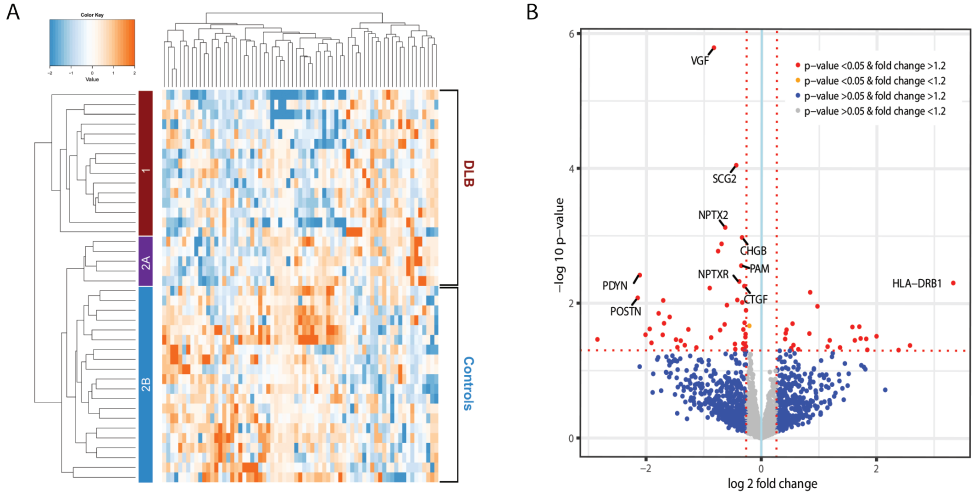

Figure 2: Results of discovery proteomics.
(A) Heatmap and cluster analysis of differentially expressed proteins (n=69) in cohort 1. The heatmap shows distinct patterns of up- and downregulated proteins in the clinical groups. The branching pattern of the dendrogram shows almost complete separation of patients with DLB from cognitively normal controls (35/40 (87.5%) were clustered correctly). 15 DLB patients were assigned to cluster 1 (red) and five DLB patients and 20 controls were assigned to cluster 2. The five DLB patients in cluster 2 clustered together in a small subgroup (cluster 2A, purple) and the controls clustered together in another subgroup (cluster 2B, blue). (B) Volcano plot representing the top biomarker candidates discriminating DLB from controls. The horizontal axis indicates log2 fold change. The vertical axis indicates -10 log p values. Each point represents a protein. Points at the far right- and left-hand sides of the plot have the largest fold changes, while those along the top of the plot are the most statistically significant. The non-axial red dotted vertical lines denote fold change thresholds of 1.2. The non-axial red dotted horizontal line denotes p value threshold of 0.05. Proteins in red have a fold change > 1.2 and p value <0.05. The top-10 biomarker candidates are highlighted in the plot.

Phase 2: Validation using identical proteomic workflow in an independent cohort

Next, we performed a replication in a completely independent second cohort (17 DLB patients, 13 controls) to validate the results (cohort 2, Table 1). Here, 1967 proteins were identified, of which 93 proteins were differentially expressed (p<0.05, Supplementary Table 2). Overlap analysis between the differentially expressed proteins (p<0.05) showed six proteins with same direction and magnitude of change in both cohorts, i.e. Neurosecretory protein (VGF), Secretogranin-2 (SCG2), Neuronal pentraxin-2 (NPTX2), Neuronal pentraxin receptor (NPTXR), Proenkephalin-B (PDYN) and PCSK1N (ProSAAS) (Table 2).

Figure 3 shows the individual levels of these six candidate biomarkers in both cohort 1 and 2. CSF levels of all these proteins were lower in DLB patients compared to controls (all p<0.05). Next, we explored whether these six candidate biomarkers were associated with age, sex and cognitive impairment. Partial Spearman correlation analysis adjusted for cohort revealed that lower CSF levels of all proteins, except PCSK1N, were associated with lower MMSE scores at time of lumbar puncture (0.37< r < 0.56, all p<0.01; Figure 4), whereas no associations were found with age and sex (data not shown).

Table 2: Overlapping differentially expressed proteins between cohort 1 and 2.

Uniprot ID	Gene name	Protein name	Fold change in discovery cohort	Fold change in validation cohort	CSF peer literature (see Suppl. Table 3 for references)
O15240	*VGF*	Neurosecretory protein VGF	-1.78	-1.41	↓ in AD, FTD and ALS ↑ in schizophrenia
P13521	*SCG2*	Secretogranin-2	-1.36	-1.30	↓ in MS ↓ in AD
P47972	*NPTX2*	Neuronal pentraxin-2	-1.55	-1.50	↓ in AD
O95502	*NPTXR*	Neuronal Pentraxin Receptor	-1.31	-1.32	↓ in AD
P01213	*PDYN*	Proenkephalin-B	-4.33	-8.78	↓ in AD
Q9UHG2	*PCSK1N*	ProSAAS	-1.22	-1.21	↓ in AD ↓ in FTD

Table lists the six CSF biomarker candidates for DLB. A positive fold change indicates that the protein is upregulated in the DLB group in contrast to the control group. A negative fold change indicates the protein is downregulated in the DLB group compared to the control group. AD: Alzheimer's disease, ALS: amyotrophic lateral sclerosis, DLB: dementia with Lewy bodies, FTD: frontotemporal dementia, MS: multiple sclerosis, NPTX2: Neuronal pentraxin 2, NPTXR: Neuronal pentraxin receptor, PCSK1N: ProSAAS, PDYN: Proenkephalin-B, SCG2: Scretogranin-2, VGF: Neurosecretory protein VGF.

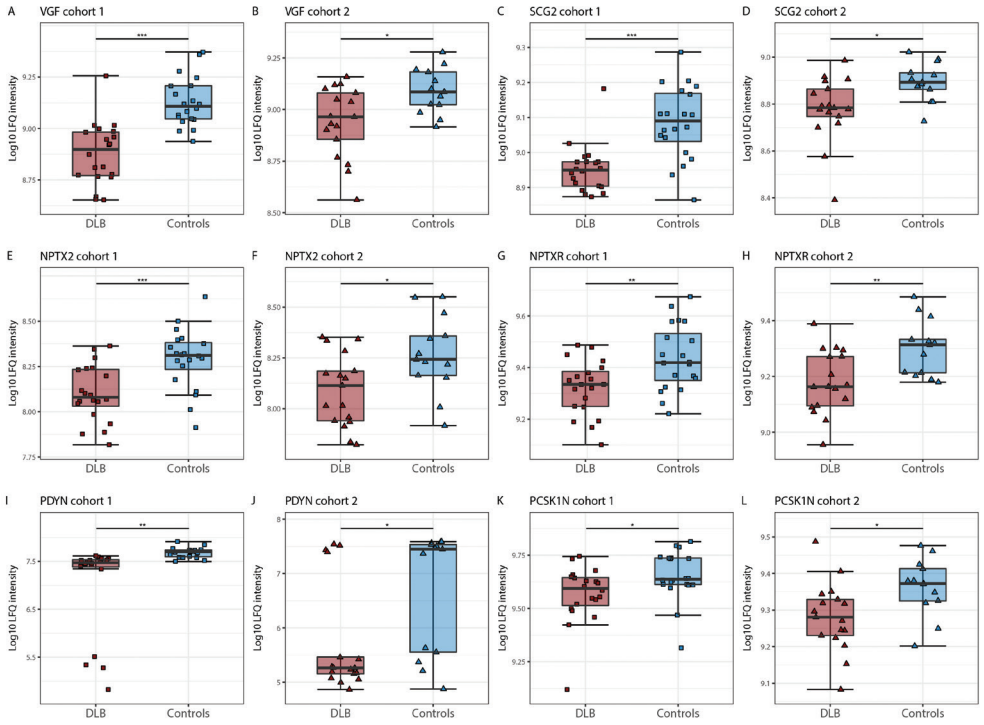

Figure 3: Box and whisker plots of candidate CSF biomarkers for DLB.
(A) Log 10 LFQ intensity of VGF in cohort 1, (B) VGF in cohort 2, (C)(D) SCG, (E)(F) NPTX2, (G) (H) NPTXR, (I)(J) PDYN, (K)(L) PCSK1N. The line through the middle of the boxes corresponds to the median and the lower and the upper lines to the 25th and 75th percentile, respectively. The whiskers extend from the 5th percentile on the bottom to the 95th percentile on top. Differences between DLB patients and controls were assessed limma package available from the Bioconductor package, * p<0.05, ** p<0.01, *** p<0.001. DLB: dementia with Lewy bodies, NPTX2: Neuronal pentraxin 2, NPTXR: Neuronal pentraxin receptor, PCSK1N: ProSAAS, PDYN: Proenkephalin-B, SCG2: Secretogranin-2, VGF: Neurosecretory protein VGF.

Table 3: Diagnostic performance of a biomarker panel for DLB versus other clinical groups.

DLB versus	Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
All non-DLB	0.82 (0.75-0.89)	0.69 (0.41-0.98)	0.83 (0.74-0.90)
Controls	0.84 (0.77-0.89)	0.77 (0.46-1.00)	0.85 (0.76-0.93)
PD	0.79 (0.69-0.87)	0.72 (0.43-0.93)	0.80 (0.66-0.90)
AD	0.89 (0.85-0.99)	0.85 (0.79-0.99)	1.00 (1.00-1.00)
FTD	0.76 (0.61-0.92)	0.73 (0.55-0.95)	0.86 (0.59-1.00)

All protein levels were Z transformed according to the mean and standard deviation in controls. AD: Alzheimer's disease, DLB: dementia with Lewy bodies, FTD: frontotemporal dementia, PD: Parkinson's disease.

Phase 3A: Validation of candidate CSF biomarkers in related neurodegenerative diseases

Next, we investigated the candidate biomarker values in related neurodegenerative diseases, including AD, PD and FTD (Figure 5A). CSF levels of the candidate biomarkers, except PCSK1N, were in general lower in all neurodegenerative patient groups compared to the control group. Protein levels were consistently lowest in DLB patients. Specifically, CSF NPTX2 levels were lower in DLB compared to both AD and PD ($p<0.05$). CSF NPTXR levels were lower in DLB than in PD ($p<0.05$). CSF levels of PCSK1N were lower in DLB compared to both PD and FTD ($p<0.05$). CSF levels of PDYN, SCG2 and VGF were lower in DLB compared to all related neurodegenerative diseases studied ($p<0.05$). CSF levels of all proteins were comparable between the other neurodegenerative disease, i.e. AD, PD and FTD, except VGF for which levels were lower in AD compared to FTD ($p<0.05$). The identified markers still showed considerable overlap between groups, suggesting limited ability for diagnostic purposes as single markers. Therefore, we performed random forest analyses to study whether a combination of biomarkers improved discrimination between DLB and all non-DLB individuals. VGF, SCG2 and PDYN best differentiated between DLB and all non-DLB, with accuracy of 0.82, specificity of 0.83 and sensitivity of 0.69 (Table 3). Subsequently, we performed pairwise comparisons between DLB versus all other clinical groups using this model. Table 3 shows a summary of the pairwise diagnostic classification results. The panel discriminated DLB other clinical groups with accuracies ranging from 76% to 89%. The specificity of all pairwise comparisons was high (0.80-1.00) while sensitivity was moderate (0.72-0.85).

Phase 3B: Validation of candidate CSF biomarkers by ELISA and SRM

Finally, VGF and NPTX2 were selected for validation in cohort 3 based on the availability of orthogonal analytical methods (ELISA and SRM). As shown in Figure 5B, decreased levels of CSF VGF ($VGF_{373-417}$ in ELISA) and NPTX2 were confirmed using these alternative analytical methods ($p<0.01$ and $p<0.001$, respectively).

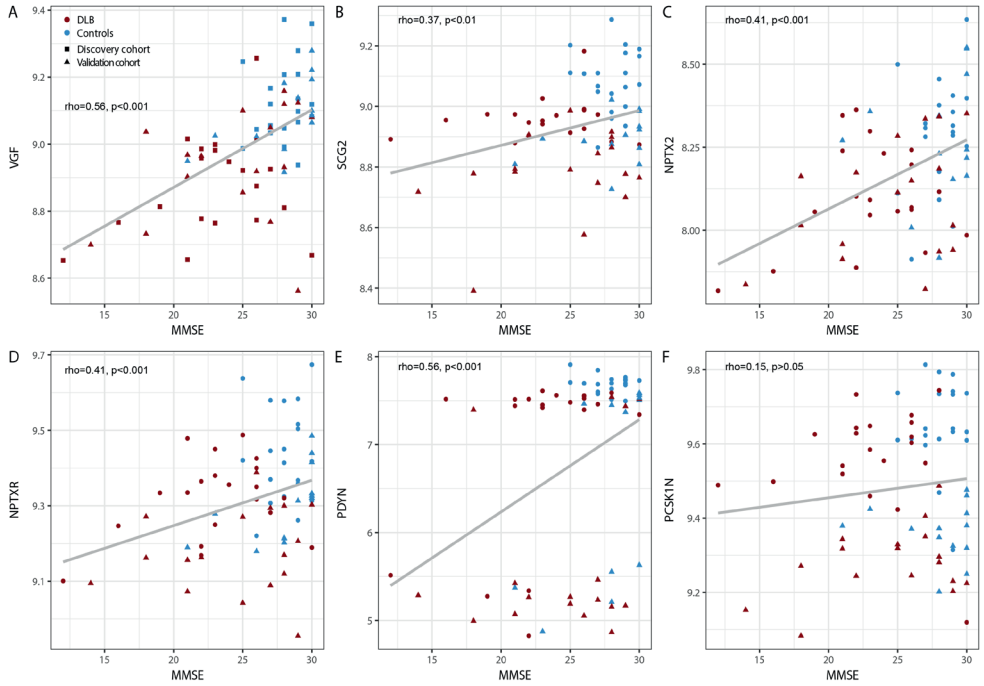

Figure 4: Associations between the six CSF candidate biomarkers for DLB and MMSE.
Scatter plots of MMSE and CSF levels of (A) VGF (B) SCG2, (C) NPTX2, (D) NPTXR, (E) PDYN, (F) PCSK1N across DLB (red) and control groups (blue). Individual subject cohort 1 are depicted as squares and individual subjects from cohort 2 are depicted as triangles. Associations were assessed using Spearman partial correlation adjusted for cohort. To correct for multiple comparisons, *p* values were corrected using a false discovery rate correction. DLB: dementia with Lewy bodies, NPTX2: Neuronal pentraxin 2, NPTXR: Neuronal pentraxin receptor, PCSK1N: ProSAAS, PDYN: Proenkephalin-B, SCG2: Scretogranin-2, VGF: Neurosecretory protein VGF.

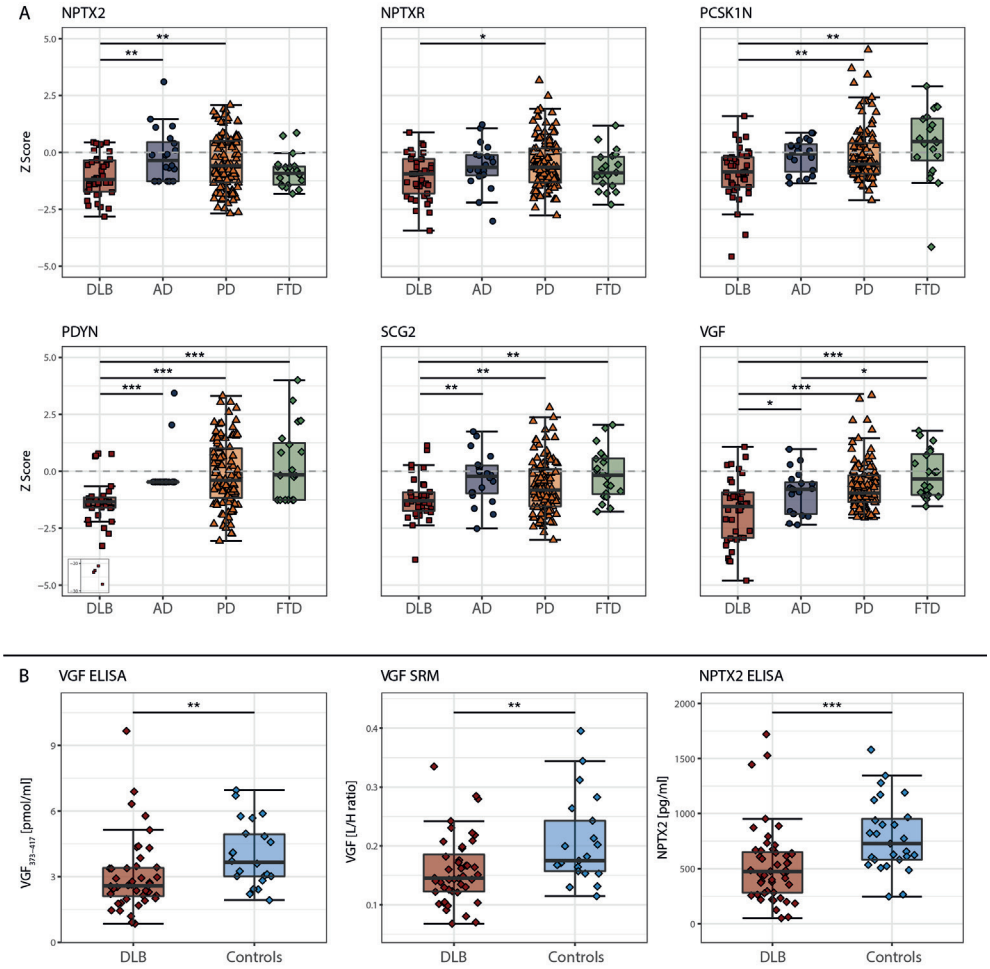

Figure 5: Validation of candidate biomarkers.
(A) Differences in levels of candidate biomarkers between DLB and related neurodegenerative diseases. All protein levels were Z transformed according to the mean and standard deviation in controls, dotted line represents average protein levels for the control group. For PDYN, 4 outliers (z-score >20) were illustrated in a box. Differences were assessed with general linear models corrected for age and a false discovery rate correction was applied. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Please note that the low variation in AD patients is caused by lack of a measurable concentration. (B) Validation of VGF and NPTX2 using orthogonal analytical methods. Levels of $VGF_{373-417}$ (pmol/ml) were determined by ELISA, levels of VGF were determined with SRM and levels of NPTX2 (pg/ml) were determined with ELISA in CSF samples from DLB patients (n=48) and controls (n=28). The line through the middle of the boxes corresponds to the median and the lower and the upper lines to the 25th and 75th percentile, respectively. The whiskers extend from the 5th percentile on the bottom to the 95th percentile on top. Differences between DLB patients and controls were assessed with general linear models. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. AD: Alzheimer's disease, DLB: dementia with Lewy bodies, ELISA: enzyme-linked immunosorbent assays, FTD: frontotemporal dementia, NPTX2: Neuronal pentraxin 2, NPTXR: Neuronal pentraxin receptor, PCSK1N: ProSAAS, PD: Parkinson's disease, PDYN: Proenkephalin-B, SCG2: Secretogranin-2, SRM: selected reaction monitoring, VGF: Neurosecretory protein VGF.

Discussion

Using a state-of-the-art rigorous proteomic approach and validation in a completely independent cohort, we identified and positively validated six promising CSF biomarker candidates for DLB, namely VGF, SCG2, NPTX2, NPTXR, PDYN and PCSK1N (proSAAS). All six biomarker candidates were downregulated in DLB and levels were consistently lowest in DLB patients compared to related neurodegenerative diseases studied, i.e. AD, PD and FTD. Additionally, we utilized machine learning to identify the biomarker panel best capable of classifying DLB patients. The combination of VGF, SCG2 and PDYN best differentiated between DLB and related neurodegenerative diseases with acceptable specificity and sensitivity. In a second validation step, we confirmed the decrease of CSF VGF (ELISA, SRM) and NPTX2 (ELISA) using orthogonal analytical techniques. Low CSF levels of all biomarker candidates, except PCSK1N, were associated with more pronounced cognitive decline. We will discuss these validated biomarker candidates below.

Three identified biomarker candidates (VGF, SCG2 and PCSK1N) are members of the chromogranin/secretogranin family and play a role in the regulated secretory pathway of peptides, hormones, neurotransmitters and growth factors. VGF topped the list of potential biomarker candidates. Biologically active peptides derived from VGF play an important role in diverse processes, for example, hormone, neurotrophin and neurotransmitter release, energy homeostasis and regulation of gastrointestinal function.[34,35] Although VGF peptides have so far not been associated with DLB, previous proteomic studies observed changes in VGF peptides in the CSF of patients affected with several neurodegenerative and psychiatric disorders. In line with our findings in DLB, multiple VGF peptides were shown to be decreased in CSF from patients with AD, FTD (VGF_{26-62}) or amyotrophic lateral sclerosis (ALS) ($VGF_{398-411}$). In addition, VGF peptides were also reduced in brain tissue of patients with AD and PD.[36] Conversely, CSF levels of VGF_{23-62} were increased in schizophrenia patients (see Supplementary Table 3 for an overview of the literature). The second biomarker candidate SCG2 is involved in the packing or sorting of peptide hormones and neuropeptides into secretory vesicles, and plays a role in inflammatory responses and in the regulation of the blood pressure.[34] Consistent with our findings, reduced levels of CSF SCG2 in AD and multiple sclerosis (MS) have been reported (Supplementary Table 3). In view of the decrease of VGF and SCG2 in CSF of patients with different neurodegenerative disorders and their localization in synaptic vesicles, we propose that VGF and SCG2 are markers for synaptic degeneration. Third, PCSK1N, an inhibitor of prohormone convertase (PC) activity[34], has also been proposed as a CSF biomarker candidate for several neurological disorders. For example, reduced levels of CSF PCSK1N in AD and FTD have been reported (Supplementary Table 3). Moreover, several lines of evidence have implicated that PCSK1N blocks aggregation of $A\beta_{1-42}$ and α-synuclein[37,38], supporting a function of PCSK1N as a neuronal secretory chaperone in DLB.

NPTX2 and NPTXR are members of the neuronal pentraxin family.[39] The neuronal pentraxin family has not previously been related to DLB. However, NPTX2 and NPTXR were also reduced in AD (Supplementary Table 3). NPTX2 promotes formation of new excitatory synapses and regulation of AMPA-type receptors clustering at established synapses.[40] The altered levels of NPTX2 and NPTXR further substantiate the importance of synaptic dysfunction in the pathophysiology of DLB. In contrast to the results in AD and DLB, both the gene and tissue expression of NPTX2 were upregulated in PD.[41] We showed that CSF NPTX2 levels in DLB were lower compared to both AD and PD patients. The reduction of NPTX2 that is correlated with cognitive decline implicates a pathophysiological mechanism - failure of the adaptive function of pyramidal neurons to modify excitatory drive of fast spiking parvalbumin interneurons - that could potentially be targeted for therapeutics.[42]

The sixth biomarker candidate that we identified and validated was PDYN. The large decrease (fold change of >4) suggest that PDYN is an on/off marker (i.e. subjects either have low PDYN levels or have high PDYN levels). More DLB patients than controls have very low PDYN levels resulting in an average decreased expression in DLB (Figure 3). PDYN is a precursor protein that is processed by PC1, PC2 and carboxypeptidase E to form different opioid neuropeptides (collectively referred to as dynorphins).[43] The effects of dynorphins are mediated through two kinds of receptors: (1) κ-opioid receptor and (2) NMDA or AMPA receptors. Dysregulation of the dynorphin/κ-opioid receptor system may contribute to behavioral abnormalities that are commonly shared by psychiatric disorders (i.e. decreased motivation and negative affect)[44], while non-opioid effects of dynorphins on NMDA or AMPA receptors could result in apoptosis and neurodegeneration[45]. Consistent with the decreased levels of PDYN in DLB in our study, reduced dynorphin levels have also been observed in CSF from AD patients (Supplementary Table 3) and in the amygdala of patients diagnosed with major depression and bipolar disorder.[46] Interestingly, a substantial loss of hypothalamic cells producing hypocretin, PDYN and NPTX2 has been found in patients with narcolepsy.[47,48] Patients with narcolepsy suffer from symptoms that are also present in DLB, such excessive daytime sleepiness, hypnagogic hallucinations and RBD.[49] The overlap in symptoms might suggests a common etiology between DLB and narcolepsy and indicates that PDYN and NPTX2 reduction may be important biological substrates underlying sleep-related symptoms in DLB.

The identification of these candidate biomarkers highlight the importance of synaptic dysfunction in DLB. This is in line with previous research indicating that this biological process is a central feature in the DLB pathogenesis.[50-52] However, the mechanisms leading to synaptic dysfunction in DLB remain elusive. Growing evidence indicates that accumulation of α-synuclein at presynaptic sides may contribute towards explaining synaptic dysfunction in DLB.[50,51,53,54] A prevailing hypothesis is that excessive α-synuclein leads to deficits in vesicular transport/trafficking resulting in functional impairment of neurotransmitter release at the synapse.[50,51,53,54] Synaptic dysfunction is thought to precede neuronal degeneration in DLB, and may correlate more directly with cognitive decline than pathological hallmarks

such as Lewy bodies.[55] Indeed, most of the identified biomarker candidates were associated with cognitive decline in DLB. Our results provide support to the link between cognitive performance and synaptic protein loss in DLB.

Loss of synapses and a decrease in synaptic proteins are constant features of neurodegenerative diseases. This is also supported by similar synaptic protein changes in post-mortem brain tissue revealed by proteomic analysis from patients with AD, PD dementia and DLB.[55] The identified candidate biomarkers may therefore not be selectively reduced in DLB. Although synaptic dysfunction and loss is evident in neurodegenerative diseases, our findings tentatively suggest that synaptic dysfunction appears to be more pronounced in DLB than in related neurodegenerative diseases, i.e. AD, PD and FTD, or may reflect differing synaptic deficits among neurodegenerative diseases. Of note, the downregulation of the identified proteins in DLB is unlikely to be caused by concomitant AD pathology, since all DLB patients in cohort 1 and most DLB patients in cohort 2B and 3B had normal CSF AD biomarker levels. Most candidate biomarkers, however, showed considerable overlap between diagnostic groups, suggesting limited ability for diagnostic purposes as single markers. Random forest analyses suggested that VGF, SCG2 and PDYN combined could best differentiate between DLB and all non-DLB individuals with a high specificity and moderate sensitivity. Despite the somewhat lower sensitivity, a vital characteristic for a biomarker (panel) is its specificity, i.e. the ability of a biomarker (panel) to correctly identify all people who not have the condition of interest, in determining disease state. Clinical symptoms are quite sensitive, but lack specificity in terms of distinguishing DLB from other types of neurodegenerative diseases, therefore, the identified biomarker panel could importantly add to the clinical work-up of DLB. Possibly, other combinations of proteins measured with modern discovery studies may further aid in differentiating between diagnoses, and future research in larger sample sizes should further investigate this question.

Among the strengths of the current study are the use of a rigorous in-depth proteomic approach, replication in an independent cohort, validation of the biomarker candidates in related neurodegenerative diseases, including AD, PD and FTD, validation of a subset of biomarker candidates using orthogonal techniques, and the strict inclusion criteria for patients and controls. For example, an abnormal DAT-SPECT scan and normal AD biomarker levels were obligatory for all DLB patients in the discovery cohort. On the other hand, DLB patients in the validation cohorts were more heterogeneous, i.e. DLB patients were more representative of DLB patients in daily memory clinic practice, as they were not selected based on their CSF AD biomarker values and almost half of the DLB patients had a CSF profile compatible with AD (in line with previous literature[56]). The use of this study design increases the generalizability of our findings. These strengths make the current study the most comprehensive proteomic analysis in DLB so far. Our study has nonetheless also limitations. A potentially important drawback is that the proteomic pipeline is biased towards the identification of more abundant proteins. This is particularly a problem in mass spectrometry-based proteomic analysis of CSF, since most proteins secreted from the brain

into the CSF (e.g. cytokines and neuropeptides) have low concentrations (~ 150 µg/mL). For example, several known key pathological determinants of DLB, including α-synuclein and $A\beta_{1-42}$, were not detected, since their concentration are below the typical limit of detection of mass spectrometry methods. In addition, these proteins can be highly post-translationally modified, which further compromises mass spectrometry-based identification by default search strategies. Hence, the possibility cannot be excluded that we may have missed some potentially interesting proteins. Furthermore, proteomic data of the PD patients were obtained from different mass spectrometry platforms, which could have introduced some noise. However, normalization of the biomarker candidate values relative to the corresponding control group could restrict the methodological differences. Additionally, the random forest classifier was generated and tested in the same cohort which may lead to over-optimistic classification results. Although we have used bootstrapping to reduce such over-fitting, larger cohorts are needed to validated these findings as well as to examine the added diagnostic value of the (combination of) candidate proteomic markers in relation to the established AD biomarkers and α-synuclein. Such a study would be of tremendous value to the field by optimizing biomarker panel for fit-for-use purposes, as well as to evaluate the role of synaptic dysfunction in the pathogenesis of neurodegeneration.

Conclusion

In conclusion, we identified and positively validated six novel proteins (VGF, SCG2, NPTX2, NPTXR, PDYN and PCSK1N) as promising biomarkers for DLB. Our results might suggest that these candidate biomarkers, particularly when used as a panel, show promise to improve diagnostic accuracy for DLB, which should be explored in future prospective validation studies. Moreover, the validation using orthogonal techniques (i.e. high-throughput immunoassays or SRM) of a subset of the candidate biomarkers revealed by the proteomics approach supports the robustness of our findings. Identification of these candidate biomarkers also strengthens the importance of synaptic dysfunction in the pathophysiology of DLB, which warrant further research as potential therapeutic target. On the applicative and clinical side, the identification of novel CSF biomarkers for DLB, can be expected to enhance clinical diagnostic accuracy, especially early in the disease course, and might thereby accelerate the development of new disease-modifying and neuroprotective agents.

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Supplementary Information

Supplementary Table 1: Differentially expressed proteins in cohort 1 based on mass spectrometry data analysis.

Uniprot Accession	Gene name	Protein name	Sequence coverage (%)	Fold change	*p* value	# DLB samples	# Control samples
O15240	***VGF***	**Neurosecretory protein VGF**	**63.4**	**-1.777**	**1.62E-06**	**20**	**20**
P13521	***SCG2***	**Secretogranin-2**	**52.2**	**-1.357**	**8.96E-05**	**20**	**20**
P47972	***NPTX2***	**Neuronal pentraxin-2**	**43.9**	**-1.548**	**0.001**	**20**	**20**
P05060	***CHGB***	**Secretogranin-1**	**71.5**	**-1.265**	**0.001**	**20**	**20**
CON__ENSEMBL:ENSBTAP00000006074			16.7	-1.618	0.001	0	1
P60842	*EIF4A*	Eukaryotic initiation factor 4A	20	-1.687	0.002	0	1
P19021;	***PAM***	**Peptidyl-glycine alpha-amidating monooxygenase**	**56.2**	**-1.276**	**0.003**	**20**	**20**
P01213	***PDYN***	**Proenkephalin-B**	**42.9**	**-4.325**	**0.004**	**16**	**20**
O95502	***NPTXR***	**Neuronal pentraxin receptor**	**67**	**-1.305**	**0.005**	**20**	**20**
P13760	***HLA-DRB1***	**HLA class II histocompatibility antigen. DRB1-4 beta chain**	**44**	**10.047**	**0.005**	**14**	**5**
P29279-	***CTGF***	**Connective tissue growth factor**	**31.7**	**-1.229**	**0.006**	**20**	**20**
P04440	*HLA-DPB1*	HLA class II histocompatibility antigen. DP beta 1 chain	29.1	-1.864	0.006	0	1
Q9UKZ9	*PCOLCE2*	Procollagen C-endopeptidase enhancer 2	6.5	1.795	0.007	1	0
Q15063	***POSTN***	**Periostin**	**45.8**	**-4.435**	**0.008**	**10**	**17**
P49747	***COMP***	**Cartilage oligomeric matrix protein**	**53.8**	**-1.337**	**0.009**	**20**	**20**
P55001	*MFAP2*	Microfibrillar-associated protein 2	12.4	-3.262	0.009	0	5
Q9BXJ3	***C1QTNF4***	**Complement C1q tumor necrosis factor-related protein 4**	**57.8**	**-1.260**	**0.010**	**20**	**20**
Q9BQ51	*PDCD1LG2*	Programmed cell death 1 ligand 2	12.6	-1.516	0.011	0	1
Q504Y2	*PKDCC*	Extracellular tyrosine-protein kinase PKDCC	17.4	1.959	0.011	2	0
Q8WXD2	***SCG3***	**Secretogranin-3**	**74.4**	**-1.204**	**0.013**	**20**	**20**
Q5VSG8	***MANEAL***	**Glycoprotein endo-alpha-1.2-mannosidase-like protein**	**51.4**	**-3.448**	**0.014**	**15**	**20**
A6NL88	*SHISA7*	Protein shisa-7	7.4	-3.020	0.016	16	20
P02452	***COL1A1***	**Collagen alpha-1(I) chain**	**26.1**	**-1.226**	**0.019**	**20**	**20**
Q9ULH4	*LRFN2*	Leucine-rich repeat and fibronectin type-III domain-containing protein 2	16	-3.229	0.020	2	9
P30456	*HLA-A*	HLA class I histocompatibility antigen. A-43 alpha chain	54	1.453	0.020	1	0
Q68DQ2	*CRYBG3*	Very large A-kinase anchor protein	1.6	-1.557	0.021	0	1
Q16610	*ECM1*	Extracellular matrix protein 1	75.7	-1.158	0.022	20	20

P15509	***CSF2RA***	**Granulocyte-macrophage colony-stimulating factor receptor subunit alpha**	**36.9**	**3.247**	**0.022**	**19**	**13**
P06703	*S100A6*	Protein S100-A6	16.7	2.985	0.022	4	0
Q6PCB0	*VWA1*	von Willebrand factor A domain-containing protein 1	41.1	-3.837	0.024	4	10
P04275;	***VWF***	**von Willebrand factor;von Willebrand antigen 2**	**48.2**	**-1.244**	**0.024**	**20**	**20**
Q16849	*PTPRN*	Receptor-type tyrosine-protein phosphatase-like N	19.6	-2.408	0.024	18	20
Q6JBY9	*RCSD1*	CapZ-interacting protein	9.4	1.352	0.025	0	1
Q4ZIN3	*TMEM259*	Membralin	3.4	-1.644	0.025	0	1
P01031	***C5***	**Complement C5**	**63.4**	**1.339**	**0.028**	**20**	**20**
P05783	*KRT18*	Keratin, type I cytoskeletal 18	24.9	1.781	0.028	1	0
Q15768	*EFNB3*	Ephrin-B3	36.5	-1.211	0.028	20	20
P01859	*IGHG2*	Ig gamma-2 chain C region	36.2	-4.036	0.029	8	15
Q14DG7	*TMEM132B*	Transmembrane protein 132B	20.2	-3.279	0.029	13	18
P61812	*TGFB2*	Transforming growth factor beta-2	27.8	3.995	0.031	13	7
P08123	*COL1A2*	Collagen alpha-2(I) chain	25.5	-1.213	0.032	20	20
P16519	*PCSK2*	Neuroendocrine convertase 2	28.9	-1.833	0.032	19	20
Q9NY56	*OBP2A*	Odorant-binding protein 2a	35.9	3.300	0.033	4	0
Q06141	*REG3A*	Regenerating islet-derived protein 3-alpha	41.7	3.528	0.033	18	12
Q9Y2E5	*MAN2B2*	Epididymis-specific alpha-mannosidase	42.8	1.330	0.034	20	20
P07478	*PRSS2*	Trypsin-2	12.1	-7.192	0.034	13	18
Q9NY33	*DPP3*	Dipeptidyl peptidase 3	40.8	-2.795	0.035	14	18
Q63HQ2	*EGFLAM*	Pikachurin	38.4	3.074	0.035	20	17
P55087	*AQP4*	Aquaporin-4	15.3	2.274	0.035	4	0
Q15493	*RGN*	Regucalcin	53.2	-2.640	0.036	15	20
P52565	*ARHGDIA*	Rho GDP-dissociation inhibitor 1	22.5	-3.752	0.039	5	11
Q9UBX7	*KLK11*	Kallikrein-11	47.6	-1.244	0.039	20	20
Q9NTU7	*CBLN4*	Cerebellin-4	37.3	-1.377	0.040	20	20
Q9UHG2	*PCSK1N*	ProSAAS	68.5	-1.224	0.040	20	20
Q8IXA5	*SPACA3*	Sperm acrosome membrane-associated protein 3	7.5	1.471	0.042	1	0
P62837	*UBE2D2*	Ubiquitin-conjugating enzyme E2 D2	14.3	5.977	0.042	11	5
Q9BUD6	*SPON2*	Spondin-2	36.3	-2.514	0.042	17	20
O14793	*MSTN*	Growth/differentiation factor 8	44.5	-1.205	0.044	20	20
Q9NS68	*TNFRSF19*	Tumor necrosis factor receptor superfamily member 19	8.9	2.217	0.044	4	1

Q9Y6C2	*EMILIN1*	EMILIN-1	20.4	2.575	0.045	17	12
Q4LDE5	*SVEP1*	Sushi. von Willebrand factor type A. EGF and pentraxin domain-containing protein 1	9.5	-2.750	0.045	8	14
Q6UY11	*DLK2*	Protein delta homolog 2	22.2	-2.187	0.045	17	20
Q30134	*HLA-DRB1*	HLA class II histocompatibility antigen. DRB1-8 beta chain	33.5	1.430	0.046	1	0
Q9UKM7	*MAN1B1*	Endoplasmic reticulum mannosyl-oligosaccharide 1.2-alpha-mannosidase	33.8	-1.250	0.048	20	20
Q92932	*PTPRN2*	Receptor-type tyrosine-protein phosphatase N2	21.5	-1.230	0.048	20	20
O60486	*PLXNC1*	Plexin-C1	7.1	-1.369	0.048	0	1
P35613	*BSG*	Basigin	39.2	1.558	0.048	1	0
O94856	*NFAS*	Neurofascin	51.1	3.576	0.049	14	9
P02788	*LTF*	Lactotransferrin	66.5	5.211	0.049	18	13

List of 69 differentially expressed proteins in CSF from patients with DLB compared to CSF from cognitively normal controls (p<0.05). Proteins in bold (n=44) also fulfilled the predefined criteria for candidate biomarkers (p<0.05, fold change >1.2, >20% sequence coverage, detected in at least 50% of DLB patients or controls).

Supplementary Table 2: Differentially expressed proteins in cohort 2 based on mass spectrometry data analysis.

Uniprot Accession	Gene name	Protein name	Sequence coverage (%)	Fold change	*p* value	# DLB samples	# control samples
P05413	***FABP3***	**Fatty acid-binding protein, heart**	**75.9**	**1.494**	**0.001**	**17**	**13**
P09936	***UCHL1***	**Ubiquitin carboxyl-terminal hydrolase isozyme L1**	**60.1**	**16.897**	**0.001**	**15**	**5**
P13489	***RNH1***	**Ribonuclease inhibitor**	**30.4**	**7.337**	**0.002**	**11**	**2**
P63104	***YWHAZ***	**14-3-3 protein zeta/delta**	**62.9**	**1.422**	**0.003**	**17**	**13**
P62258	***YWHAE***	**14-3-3 protein epsilon**	**79.2**	**1.309**	**0.003**	**17**	**13**
P18669	***PGAM1***	**Phosphoglycerate mutase 1**	**57.1**	**1.212**	**0.004**	**17**	**13**
P52565	***ARHGDIA***	**Rho GDP-dissociation inhibitor 1**	**22.5**	**4.740**	**0.005**	**17**	**8**
P35080	***PFN2***	**Profilin-2**	**37.9**	**1.253**	**0.005**	**17**	**13**
P31946	***YWHAB***	**14-3-3 protein beta/alpha**	**53.7**	**1.276**	**0.005**	**17**	**13**
O43396	***TXNL1***	**Thioredoxin-like protein 1**	**23.5**	**6.869**	**0.005**	**12**	**3**
P00338	***LDHA***	**L-lactate dehydrogenase A chain**	**62**	**1.297**	**0.006**	**17**	**13**
Q9H008	***LHPP***	**Phospholysine phosphohistidine inorganic pyrophosphate phosphatase**	**34.8**	**4.668**	**0.007**	**17**	**9**
O95502	***NPTXR***	**Neuronal pentraxin receptor**	**67**	**-1.321**	**0.008**	**17**	**13**
Q9Y6R7	***FCGBP***	**IgGFc-binding protein**	**50.6**	**1.523**	**0.008**	**17**	**13**

Q9H7C9	***AAMDC***	**Mth938 domain-containing protein**	**53.3**	**6.141**	**0.010**	**9**	**1**
Q9H4F8	***SMOC1***	**SPARC-related modular calcium-binding protein 1**	**35.3**	**1.406**	**0.010**	**17**	**13**
Q99969	*RARRES2*	Retinoic acid receptor responder protein 2	74.2	1.189	0.011	17	13
Q13591	*SEMA5A*	Semaphorin-5A	7.9	-1.715	0.011	0	1
O15240	***VGF***	**Neurosecretory protein VGF**	**63.4**	**-1.413**	**0.012**	**17**	**13**
P09493	*TPM1*	Tropomyosin alpha-1 chain	25.7	1.726	0.012	1	0
P61981	***YWHAG***	**14-3-3 protein gamma**	**58.3**	**4.816**	**0.012**	**17**	**10**
Q9UBW5	*BIN2*	Bridging integrator 2	3.2	-1.851	0.013	0	1
Q9HD45	*TM9SF3*	Transmembrane 9 superfamily member 3	14.1	4.770	0.013	5	0
P29622	***SERPINA4***	**Kallistatin**	**65.1**	**1.300**	**0.013**	**17**	**13**
P47972	***NPTX2***	**Neuronal pentraxin-2**	**43.9**	**-1.503**	**0.014**	**17**	**13**
P17936	***IGFBP3***	**Insulin-like growth factor-binding protein 3**	**44.7**	**1.317**	**0.016**	**17**	**13**
P13521	***SCG2***	**Secretogranin-2**	**52.2**	**-1.304**	**0.017**	**17**	**13**
Q16787	*LAMA3*	Laminin subunit alpha-3	3.6	1.503	0.017	1	0
P02647	***APOA1***	**Apolipoprotein A-I**	**78.3**	**1.581**	**0.017**	**17**	**13**
P01011	***SERPINA3***	**Alpha-1-antichymotrypsin**	**81.1**	**1.251**	**0.019**	**17**	**13**
P01213	***PDYN***	**Proenkephalin-B**	**42.9**	**-8.781**	**0.019**	**4**	**8**
P14174	***MIF***	**Macrophage migration inhibitory factor**	**20.9**	**1.268**	**0.019**	**17**	**13**
P07195	*LDHB*	L-lactate dehydrogenase B chain	56.6	1.171	0.020	17	13
Q15459	*SF3A1*	Splicing factor 3A subunit 1	8.1	6.508	0.020	13	5
P02763	***ORM1***	**Alpha-1-acid glycoprotein 1**	**41.3**	**5.683**	**0.022**	**16**	**9**
P16152	***CBR1***	**Carbonyl reductase [NADPH] 1**	**75.1**	**1.262**	**0.022**	**17**	**13**
P12955	***PEPD***	**Xaa-Pro dipeptidase**	**45**	**1.301**	**0.022**	**17**	**13**
P50453	***SERPINB9***	**Serpin B9**	**53.7**	**-5.718**	**0.023**	**7**	**11**
P51665	*PSMD7*	26S proteasome non-ATPase regulatory subunit 7	7.4	1.515	0.023	1	0
Q99832	*CCT7*	T-complex protein 1 subunit eta	8.6	1.588	0.023	1	0
Q16539	*MAPK14*	Mitogen-activated protein kinase 14	5.1	-1.729	0.024	0	1
P0C7U0	*ELFN1*	Protein ELFN1	4.6	-1.978	0.024	0	1
Q15818	*NPTX1*	Neuronal pentraxin-1	56.9	-1.193	0.024	17	13
Q01469	***FABP5***	**Fatty acid-binding protein, epidermal**	**80.7**	**1.239**	**0.024**	**17**	**13**
P0C6S8	***LINGO3***	**Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 3**	**20.4**	**-3.082**	**0.025**	**12**	**13**

P10644	*PRKAR1A*	cAMP-dependent protein kinase type I-alpha regulatory subunit	36.5	2.061	0.026	1	0
Q9HC38	*GLOD4*	Glyoxalase domain-containing protein 4	52.7	1.185	0.026	17	13
Q15262	***PTPRK***	**Receptor-type tyrosine-protein phosphatase kappa**	**16.9**	**-1.207**	**0.026**	**17**	**13**
Q7Z7M8	***B3GNT8***	**UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8**	**26.7**	**4.703**	**0.028**	**12**	**4**
P10646	*TFPI*	Tissue factor pathway inhibitor	17.1	2.023	0.028	2	0
O15540	***FABP7***	**Fatty acid-binding protein, brain**	**68.9**	**2.687**	**0.028**	**17**	**11**
P30508	***HLA-C***	**HLA class I histocompatibility antigen, Cw-12 alpha chain**	**59**	**-6.500**	**0.028**	**4**	**8**
P01009	***SERPINA1***	**Alpha-1-antitrypsin;Short peptide from AAT**	**72**	**2.543**	**0.029**	**17**	**13**
Q9UHG2	***PCSK1N***	**ProSAAS**	**68.5**	**-1.208**	**0.029**	**17**	**13**
P61278	*SST*	Somatostatin	20.7	-2.235	0.029	2	5
Q14520	***HABP2***	**Hyaluronan-binding protein 2**	**46.3**	**1.316**	**0.030**	**17**	**13**
P59665	***DEFA1***	**Neutrophil defensin 1**	**28.7**	**4.850**	**0.030**	**17**	**10**
P02787	***TF***	**Serotransferrin**	**83.4**	**1.799**	**0.030**	**17**	**13**
P62937	***PPIA***	**Peptidyl-prolyl cis-trans isomerase A**	**70.9**	**1.240**	**0.031**	**17**	**13**
Q01151	*CD83*	CD83 antigen	4.9	1.590	0.031	1	0
P00558	***PGK1***	**Phosphoglycerate kinase 1**	**77.5**	**1.256**	**0.033**	**17**	**13**
A0A0C4DH73	*IGKV1-12*	Immunoglobulin kappa variable 1-12	13.7	-1.603	0.033	0	1
P0DP25	*CALM3*	Calmodulin-3	61.1	1.186	0.033	17	13
Q8WY21	***SORCS1***	**VPS10 domain-containing receptor SorCS1**	**38.1**	**-1.273**	**0.034**	**17**	**13**
Q7Z7G0	***ABI3BP***	**Target of Nesh-SH3**	**22.2**	**1.233**	**0.035**	**17**	**13**
CON__Q3MHN5			19,2	-1.465	0.035	0	1
P01008	***SERPINC1***	**Antithrombin-III**	**71.8**	**1.239**	**0.035**	**17**	**13**
Q96GD0	*PDXP*	Pyridoxal phosphate phosphatase	28.7	3.596	0.037	8	2
Q4LDE5	*SVEP1*	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	9.5	3.207	0.037	11	4
P36980	***CFHR2***	**Complement factor H-related protein 2**	**68.3**	**1.517**	**0.037**	**17**	**13**
P13497	*BMP1*	Bone morphogenetic protein 1	9.3	3.890	0.038	15	7
P01042	***KNG1***	**Kininogen-1**	**67.9**	**1.463**	**0.039**	**17**	**13**
Q9BZ76	***CNTNAP3***	**Contactin-associated protein-like 3**	**30.4**	**-3.497**	**0.041**	**9**	**11**
Q08554	***DSC1***	**Desmocollin-1**	**24.6**	**2.691**	**0.042**	**17**	**11**
P0C0L5	***C4B***	**Complement C4-B**	**80**	**1.219**	**0.044**	**17**	**13**
P06744	***GPI***	**Glucose-6-phosphate isomerase**	**44.4**	**1.250**	**0.044**	**17**	**13**

Q6ZN30	*BNC2*	Zinc finger protein basonuclin-2	4.3	-21.430	0.044	7	10
P00568	***AK1***	**Adenylate kinase isoenzyme 1**	**59.8**	**3.112**	**0.044**	**17**	**10**
Q16853	***AOC3***	**Membrane primary amine oxidase**	**19.8**	**-3.288**	**0.045**	**9**	**11**
Q9UIW2	***PLXNA1***	**Plexin-A1**	**19.5**	**-2.576**	**0.045**	**14**	**13**
Q5BIV9	*SPRN*	Shadow of prion protein	22.5	3.455	0.045	6	1
Q14563	***SEMA3A***	**Semaphorin-3A**	**27.8**	**-3.326**	**0.045**	**9**	**11**
P27348	***YWHAQ***	**14-3-3 protein theta**	**43.7**	**3.297**	**0.045**	**14**	**7**
Q16473	*TNXA*	Putative tenascin-XA	62.4	-1.597	0.046	0	1
Q9ULB1	*NRXN1*	Neurexin-1	48.5	-1.171	0.046	17	13
P31150	***GDI1***	**Rab GDP dissociation inhibitor alpha**	**69.4**	**1.205**	**0.046**	**17**	**13**
Q9NZL9	*MAT2B*	Methionine adenosyltransferase 2 subunit beta	17.4	2.582	0.047	3	0
Q9HDC9	*APMAP*	Adipocyte plasma membrane-associated protein	28.4	2.333	0.047	3	0
Q9GZQ8	***MAP1LC3B***	**Microtubule-associated proteins 1A/1B light chain 3B**	**27.2**	**6.112**	**0.048**	**12**	**5**
P04899	*GNAI2*	Guanine nucleotide-binding protein G(i) subunit alpha-2	10.4	-1.871	0.049	0	1
P07307	*ASGR2*	Asialoglycoprotein receptor 2	33.8	-1.695	0.049	0	1
P49862	***KLK7***	**Kallikrein-7**	**50.2**	**-4.699**	**0.049**	**10**	**12**
P35612	*ADD2*	Beta-adducin	6.1	1.375	0.050	1	0

List of 93 differentially expressed proteins in CSF from patients with DLB compared to CSF from cognitively normal controls ($p<0.05$). Proteins in bold (n=60) also fulfilled the predefined criteria for candidate biomarkers ($p<0.05$, fold change >1.2, >20% sequence coverage, detected in at least 50% of DLB patients or controls).

Supplementary Table 3: Comprehensive overview of CSF studies of the identified candidate biomarkers for DLB.

Neurosceretory protein VGF			
Study	**Method**	**Participants**	**Results**
Brinkmalm (2018)[1]	Targeted Mass Spectrometry (PRM-MS)	10 AD patients 13 healthy controls	VGF ↓ in AD vs controls
Carrette (2003)[2]	Quantitative Mass Spectrometry	9 AD 10 healthy controls	VGF ↓ in AD vs controls
Duits (2018)[3]	Targeted Mass Spectrometry (PRM-MS)	40 AD 40 MCI 40 non-demented controls	VGF ↓ in AD vs controls VGF ↑ in MCI vs AD
Hendrikson (2015)[4]	Targeted Mass Spectrometry (SRM)	30 AD 30 controls	VGF ↓ in AD vs controls
Holtta (2015)[5]	Quantitative Mass Spectrometry	8 AD 8 non-demented controls	VGF ↓ in AD vs controls
Jahn (2011)[6]	Quantitative Mass Spectrometry	34 AD 17 controls	VGF ↓ in AD vs controls
Llano (2017)[7]	Targeted Mass Spectrometry (MRM)	66 AD 135 MCI 86 normal aged controls	VGF ↓ in AD vs controls
Selle (2005)[8]	Quantitative Mass Spectrometry	127 AD 86 non-demented controls 66 non-AD demented patients	VGF ↓ in AD vs controls
Simonsen (2007)[9]	Quantitative Mass Spectrometry	85 AD 20 FTD 32 healthy controls	VGF ↓ in AD vs controls
Wijte (2012)[10]	Quantitative Mass Spectrometry	20 AD 20 non-demented controls NB. Postmortem CSF	VGF ↓ in AD vs controls
Ruetschi (2012)[11]	Quantitative Mass Spectrometry	16 FTD 12 non-demented controls	VGF_{26-62} ↓ in FTD vs controls
Pasinetti (2006)[12]	Quantitative Mass Spectrometry	36 ALS 21 healthy controls	$VGF_{398-411}$ ↓ in ALS vs controls
Zhao (2008)[13]	ELISA	17 ALS 21 healthy controls	$VGF_{398-411}$ ↓ in ALS vs controls
Huang (2006)[14]	Quantitative Mass Spectrometry	41 Schizophrenia 40 healthy controls	VGF_{23-62} ↑ in schizophrenia vs controls

Brinkmalm (2018)[1]	Targeted Mass Spectrometry (PRM-MS)	10 AD patients 13 healthy controls	SCG2 ↓ in AD vs controls
Llano (2017)[7]	Targeted Mass Spectrometry (MRM)	66 AD 86 normal aged controls	SCG2 ↓ in AD vs controls
Mattsson (2007)[15]	Quantitative Mass Spectrometry	46 MS 46 healthy siblings 50 healthy controls	SCG2 ↓ in MS vs siblings and controls
PCSK1N (ProSAAS)			
Study	**Method**	**Participants**	**Results**
Abdi (2006)[16]	Quantitative Mass Spectrometry	10 AD 10 PD 5 DLB 10 normal aged controls	ProSAAS ↓ in AD vs controls No significant changes in DLB and PD
Jahn (2011)[6]	Quantitative Mass Spectrometry	34 AD 17 controls	ProSAAS ↓ in AD vs controls
Wang (2016)[17]	Quantitative Mass Spectrometry	8 AD 4 non-demented controls	ProSAAS ↓ in AD vs controls
Davidsson (2002)[18]	Quantitative Mass Spectrometry	15 FTD 12 non-demented controls	ProSAAS ↓ in FTD vs controls
NPTX2 and NPTXR			
Study	**Method**	**Participants**	**Results**
Hendrikson (2015)[4]	Targeted Mass Spectrometry (SRM)	30 AD 30 controls	NPTXR ↓ in AD vs controls
Llano (2017)[7]	Targeted Mass Spectrometry (MRM)	66 AD 135 MCI 86 normal aged controls	NPTX2 and NPTXR ↓ in AD vs controls NPTX2 and NPTXR ↓ in MCI converters vs non-converters
Perrin (2011)[19]	Quantitative Mass Spectrometry	24 AD 24 non-demented controls	NPTXR ↓ in AD vs controls
Spellman (2015)[20]	Targeted Mass Spectrometry (MRM)	66 AD 134 MCI 85 healthy controls	NPTX2 and NPTXR ↓ in AD vs controls
Xiao (2017)[21]	ELISA and Westernblot	30 AD 36 healthy controls	NPTX2 and NPTXR ↓ in AD vs controls
Llano (2017)[7]	Targeted Mass Spectrometry (MRM)	66 AD 135 MCI 86 normal aged controls	PDYN ↓ in AD vs controls

Table lists CSF studies of the six identified candidate biomarkers for DLB in neurological and psychiatric diseases. AD: Alzheimer's disease, ALS: amyotrophic lateral sclerosis, DLB: dementia with Lewy bodies, FTD: frontotemporal dementia, MRM: multiple reaction monitoring, MS: multiple sclerosis, NPTX2: Neuronal pentraxin 2, NPTXR: Neuronal pentraxin receptor, PCSK1N: ProSAAS, PRM-MS: Parallel Reaction Monitoring Mass Spectrometry, PDYN: Proenkephalin-B, SCG2: Scretogranin-2, SRM: Selected Reaction Monitoring; VGF: Neurosecretory protein VGF.

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5.1

Appendix to Chapter 5.1

Combining proteomics and whole exome sequencing to find novel genes associated with dementia with Lewy bodies: a pilot study

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Introduction

Increasing evidence shows that genetic factors play a considerable role in dementia with Lewy bodies (DLB). The heritability based on common variants has even been estimated to be around 60%.[1] However, well-established genetic factors explain only a very small part of the total heritability[1], which suggests that many genetic factors still have to be discovered. Thus far, unbiased whole exome sequencing (WES) studies have not been reported for DLB. This is probably due to the large amount of candidate genes that remain after filtering for possible pathogenicity. The amount of candidate genes could be diminished by combining WES data with other omics data[2,3], such as cerebrospinal fluid (CSF) proteomics data. The hypothesis behind this approach is that a pathogenic genetic variant will lead to a differential expression of the corresponding protein in the CSF.

In this pilot study we try to identify novel candidate genes for DLB by combining the data from the CSF proteomics study (Chapter 5.1) with whole exome sequencing in familial patients. This pilot study focuses on patients with a positive family history of dementia or Parkinson's disease (PD) and therefore with presumably a higher genetic load, to increase the chance of disease-associated findings.

Methods

Seven of the 37 patients enrolled in our CSF proteomics study (Chapter 5.1) were selected for this pilot study. These patients were selected based on the presence of a positive family history (at least one first- or second-degree relative with DLB, PD or dementia). Affected and non-affected relatives of the 7 index patients were also included in the genetic part of this pilot study.

For the genetic analysis, genomic DNA was isolated from blood using standard methods. WES was performed using the Nimblegen SeqCap EZ Exome v.2.0 44Mb kit (Roche Nimblegen, Inc., Madison, WI) on a HiSeq2000 sequencer (paired-end 2x100). Reads were aligned to the human reference genome (hg19) using the Burrows-Wheeler alignment[4] tool and processed using Picard (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK)[5] following standard procedures[6]. Single nucleotide variations were determined using GATKs Haplotype Caller and annotated using ANNOVAR[7]. Heterozygous, non-synonymous, stop-gain or stop-loss variants in exons, and variants near splice sites with a mean read depth of >20 were extracted from the WES data. Subsequently, variants were selected based on a minor allele frequency (MAF) of <0.1% in the ExAC-NFE (Exome Aggregation Consortium-Non Finnish Europeans) database, GoNL (Genome of the Netherlands) database and 1000Genomes database (Europeans). We used the raw CSF proteomics data as described in Chapter 5.1. We searched for overlap between differentially expressed proteins and genes with possibly pathogenic variants on group level and individual

level. Differentially expressed proteins on group level were identified after repeating the statistical analysis described in Chapter 5.1 in the 7 selected patients and in 7 sex- and age-matched controls. A p value of <0.05 was considered a statistically significant differential expression. Proteins were considered differentially expressed on individual level when they had a fold change (intensity of one patient divided by the mean of the intensity of the 7 controls) of >1.2 or <0.83, or when they were uniquely present or absent in the patient.

The study was performed according to the ethical principles of the Declaration of Helsinki approved by the relevant Medical Ethical Authorities. All patients or their legal representative signed informed consent for use of clinical records, DNA, and pathological data for research purposes.

Results

Table 1 shows an overview of the identified genetic variants on group level. This table also shows the function and expression of corresponding proteins and available data of the cosegregation analyses. Forty two proteins were differentially expressed between the 7 patients and 7 controls. Seven genetic variants in 5 corresponding genes were identified in 6 patients. Two variants in 2 genes (*HEG1*, *FREM2*) were predicted to be pathogenic in more than half of *in-silico* software. These proteins may be involved in heart and vessel formation and integrity and the maintenance of skin and renal epithelia.[8] Cosegregation studies could be performed for these 2 variants, showing no evidence of cosegregation.

Table 2 shows an overview of the identified genetic variants on individual level. This table also shows the function and expression of corresponding proteins and available data of the cosegregation analyses. In total, 11 proteins with a fold change of >1.2 or <0.83 were identified in the 7 patients. No variants were identified in the corresponding genes. Fifty proteins were uniquely absent. One variant in 1 corresponding gene (*EXT2*) was identified. EXT2 might be involved in heparin sulfate biosynthesis.[8] The identified variant was predicted to be pathogenic in more than half of the *in-silico* software and was not found in his unaffected sister aged 78. A total of 347 proteins were uniquely present. Two variants in 2 corresponding genes (*SYNE1*, *AHNAK*) were identified. SYNE1 may be involved in the maintenance of subcellular spatial organization.[8] The variant in *SYNE1* was not predicted to be pathogenic in more than half of the *in-silico* software, but was found in his sister with dementia. AHNAK may be involved in neural cell differentiation.[8] The variant in *AHNAK* was predicted to be pathogenic in more than half of the *in-silico* software, and was present in his unaffected sister aged 78.

Table 1: Properties of identified genetic variants, genes and proteins (analysis on group level).

Patient	Genomic position	Gene	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP accession number	MAF GoNL	MAF ExAC (NFE)	MAF 1000Genomes (EUR)	MAF GnomAD	Functional predictions: pathogenic (total)	Depth
1	3:124696763	*HEG1*	c.C3761A	p.A1254E	15	nonsynonymous	.	.	.	.	.	8/10	79
2	12:53002170	*KRT73*	c.G1433C	p.G478A	9	nonsynonymous	rs377281547	.	0.0002	.	0.0002	2/11	46
3	13:39425958	*FREM2*	c.A6878G	p.K2293R	11	nonsynonymous	.	.	.	.	2.0e-05	5/11	108
4	13:39343798	*FREM2*	c.C5494A	p.Q1832K	4	nonsynonymous	rs114087410	.	0.0001	0.001	5.7e-05	8/11	103
5	17:57763116	*CLTC*	c.A4774G	p.M1592V	30	nonsynonymous	rs143886947	.	0.0002	.	0.0001	2/11	75
6	19:40377117	*FCGBP*	c.G11305A	p.A3769T	24	nonsynonymous	rs534122657	.	0.0005	0.001	0.0006	1/8	34
6	19:40398322	*FCGBP*	c.C6645A	p.N2215K	14	nonsynonymous	.	.	.	.	2.5e-05	NA	40

Table 1: Continued.

Patient	Genomic position	Gene	Name	Amino acid change	Protein expression in brain (human protein atlas)	May be involved in: (Uniprot function)	Present in affected family member?	Absent in unaffected family member?
1	3:124696763	*HEG1*	Heart development protein with EGF like domains 1	p.A1254E	yes	heart and vessel formation and integrity	yes (n=1), no (n=2)	yes (n=1), no (n=1)
2	12:53002170	*KRT73*	Keratin 73	p.G478A	NA	hair formation	no (n=1)	n.a.
3	13:39425958	*FREM2*	FRAS1 related extracellular matrix protein 2	p.K2293R	NA	maintenance of skin and renal epithelia	n.a.	yes (n=1)
4	13:39343798	*FREM2*	FRAS1 related extracellular matrix protein 2	p.Q1832K	NA	maintenance of skin and renal epithelia	no (n=1)	n.a.
5	17:57763116	*CLTC*	Clathrin heavy chain	p.M1592V	yes	stabilization of kinetochore fibers of mitotic spindle	yes (n=1)	no (n=1)
6	19:40377117	*FCGBP*	Fc fragment of IgG binding protein	p.A3769T	NA	maintenance of mucosal structure	no (n=1)	n.a.
6	19:40398322	*FCGBP*	Fc fragment of IgG binding protein	p.N2215K	NA	maintenance of mucosal structure	no (n=1)	n.a.

Variants which are predicted to be pathogenic in more than half of *in-silico* software are depicted in grey. MAF: minor allele frequency, NA: not available, n.a.: not applicable.

Table 2: Properties of identified genetic variants, genes and proteins (analysis on individual level).

Patient	Genomic position	Gene	Nucleotide change	Amino acid change	Exon	Coding effect	dbSNP accesion number	MAF GoNL	MAF ExAC (NFE)	MAF 1000Genomes (EUR)	MAF GnomAD	Functional predictions: pathogenic (total)	Depth
Absent													
3	11:44129707	*EXT2*	c.C445T	p.R149W	2	nonsynonymous	.	.	6.0e-5	.	6.5.e-05	11/11	70
Present													
6	6:152469390	*SYNE1*	c.A24766C	p.N8256H	137	nonsynonymous	.	.	.	.	.	2/11	42
3	11:62288639	*AHNAK*	c.C13250G	p.P4417R	5	nonsynonymous	.	.	0.0002	.	8.1e-05	7/11	77

Table 2: Continued.

Patient	Genomic position	Gene	Name	Amino acid change	Protein expression in brain (human protein atlas)	May be involved in (Uniprot function):	Present in affected family member?	Absent in unaffected family member?
Absent								
3	11	*EXT2*	Exostosin Glycosyltransferase 2	p.R149W	NA	heparan sulfate biosynthesis	n.a.	yes (n=1)
Present								
6	chr6	*SYNE1*	Spectrin Repeat Containing Nuclear Envelope Protein 1	p.N8256H	yes	maintainance of subcellular spatial organization	yes (n=1)	n.a.
3	chr11	*AHNAK*	AHNAK Nucleoprotein	p.P4417R	yes	neuronal cell differentiation	n.a.	no (n=1)

Variants which are predicted to be pathogenic in more than half of *in-silico* software are depicted in grey. MAF: minor allele frequency, NA: not available, n.a.: not applicable.

Discussion

In this pilot study we searched for novel candidate genes by combining CSF proteomics and WES data. Although we identified an overlap between 8 genes and proteins, evidence that these genes or proteins are related to DLB is very limited. There are several points to consider regarding our analyses. First, combining different datasets with many variables could lead to overlapping results based on chance. Second, we were searching for rare genetic defects with a large effect on disease development. However, these variants may not be present at all in our patient group as a clear Mendelian inheritance pattern was not seen in any of our patients. Third, the protein composition of the CSF may not be changed by genetic defects. Fourth, mass spectrometry has a bias towards identification of peptides with higher concentrations.[9] Therefore, it is possible that we did not detect small differences in protein concentrations. Fifth, due to genetic defects peptide sequences could be altered. These peptides may not be detected, because of the absence of these peptide sequences in the reference protein sequence database.

In our view, future research combining genetics and proteomics with the aim to identify novel genes associated with disease is still promising when: 1) a strong family history is present, 2) DNA of multiple relatives is available, 3) a small set of possibly pathogenic genetic variants is obtained after genetic analysis, 4) tissue of interest is available in preferably >1 relative and 5) the protein reference database is adjusted to recognize mutated peptides.

In conclusion, this pilot study did not show convincing evidence for a novel candidate gene for DLB. Nonetheless, we suggest that the combination of WES and proteomics to find novel genes could still be a promising approach in future research. This in turn, may lead to a better understanding of which genes and molecular mechanisms are involved in the development of DLB and may in potential lead to novel biomarkers and disease-modifying therapy in the future.

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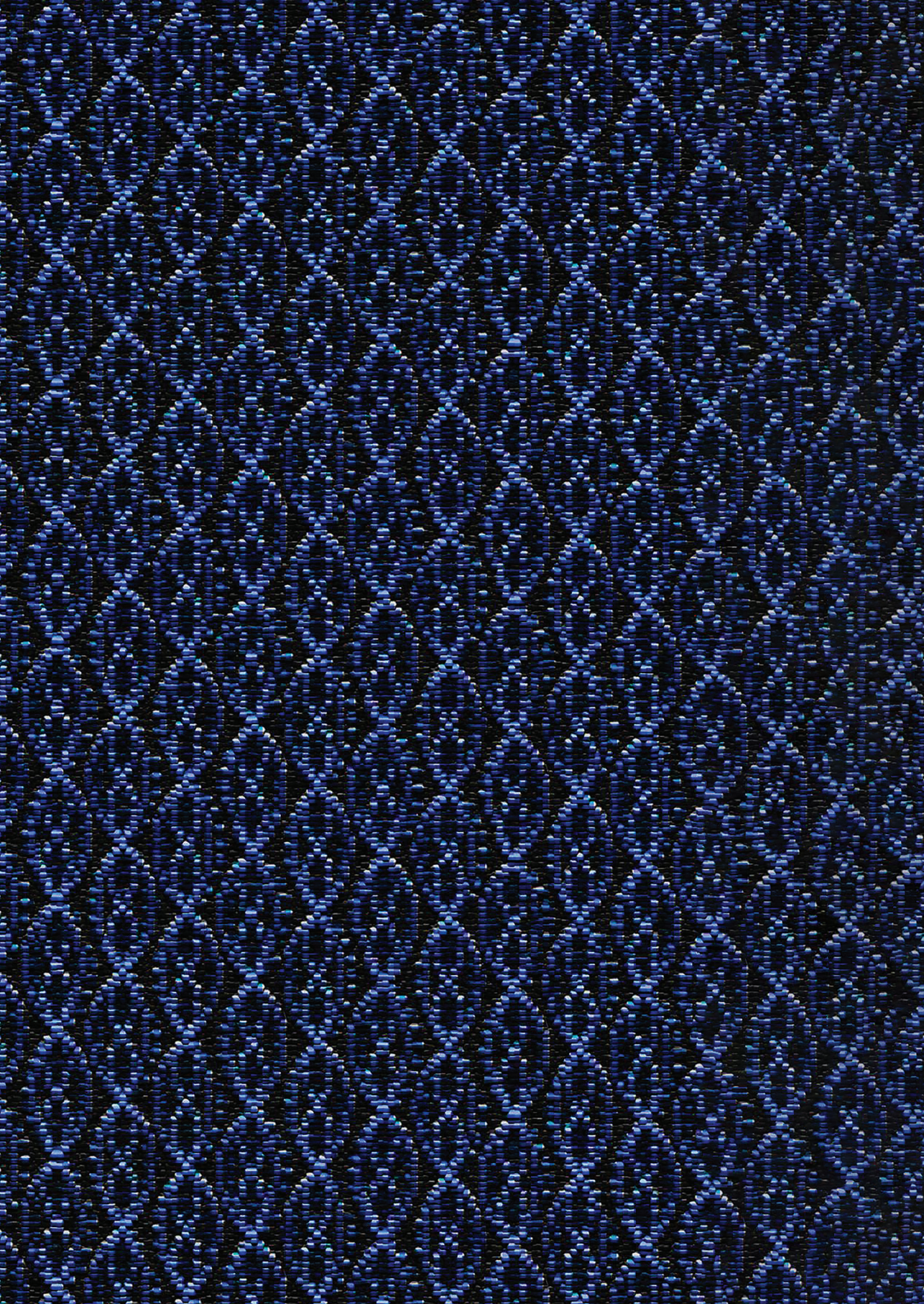

Part 6

General discussion

Dementia with Lewy bodies (DLB) is a common neurodegenerative disease of the elderly[1] and is characterized by progressive cognitive decline, parkinsonism, visual hallucinations, fluctuating cognition, and REM-sleep behavior disorders.[2] Clinical features, pathological hallmarks and genetic factors of DLB overlap with those of Alzheimer's disease (AD) and Parkinson's disease (PD).[1] Genetic factors seem to play a considerable role in DLB, as the heritable component of DLB has been estimated to be approximately 60%.[3,4] However, few genetic studies, often with small sample sizes and candidate gene-based (biased) approaches, have been undertaken in DLB (as described in our review in **Chapter 1.2)**.[5] The first genome wide association (GWA) studies of DLB have only been performed recently, and whole exome sequencing (WES) studies have not been published yet. The paucity of genetic studies in DLB could be due to several reasons. Firstly, DLB has only been recognized as a separate disease since 1984. Up until that time, DLB was referred to as the Lewy body variant of AD or grouped under the umbrella term Lewy body diseases, which is comprised of PD, PD dementia (PDD) and DLB. Secondly, the accuracy of the clinical criteria for DLB is relatively low and many DLB patients are misdiagnosed or overlooked.[6] This could have led to small and heterogeneous study groups. Thirdly, DLB patients with multiple relatives suffering from DLB, AD, or PD have only rarely been recognized, which hampers the identification of rare, highly-penetrant genetic variants in this disease. As a consequence, only several genetic factors (the *APOE* ε4 allele, specific disease-associated variants in *GBA* and *SNCA*) have been associated with DLB up until now. These genetic factors seem to explain only a small percentage of the total heritability[4], which suggests that many genetic factors still remain to be discovered.

This thesis describes our findings on the familial aggregation in DLB, the role for variants in known AD and PD genes in DLB, and the search for novel genes associated with DLB. This Part begins with a discussion of these findings in light of current literature. Subsequently, an overview is provided on how the identified genes may play a role in the molecular mechanisms leading to DLB, and on how these genes may aid in developing biomarkers and disease-modifying treatment options in the future. Hereafter, the strengths and limitations of the studies contained in this thesis are discussed. Finally, suggestions for future study directions are provided.

Familial aggregation

The precise frequencies of DLB patients with relatives with DLB, AD, or PD are currently unknown. Nonetheless, it has been noted that AD and PD occur more frequently in relatives of DLB patients than in relatives of controls[7,8], and that DLB has a higher prevalence in relatives of DLB patients than in relatives of AD patients.[9] Families with multiple members with DLB, compatible with an autosomal dominant pattern of inheritance, have rarely been described. Mutations in *SNCA, LRRK2, PSEN1, PSEN2,* and *APP* have been identified in a handful of DLB patients with relatives with DLB, PD, or dementia.[5] These genes have

previously been associated with PD (*SNCA, LRRK2*) or AD (*PSEN1, PSEN2, APP*). Genetic factors associated with DLB, PD, and AD seem, therefore, to overlap, but little is known about the magnitude of this overlap and its effect on different phenotypes.

In this thesis, we studied possible differences in phenotype between DLB patients with relatives with dementia or PD (familial patients) and DLB patients without relatives with these diseases (sporadic patients) (**Chapter 2.1**) and between DLB patients with and without known, well-established genetic risk factors (**Chapter 3.1**). In **Chapter 2.1** we showed that familial DLB patients have a shorter survival than sporadic DLB patients. We also found a higher percentage of familial DLB patients with elevated AD biomarkers in their cerebrospinal fluid (CSF) compared to sporadic DLB patients. This suggests that genetic factors influence the course of the disease, possibly by affecting the amount of concomitant AD pathology. The exploratory study elaborated in **Chapter 3.1** suggested that DLB patients with the *APOE* ε4 allele more often have a positive family history of AD or dementia than non-carriers. Furthermore, a family history of PD or PDD was present at a greater frequency in DLB patients with disease-associated *GBA* variants than in DLB patients with the *APOE* ε4 allele. Interestingly, we also found that DLB patients with relatives with PD more often show parkinsonism as first symptom than DLB patients with a family history of dementia (**Chapter 2.1**).

These findings suggest that different subgroups may be identified within the DLB population: patients with a phenotype which more closely resembles PD, with possibly underlying PD-associated genetic factors, and patients with a phenotype which more closely resembles AD, with possibly underlying AD-associated genetic factors and mixed neuropathology.

In the next paragraph, I will discuss which well-established genes are associated with DLB and how they overlap with genes associated with AD and PD.

Well-established genes associated with DLB

There are three well-established genes associated with DLB: *APOE*, *GBA* and *SNCA*. Variants in these genes have been associated with DLB in multiple different candidate gene studies (**Chapter 3.1** and **Chapter 3.2**)[10,11] and in two recent GWA studies.[12,13] The *APOE* ε4 allele is the most common risk factor for DLB.[10,12-14] An odds ratio of 2.9 has been reported for carriers of one *APOE* ε4 allele and of 5.9 for carriers of two *APOE* ε4 alleles for developing DLB.[15] DLB patients with an *APOE* ε4 allele have a shorter survival than DLB patients without this allele.[16-18] Conversely, *APOE* ε2 allele carriers have a reduced risk of developing DLB compared to controls.[15] For *GBA*, the risk of developing DLB is dependent on the specific disease-associated variant.[19,20] An odds ratio of 8 for developing DLB has been reported for carriers of disease-associated *GBA* variants in one study.[11] DLB patients with disease-associated *GBA* variants display an earlier age of onset and earlier age of death than patients without these variants.[11,14,21,22] Disease-associated variants in *SNCA* can modify

the risk of developing DLB, and can be, very rarely, the cause of DLB. Families with *SNCA* mutations show a large heterogeneity in diagnosis (DLB, PDD, PD), age of onset and disease progression.[23-26] The *SNCA* locus has been found and replicated in a GWA study of DLB, including 1743 cases and 4454 controls.[12] However, the exact *SNCA* variants which increase the risk of developing DLB remain controversial. Furthermore, the magnitude of this risk and the effect of these genetic variants on phenotype remain unexplored.

The three well-established genes which are associated with DLB (*APOE*, *GBA* and *SNCA*), also play a role in the development of AD or PD. The *APOE* ε4 allele is the most important genetic risk factor for AD, and disease-associated *GBA* variants increase the risk of developing PD and cognitive decline in PD.[27,28] Rare causal variants and common disease-associated variants in *SNCA* have repeatedly been identified in PD patients.[29-31] Intriguingly, different association profiles in *SNCA* have been found for PD and DLB.[12] The single nucleotide polymorphism (SNP) showing the highest association with DLB for the *SNCA* locus (rs7681440) was not significantly associated with PD. Equally, the SNP showing the highest association with PD for the *SNCA* locus (rs356182) was not significantly associated with DLB. Thus, it may be hypothesized that these differences lead to different gene expression profiles and different molecular mechanisms between DLB and PD.

Besides *APOE*, *GBA* and *SNCA*, other genetic loci associated with AD or PD, have not been identified by GWA studies in DLB.[12,13] Furthermore, a recent study has shown that known AD and PD genetic risk loci only explain a very small percentage (~1.3% and ~0.4%, respectively) of the phenotypic variance in DLB[4].

To summarize, some genetic factors are shared between DLB and AD or PD, which may lead to similar molecular mechanisms and possible clinical features; however, the majority of genetic factors associated with DLB might be independent of those associated with AD and PD and still have to be discovered.

Novel genes associated with DLB

LRP10

Rare variants in the *low-density lipoprotein receptor related protein 10* gene (*LRP10)* were reported for the first time in familial forms of PD, PDD, and DLB by Quadri et al., in 2018.[32] In this study, genome-wide linkage analysis in a family with dominantly inherited PD (10 affected members) was performed to map the disease locus to a region in chromosome 14. Subsequently, WES identified a single nucleotide substitution in *LRP10,* leading to a missense change (Gly603Arg) as the most likely disease-causing variant. This was followed by the sequencing of the whole *LRP10* coding region in 660 unrelated PD, PDD, and DLB patients and 645 controls, and by the sequencing of specific possibly pathogenic *LRP10* variants in additional series of 1448 PD patients and 811 controls. Eight patients and one control from the first series and three patients and no controls from the second series were identified with a possibly pathogenic *LRP10* variant. Furthermore, the variant present in the

index cases was also present in 9 out of 10 additional affected relatives with DNA available for testing. Three patients carrying one of the possibly pathogenic *LRP10* variants could be studied post-mortem and they all had a severe burden of Lewy pathology. Functional studies in cell cultures showed that the *LRP10* variants affected mRNA stability, protein stability or protein localization.

Several studies have tried to replicate these initial findings. Five studies failed to find evidence for an association between *LRP10* variants and PD, PDD, DLB, progressive supranuclear palsy (PSP), multiple system atrophy (MSA), or frontotemporal dementia (FTD).[33-37] However, the absence of evidence in these studies does not allow to conclude for the absence of association. There are several reasons why these replication studies might have failed to detect a role of *LRP10* in these diseases.

First, and most importantly, case-control association studies are not a powerful strategy to detect or replicate the role of rare, highly-penetrant variants in the disease etiology, unless only large series of patients with familial forms of the disease are considered. In some of the above-mentioned studies, the power to detect rare variants was not optimal because of limited sample sizes. In the original paper, the research sample was comprised of predominantly familial patients with late-onset disease. Three of the replication studies included sporadic patients[33-35], and one analyzed patients with young-onset disease.[33] Second, possibly pathogenic *LRP10* variants may be population specific. The ethnicity of the patients in two of the replication studies were substantially different than the original study.[33,37] Third, the control groups may have introduced bias, as they also included relatively young, unaffected participants who could still develop a late-onset neurodegenerative disease like PD or DLB.[33,34] Furthermore, some control groups also contained participants who had not been clinically examined.[34] Fourth, almost all replication studies adopted more relaxed filtering criteria for possibly pathogenic variants than those used in the original paper. This may have biased the results, as truly pathogenic and benign variants might have been grouped together. It is also possible that only some, specific variants are truly pathogenic and that these variants are more frequently found in patients than in controls. These variants will not be recognized as such with the used analysis methods.

We (**Chapter 4.1, Chapter 4.2** and **Chapter 4.3**) and three other research groups[36,38,39] found further evidence for an involvement of *LRP10* in PD, dementia with parkinsonism without Lewy pathology, dementia with Lewy pathology, and PSP. Of particular interest is one of the variants, p.Tyr307Asn, as this variant was identified in the original study in a familial PDD patient from Italy, and subsequently in a mother and daughter with PD from France, and then in a PD and PDD patient with a shared ancestor from the Netherlands (**Chapter 4.1**).[36,40,41] Considering all these studies together, the frequency of this specific variant seems significantly higher in familial PD and PDD patients in comparison to large control groups such as the Genome Aggregation Database (total 15 alleles in 251,270).[41] The p.Tyr307Asn variant has also been shown in functional studies to decrease LRP10 protein stability.[40] However, it should be noted that the p.Tyr307Asn variant was not found in another

two relatives affected with PD in the same French family mentioned earlier, and they may represent phenocopies. In our study of patients with dementia and parkinsonism without Lewy pathology, and patients with dementia and Lewy pathology, we identified three rare and possibly pathogenic *LRP10* variants (**Chapter 4.2**). Interestingly, in previous studies, all these three variants were identified in additional unrelated patients with PDD, PD, MSA and in a single unaffected person (not neurologically examined)[33,35,40,41]. Interestingly, no Lewy pathology was observed in one of our patients with AD and parkinsonism carrying a possibly pathogenic *LRP10* variant. Our PSP study (**Chapter 4.3**) showed that an increased fraction of these patients carries a possibly pathogenic *LRP10* variant compared to controls. These results suggest that *LRP10* variants may be associated with several neurodegenerative diseases with different underlying neuropathologies.

In conclusion, our studies provide additional evidence for a role of *LRP10* in PD, DLB, PSP and possibly AD. Nonetheless, there is still no firm consensus about the role of *LRP10* in these neurodegenerative diseases, and additional robust and independent replication is warranted, preferably from studies in large families with co-segregation of the variant in multiple affected members.

A multimodal approach to find novel genes

In a parallel effort, we tried to find novel genes for DLB by combining WES data and CSF proteomics data. We described the results of the CSF proteomics analysis in **Chapter 5.1**, and the combination of the WES data and CSF proteomics data in the **Appendix to Chapter 5.1**. Besides the use of CSF proteomics to find novel genes for DLB, this analysis is also very interesting to find novel fluid biomarkers for the disease. In **Chapter 5.1** we identified and validated six novel candidate CSF biomarkers (VGF, SCG2, NPTX2, PDYN and PCSK1N) for DLB. These proteins are, amongst others, involved in neurotransmitter release, the packaging of neuropeptides in secretory vesicles and the formation of new excitatory synapses[42-44], and may be markers of synaptic dysfunction. Synaptic dysfunction is observed in many neurodegenerative diseases, which led to the question of whether the identified biomarkers are specific for DLB. We analyzed these markers, therefore, in cohorts of AD, PD and FTD patients, which showed that the CSF levels of PDYN, SCG2 and VGF are significantly lower in DLB than in the other groups. To improve the discrimination of DLB and the other neurodegenerative diseases, machine learning was used to identify the most optimal biomarker panel. This resulted in a panel of PDYN, SCG2 and VGF with an accuracy of 0.82, specificity of 0.83 and sensitivity of 0.69. Further studies in larger and independent cohorts are needed to investigate the possible clinical usefulness of these markers.

In the pilot study described in the **Appendix to Chapter 5.1** we combined these CSF proteomics data and WES data to identify novel candidate genes associated with DLB. The hypothesis behind this approach is that a pathogenic genetic variant will lead to a differential expression of the corresponding protein in the CSF. Our pilot study focused on DLB patients with relatives with dementia or PD to increase the likelihood of detecting disease-associated

findings. Although we identified an overlap between eight genes and proteins, the evidence of an association with DLB is limited. We discuss the limitations of this study and suggestions for improving such study designs in the future in the 'Strenghts and limitations' and 'Future study directions' sections.

Molecular mechanisms underlying DLB

The identification of genes associated with DLB might contribute to a better understanding of the underlying molecular disease mechanisms. Multiple molecular pathways are likely implicated in the development of neurodegeneration due to primary defects in *APOE*, *GBA*, *SNCA,* and *LRP10,* but many of these molecular mechanisms are not fully elucidated. Here, I describe the key molecular mechanisms in which these genes have been implicated up until now.

APOE encodes the protein Apolipoprotein E, which plays a role in lipid transport, synaptic integrity, neuroplasticity, neuroinflammation, tau phosphorylation, glucose metabolism, and cerebrovascular function.[45] APOE has been shown to affect amyloid-β clearance and aggregation leading to AD pathology. Several studies have indicated an APOE isoform-dependent effect, in which the *APOE* ε4 isoform is less efficient in amyloid-β trafficking and degradation than the *APOE* ε3 isoform.[46,47] It has also been demonstrated that the *APOE* ε4 isoform promotes the formation of amyloid-β fibrils and amyloid-β production.[45] At autopsy, the majority of patients with DLB have both Lewy as well as AD pathology.[48,49] Interestingly, one study has indicated that the *APOE* ε4 allele is not only associated with DLB with AD pathology, but also with DLB without AD pathology.[10] This suggests that the *APOE* ε4 allele also plays a role in amyloid-β independent mechanisms leading to DLB.

GBA encodes the protein Glucocerebrosidase (GCase), a lysosomal enzyme. GCase dysfunction has been shown to be related to an impaired autophagy-lysosomal system, increased endoplasmic stress, mitochondrial dysfunction and α-synuclein aggregation.[50-52] GCase hydrolyses glucosylceramide (GlcCer) and glucosylsphingosine (GLSph). Mutations in *GBA* result in a reduction of the enzymatic function of GCase, which leads to the accumulation of GlcCer and GLSph in lysosomes. This could lead to autophagy-lysosomal and mitochondrial dysfunction, and the accumulation of α-synuclein. Conversely, α-synuclein accumulation may result in the retention of GCase in the endoplasmic reticulum, leading to impaired GCase trafficking to the lysosome and to endoplasmic reticulum stress.[53] This indicates that GCase and α-synuclein might be part of a bidirectional pathogenic loop.[54]

SNCA encodes the protein α-synuclein, one of the major constituents of Lewy bodies and neurites.[55] α-Synuclein has been implicated in synaptic plasticity, neurotransmitter release, and synaptic vesicle pool maintenance.[56-58] Disease-causing mutations in *SNCA* seem to increase the susceptibility of the protein to aggregate.[59]

LRP10 encodes a plasma membrane receptor (LRP10) whose function remains largely elusive. It has been suggested that LRP10 plays a role in the metabolism of

APOE lipoproteins[60] and APP trafficking and processing, which may influence amyloid-β homeostasis.[61] Interestingly, a decreased expression of LRP10 in the brains of AD patients compared to controls has been reported, even if caution is warranted here, as the specificity of the antibody used to detect the LRP10 protein in the human brain tissue was not adequately demonstrated.[61] LRP10 may also interact with VPS35, a component of the retromer complex and the product of another PD-causing gene, and with GGA proteins, which have been shown to promote α-synuclein aggregation.[62-64] Further research is warranted to study if LRP10 expression is also modified in the brains of PD or DLB patients, and to elucidate the interactions between LRP10, APOE, APP, and α-synuclein aggregation.

In conclusion, the current evidence suggest that *APOE*, *GBA*, *SNCA* and *LRP10* may be involved in similar or interacting neurobiological pathways driving Lewy and AD pathology. Further studies are needed to gain more information about the different molecular mechanisms leading to DLB. This is important for future development of biomarkers and disease-modifying treatments.

Future applications

Biomarkers

The identification of genes associated with DLB could lead to the implementation of these genes or their derivatives (e.g. encoded or interacting proteins in CSF or blood) as biomarkers in clinical practice. Biomarkers can be useful in 1) the diagnostic process, 2) the prognostic process, 3) the identification of at-risk persons, and 4) personalized medicine.

Diagnostic biomarkers which improve the accuracy of the DLB diagnosis are highly needed, as the accuracy of current clinical criteria for DLB is still relatively low.[6] GCase and α-synuclein in blood or CSF are interesting candidate biomarkers, which could possibly be used for this purpose in the future. Several studies have indicated that CSF levels of GCase are not only decreased in PD patients with disease-associated *GBA* variants, but also in PD patients without these genetic variants.[65,66] GCase has also been found at decreased levels in the blood of sporadic PD patients as compared to controls. Furthermore, GCase correlated with α-synuclein levels in the brain of PD patients, possibly due to the effects of α-synuclein on GCase.[54] GCase CSF levels are also decreased in DLB patients with disease-associated *GBA* variants compared to controls.[67] However, it is currently unknown whether GCase CSF levels are also downregulated in DLB patients without disease-associated genetic variants, and in the blood of DLB patients. In addition, CSF levels of total α-synuclein have been found to be decreased in DLB patients compared to AD patients and controls, but not in comparison to PD patients.[68,69] Conversely, CSF levels of oligomeric α-synuclein are increased in DLB patients compared to AD patients and controls, but not compared to PD patients.[13] These findings suggest that levels of GCase, total α-synuclein, and oligomeric α-synuclein may be useful in differentiating DLB patients from AD patients and controls.

Prognostic biomarkers based on genetic factors are not yet used in clinical practice. The

main reason for this is that the disease course of carriers of a specific genetic variant is very variable. Indeed, even in families with *SNCA* mutations the disease course is largely unpredictable.[23,26] However, several differences between genetic variant carriers and non-carriers have been found at group level: DLB patients carrying the *APOE* ε4 allele have a shorter survival than non-carriers[16-18], and DLB patients carrying disease-associated *GBA* variants have an earlier onset and an earlier death than non-carriers.[11,14,21,22] Our study described in **Chapter 2.1** also showed a different disease course between familial and sporadic DLB patients. This provides further evidence that genetic factors can be useful to estimate the prognosis in different patient groups. Further studies are needed to clarify whether genetic factors can also be used to generate individual predictions of disease course.

In DLB, the prediction of disease development and the selection of at-risk persons for medical trials by using biomarkers, is not yet a reality. One exception to this is in the testing of causal mutations in *SNCA* in families with multiple patients with DLB, PDD, and PD. The results of this test could identify individuals who have a high risk of developing DLB, PDD or PD. *LRP10* genetic testing in clinical practice awaits conclusive replication of the role of variants in this gene in independent studies. Furthermore, current evidence points to a reduced penetrance for at least some *LRP10* variants, which limits the predictive value for an individual person. Genetic testing for risk factors of DLB, such as the *APOE* ε4 allele and disease-associated *GBA* variants, is also not currently recommended for clinical purposes. Results of such testing explain only a very small fraction of the total prediction, which is not informative by itself. However, polygenic risk scores, in which the combined risk of multiple genetic factors is considered, may ultimately be useful to predict the risk of disease development. The general belief is that the pathological process that leads to neurodegenerative diseases can only be reversed or prevented when medication is taken in the early pre-symptomatic phase of the disease. The identification of at-risk persons is, therefore, of utmost importance to detect alterations in molecular mechanisms and apply novel treatment options, before the onset of clinical symptoms. Furthermore, genetic status may be crucially important if treatment options are only found to be effective in the carriers of specific genetic variants.

Treatment

The ultimate goal of studying genes associated with DLB is to increase our understanding of the disease pathogenesis, which could eventually aid in the development of disease-modifying treatments. Treatment options targeting genes or related molecular mechanisms associated with DLB may not only be beneficial to patients with disease-associated genetic variants, but to all DLB patients. Unfortunately, no clinical trials have yet been performed or are currently underway in DLB specifically, targeting well-established genes associated with DLB or related molecular mechanisms. However, related clinical trials are presently being performed on patients with AD, patients with mild cognitive impairment (MCI), PD and PDD concerning *APOE*, *GBA,* and *SNCA*.

Recently, two phase 1 clinical trials have commenced targeting the *APOE* gene in patients with AD and MCI due to AD pathology. The target of one of these trials is to assess the safety and toxicity of the intracisternal administration of adeno-associated viral vectors (AAVs) expressing the cDNA coding *APOE* ε2 in patients with two *APOE* ε4 alleles (ClinicalTrials.gov identifier NCT03634007). In the other trial, the safety of plasma infusion from young adults with two *APOE* ε3 alleles to MCI patients with two *APOE* ε4 alleles will be determined (ClinicalTrials.gov identifier NCT03887741). The rationale behind these studies is that the detrimental effects of carrying two *APOE* ε4 alleles will be diminished.[70]

At present, several clinical trials are being performed in PD and PDD, which target *GBA* and associated molecular mechanisms. One such phase 2 trial in PD patients with disease-associated *GBA* variants assesses the drug dynamics, efficacy, and safety of Venglustat, a GlcCer synthase inhibitor (ClinicalTrials.gov identifier NCT02906020).[71] Two other phase 2 clinical trials, one in PD and one in PDD patients with disease-associated *GBA* variants, test the safety, tolerability, pharmacodynamics, and clinical effects of Ambroxol (ClinicalTrials.gov identifier NCT02941822 and NCT02914366). Ambroxol is a small molecular chaperone, which aids physiological posttranslational folding of mutant GCase, resulting in the upregulation of GCase trafficking to the lysosome.[72] Another potential disease-modifying treatment option for disease-associated *GBA* carriers is gene therapy, in which AAVs coding for *GBA* increases GCase activity. A phase 1/2a trial has recently begun to evaluate the safety of intracisternal administration of AAVs in PD patients with at least one *GBA* disease-associated variant (ClinicalTrials.gov identifier NCT04127578). The results of these trials are still pending.

For *SNCA*, different compounds have been examined with the aim to decrease α-synuclein aggregation, increase α-synuclein degradation, or decrease extracellular α-synuclein. For instance, one phase 2 trial concerned the small molecule, Nilotinib, an inhibitor of c-Abl tyrosine kinase, which is involved in many essential cellular processes, such as cell growth, neurogenesis and neurite extension.[73] The non-blinded, non-placebo-controlled safety trial with the aforementioned drug showed some improvement in motor function of DLB patients and PDD patients.[74] Different trials utilizing both passive and active immunotherapies to reduce extracellular α-synuclein are currently ongoing. Other promising therapies involving *SNCA* involve the reduction of the production of α-synuclein by gene silencing mechanisms using antisense oligonucleotide therapy and altering histone acetylation of the promotor and enhancer regions of *SNCA*.[71]

Positive results originating from these trials will stimulate the development and testing of treatment options in DLB. Personalized medicine, in which a combination of different drugs targets different genes and molecular mechanisms based on the genetic profile of the patient, is a very promising approach in the future.

Strengths and limitations of the studies described in this thesis

The first strength of the described studies is the relatively large sample size of the studies in **Part 2** and **Part 4**. We collaborated with several research groups to obtain such a large quantity of patients. It should be noted, nonetheless, that the sample sizes of the patient groups in **Part 3** and **Part 5** were relatively small. However, these study groups were carefully selected subgroups of patients, which had never been studied in such a comprehensive or novel manner before. As the accuracy of the clinical diagnosis of DLB remains suboptimal[6], we did not only study clinical diagnosed DLB patients, but also pathologically confirmed DLB patients. We have found similar results in clinical as well as in pathologically confirmed patients (**Part 3**), which underpins the reliability of our findings. Other strengths include the use of Sanger sequencing for the initial analyses or validation of genetic variants in **Part 3** and **Part 4**, and the replication of our findings in independent cohorts in **Chapter 4.3** and **Chapter 5.1**.

The marked limitation of the described studies is that bias may have been introduced by relying on retrospective clinical data. Only a portion of the data on family history was obtained by means of a structured patient/caregiver interview, and patient/caregivers were often not able to provide accurate information on which diagnoses were made in their relatives. A bias in family history may also be introduced by relatives who died before symptom onset, and by asymptomatic relatives at time of inclusion who developed the disease later in life (**Part 2** and **Part 3**). Another limitation is that co-segregation studies could only be performed in a small percentage of patients with relatives with neurodegenerative diseases (**Part 3, Part 4,** and **Appendix to Chapter 5.1)**. The main reason for this is that often no DNA was available from deceased relatives and that younger relatives were not (yet) affected by disease. Limitations of the CSF proteomics study (**Part 5**) are, amongst others, that proteins could have been missed due to a bias towards the identification of peptides with higher concentrations in mass spectrometry studies, and that the control group consisted of individuals with subjective cognitive complaints. Furthermore, a bias could have been introduced by comparing the levels of the candidate biomarkers between DLB patients and the patients with other neurodegenerative diseases for which different control groups and techniques were used. The limitations of the pilot study (**Appendix to Chapter 5.1**) are, amongst others, that it is possible that CSF protein levels may not be altered at all by pathogenic genetic defects, and that we did not use an adjusted reference protein database, which may have led to the missing of altered peptides.

Future study directions

Although this thesis has contributed to the knowledge of the genetics of DLB, many genetic factors associated with the disease remain unknown. In general, future genetic studies will benefit from a more accurate clinical diagnosis, a better documentation of family history,

larger samples sizes and the use of novel research strategies and techniques.

In clinical practice, DLB remains markedly under-recognized. This could be due to unacquaintance of family doctors and specialists with the concept and the clinical criteria of DLB, resulting in a failure to ask and examine the patients for all the possible symptoms and signs of DLB.[1,75] Additionally, the accuracy of clinical diagnostic criteria for DLB is still relatively low and DLB is often misdiagnosed as AD.[6,76] Especially the sensitivity of the clinical criteria of DLB should increase to improve an early diagnosis. Enhancing these aspects of disease identification will ultimately lead to larger and more homogeneous study groups. Furthermore, it is important to estimate how often DLB patients have relatives with DLB or related disorders. This has yet to be understood and could alter our perception of the genetics of DLB, leading to more refined and powered genetic research into rare variants associated with DLB. Therefore, taking a careful and structured family history in DLB is essential and should be promoted among health care providers.

To find novel, common variants associated with DLB, samples sizes of GWA studies have to increase drastically. To date, the largest GWA study performed in DLB included 1743 patients and 4454 controls[12], which are relatively small numbers for a GWA study. The statistical power to find novel risk factors with low effect sizes is limited with these relatively low sample sizes. Larger GWA studies are necessary to find new genetic factors with a small to moderate effect size. In AD and PD, GWA studies reach sample sizes of around 15.000 patients and >40.000 controls. Such large cohorts are necessary to identify risk factors with effect sizes as low as 1.1 - 1.4.[30,77] GWA studies with more patients are, therefore, essential to find more risk factors with small effect sizes associated with DLB. National and international collaborations are essential in these efforts.

To find novel, rare variants associated with DLB different study approaches can be pursued. For instance, WES or whole genome sequencing (WGS) could be performed in large cohorts or in subgroups of patients in which the chances of finding rare variants is higher than in the total DLB population, such as families with multiple patients with DLB, PD(D), or dementia. Another possibility is to use a multimodal approach, such as the combination of genetics and proteomics, as shown in our pilot study (**Appendix to Chapter 5.1**). Future research combining genetics and proteomics with the aim to identify new genes associated with disease are promising if: 1) a strong family history is present, 2) DNA of multiple relatives is available, 3) a small set of possibly pathogenic genetic variants is obtained after genetic analysis, 4) tissue of interest is available in preferably >1 relative, and 5) the protein reference database is adjusted to recognize mutated peptides. The preferable tissue of interest when combining genetic and proteomic data in DLB might be neurons or brain inclusions. However, it should be considered that these data represent proteomic changes in late stage disease, which may not reflect protein differences due to a primary genetic defect. Other omics data, such as epigenomic, transcriptomics and metabolomic data, could also be used in multimodal approaches.

Novel common and rare variants identified by future GWA, WES and WGS studies will contribute to a higher percentage of explained heritability for DLB. Furthermore, a part of the

heritability will probably be explained by copy number variation (CNV) and epigenetics, such as enhancers and other regulatory elements, gene-gene interactions and gene-environment interactions. Unbiased studies into these domains are still in its infancy concerning DLB.

Conclusion

Despite the appreciation that DLB has a substantial heritable component, only few genetic studies have been performed. This thesis adds to the understanding of the genetics of DLB. First, it showed that DLB patients with relatives with dementia or PD have a different phenotype than DLB patients without a positive family history. Second, we confirmed that the *APOE* ε4 allele and disease-associated variants in *GBA* are important genetic factors associated with familial DLB and DLB with a rapidly progressive disease course. Third, we found more evidence that the recently identified *LRP10* gene is associated with PD, DLB, PSP and possibly AD. Fourth, we found several new candidate CSF biomarkers associated with DLB, which could potentially increase the accuracy of the DLB diagnosis.

Nonetheless, known genetic factors associated with DLB still explain only a small part of the total heritability of DLB. The majority of genetic factors associated with DLB still remain to be discovered. Large, homogeneous cohorts of DLB patients and very well-defined families with DLB and related disorders are needed to find new genetic variants associated with DLB. These genetic factors, in combination with environmental, epigenetic, and stochastic factors, will likely all play important roles to determine the resulting individual risk of developing DLB.[78]

The coming years promise to be exciting as many new genetic factors are likely to be discovered. These findings may lead to more accurate diagnosis, a better prediction of prognosis, and, ultimately the development of disease-modifying treatments.

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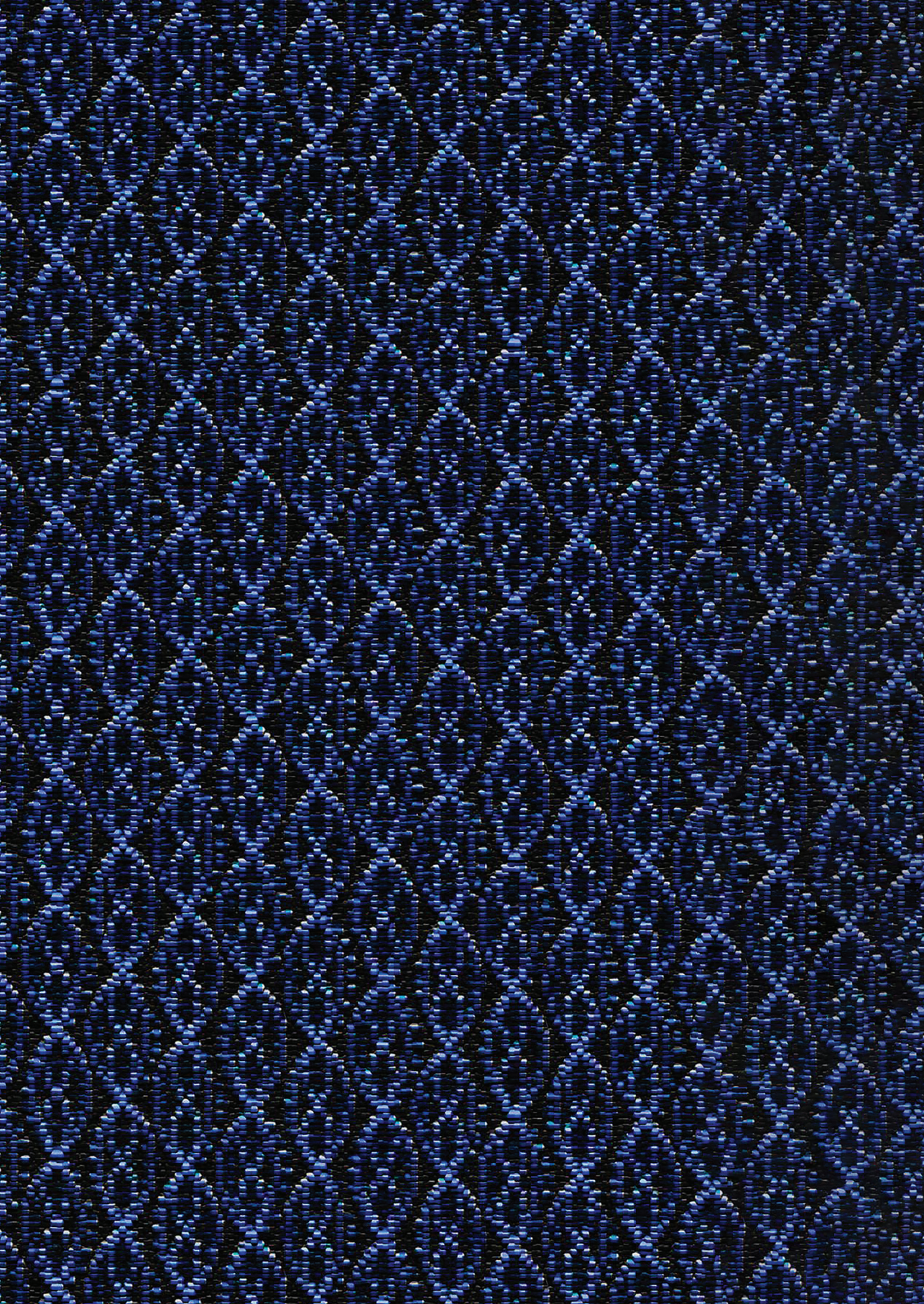

Part 7

Summary

Dementia with Lewy bodies (DLB) is a common neurodegenerative disease in the elderly. The disease is characterized by progressive cognitive decline accompanied by parkinsonism, hallucinations, fluctuating cognition, and REM-sleep behavior disorders. To date, no disease-modifying treatment options are available and the median survival of patients with DLB is approximately four years from diagnosis. The pathological hallmarks of DLB are cortical Lewy bodies and Lewy neurites. However, Alzheimer's disease (AD) pathology is also observed in the majority of patients. In addition to these pathological features, clinical symptoms and genetic factors also overlap with those of AD and Parkinson's disease (PD).

It is likely that genetic factors play a considerable role in DLB. Recently, the heritable component has been estimated to be approximately 60%. However, to date, only few genetic studies have been performed in DLB. The aim of this thesis was to shed more light on the genetics of DLB by studying 1) familial aggregation in DLB, 2) known AD and PD genes associated with DLB, and 3) novel genes associated with DLB.

Part 1 provides a general introduction to the thesis and summarizes the genetic research on DLB up until the beginning of this work in 2016. Up until that time, genetic studies were only performed in small study groups with candidate gene-based (biased) approaches. Unbiased genome wide association studies and whole exome sequencing (WES) studies had not yet been performed by then. The candidate gene studies showed that the *APOE* ε4 allele and specific variants in *GBA* and *SNCA* are associated with DLB. These genetic factors had previously been shown to play a role in AD (*APOE* ε4 allele) and PD (*GBA* and *SNCA*). Besides the fact that little was known about which genetic factors play a role in DLB, it was also largely unknown what effect these genetic factors have on the phenotype of DLB patients.

In **Part 2** we, therefore, examined the differences in phenotype between DLB patients with relatives with dementia or PD (familial patients) and DLB patients without relatives with these diseases (sporadic patients). We showed that familial DLB patients have a shorter survival than sporadic DLB patients. Furthermore, we demonstrated that a higher percentage of familial DLB patients have elevated AD biomarkers in their cerebrospinal fluid (CSF) compared to sporadic DLB patients. These findings suggest that genetic factors contribute to disease course, possibly by influencing the amount of concomitant AD pathology.

Owing to the genetic overlap between DLB, PD and AD, we investigated more profoundly if known genes associated with PD and AD also play a role in DLB. **Part 3** describes these genetic analyses in carefully selected study groups, namely familial DLB patients (**Chapter 3.1**) and pathologically confirmed DLB patients with a rapidly progressive disease course (**Chapter 3.2**). We showed that the *APOE* ε4 allele and specific variants in *GBA* are also important genetic risk factors in these patient groups.

The search for novel genes associated with DLB is described in **Part 4** and **Part 5**.

In **Part 4** we studied the association between the *LRP10* gene and DLB, and other related disorders. The *LRP10* gene was recently nominated as a novel gene associated with PD, PD dementia, and DLB. The function of LRP10 is largely elusive. However, the protein has been suggested to play a role in the aggregation of amyloid-β and α-synuclein. **Chapter 4.1** illustrates that rare, possibly pathogenic variants in *LRP10* are detected in clinically diagnosed PD and PD dementia patients from the South West of the Netherlands. In **Chapter 4.2** and **Chapter 4.3** we described that rare, possibly pathogenic *LRP10* variants may also play a role in patients with dementia and Lewy pathology, dementia patients with parkinsonism without Lewy pathology, and patients with progressive supranuclear palsy. In **Part 5**, we searched for novel genes by combining WES and CSF proteomics. **Chapter 5.1** outlines the identification of six novel CSF candidate biomarkers for DLB: VGF, SCG2, NPTX2, NPTXR, PDYN, and PCSK1N. These proteins play a role in, amongst others, synaptic dysfunction, and show promise to improve the diagnostic accuracy of DLB. The data from **Chapter 5.1** and the WES data were combined in the pilot study described in the **Appendix to Chapter 5.1.** The hypothesis behind this approach was that a pathogenic genetic variant will lead to a differential expression of the corresponding protein in the CSF. Although we identified overlap between eight genes and proteins, the evidence that these genes or proteins are associated with DLB remains very limited at this stage.

Part 6 provides a general discussion of the findings of this thesis in the context of the current literature and provides suggestions for future study directions.

Samenvatting

Dementie met Lewy bodies (DLB) is een vaak voorkomende neurodegeneratieve ziekte bij ouderen. De ziekte wordt gekenmerkt door progressieve cognitieve achteruitgang met hierbij parkinsonisme, een fluctuerend bewustzijn en REM-slaap gedragstoornissen. Er is geen medicatie die aangrijpt op het ziekteproces en de mediane overleving van patiënten met DLB is ongeveer 4 jaar vanaf de diagnose. De pathologische kenmerken van DLB zijn corticale Lewy bodies en Lewy neurieten. De meeste patiënten hebben daarnaast ook Alzheimer pathologie. Niet alleen pathologische kenmerken, maar ook klinische en genetische kenmerken overlappen met kenmerken die voorkomen bij de ziekte van Alzheimer (ZvA) en de ziekte van Parkinson (ZvP).

Genetische factoren lijken een belangrijke rol bij DLB te spelen. Recent is de erfelijke component zelfs geschat op ongeveer 60%. Er is echter nog maar weinig genetisch onderzoek bij DLB verricht. Het doel van dit proefschrift was om meer inzicht te krijgen in de genetica van DLB door het bestuderen van 1) het familiair voorkomen van DLB, 2) bekende ZvA en ZvP genen die geassocieerd zijn met DLB, en 3) nieuwe genen die geassocieerd zijn met DLB.

Deel 1 bevat een algemene introductie van het proefschrift en vat het genetisch onderzoek bij DLB samen tot de start van dit promotieonderzoek in 2016. Tot die tijd waren er alleen onderzoeken uitgevoerd in kleine studiegroepen waarbij gezocht werd naar varianten in kandidaatgenen. Genome wide association studies en whole exome sequencing (WES) studies waren tot dan toe nog niet uitgevoerd. De onderzoeken naar varianten in kandidaatgenen hebben aangetoond dat het *APOE* ε4 allel en specifieke varianten in *GBA* en *SNCA* geassocieerd zijn met DLB. Deze variaties zijn tevens genetische risicofactoren voor de ZvA *(APOE* ε4 allel) en de ZvP (*GBA* en *SNCA*). Naast dat er nog maar weinig bekend was over welke genetische factoren een rol spelen bij DLB, was het ook grotendeels onbekend welk effect deze genetische factoren hebben op de klinische kenmerken (fenotype) van patiënten met DLB.

In **Deel 2** hebben wij daarom de verschillen in fenotype tussen DLB patiënten met familieleden met dementie of de ZvP (familiaire patiënten) en DLB patiënten zonder familieleden met deze ziektes (sporadische patiënten) onderzocht. We hebben laten zien dat familiaire patiënten een kortere overleving hebben dan sporadische patiënten. Hiernaast hebben we laten zien dat relatief meer familiaire patiënten dan sporadische patiënten Alzheimer eiwitten in het hersenvocht hebben. Deze resultaten suggereren dat genetische factoren invloed hebben op het ziektebeloop, mogelijk door het beïnvloeden van Alzheimer pathologie.

Aangezien er een genetische overlap is tussen DLB, de ZvA en de ZvP, hebben wij verder onderzocht welke bekende genen die geassocieerd zijn met de ZvA en de ZvP ook een rol spelen bij DLB. **Deel 3** beschrijft deze genetische analyses bij zorgvuldig geselecteerde

groepen, namelijk bij familiaire DLB patiënten (**Hoofdstuk 3.1**) en pathologisch bevestigde DLB patiënten met een snel progressief ziektebeloop (**Hoofdstuk 3.2**). We hebben aangetoond dat het *APOE* ε4 allel en dat specifieke varianten in *GBA* ook belangrijke risicofactoren zijn in deze patiëntengroepen.

In **Deel 4** en **Deel 5** wordt onderzoek naar nieuwe genen voor DLB beschreven. In **Deel 4** onderzochten we de associatie tussen het *LRP10* gen en DLB, en andere gerelateerde ziektes. Recent is ontdekt dat het *LRP10* gen waarschijnlijk geassocieerd is met de ZvP, dementie bij de ZvP en DLB. De functie van LRP10 is grotendeels onbekend, maar het eiwit speelt mogelijk een rol bij de aggregatie van amyloïd-β en α-synucleïne. In **Hoofdstuk 4.1** wordt beschreven dat zeldzame, mogelijk pathogene varianten in *LRP10* voorkomen in klinisch gediagnosticeerde patiënten met de ZvP en dementie bij de ZvP uit het zuidwesten van Nederland. In **Hoofdstuk 4.2** en **Hoofdstuk 4.3** wordt beschreven dat zeldzame, mogelijk pathogene *LRP10* varianten mogelijk ook een rol spelen bij patiënten met dementie en Lewy pathologie, dementie met parkinsonisme zonder Lewy pathologie en progressieve supranucleaire verlamming. In **Deel 5** zijn we op zoek gegaan naar nieuwe genen door WES en eiwitonderzoek ('proteomics') van hersenvocht te combineren. **Hoofdstuk 5.1** zet de identificatie van zes nieuwe kandidaat biomarkers (VGF, SCG2, NPTX2, NPTXR, PDYN en PCSK1N) in het hersenvocht voor DLB uiteen. Deze eiwitten spelen onder andere een rol bij synaptische dysfunctie en kunnen mogelijk de correctheid van de diagnose DLB verbeteren. De data uit **Hoofdstuk 5.1** en de WES data werden gecombineerd in een pilot studie, die beschreven is in de **Appendix bij Hoofdstuk 5.1**. De hypothese bij dit onderzoek was dat een pathogene genetische variant zal leiden tot een differentiële expressie van corresponderend eiwit in het hersenvocht. Hoewel we een overlap hebben gevonden tussen acht genen en eiwitten, blijft het bewijs dat deze genen of eiwitten geassocieerd zijn met DLB tot op heden beperkt.

Deel 6 bevat de algemene discussie van de bevindingen uit dit proefschrift in het kader van de huidige literatuur en bevat suggesties voor toekomstig onderzoek.

Curriculum vitae

Leonie Vergouw was born on the 6th of November 1985 in Nieuwegein, the Netherlands. She went to the Gymnasium at the Oosterlicht College in Nieuwegein (secondary school). After secondary school, she obtained a Bachelor's degree in Chemical Engineering and Bioprocess Technology at the Technical University of Delft (2007), and a Bachelor's degree in Psychology at the University of Leiden and the University of Utrecht (2010). She also studied Medicine at the University of Utrecht and the Erasmus Medical Center in Rotterdam (2008-2014). She traveled a lot and did internships in Australia and Kenia. After obtaining her Master's degree in Medicine, she worked as medical doctor in Neurology (2014-2015) at the Sint Franciscus Gasthuis in Rotterdam. She started her PhD on the genetics of dementia with Lewy bodies in 2015, under supervision of Dr. F.J. de Jong, Prof. dr. J.C. van Swieten and Prof. dr. V. Bonifati. Leonie currently lives in Stellendam with her husband Michael and daughter Liva and started as medical doctor at the Clinical Genetics department in the Erasmus Medical Center in February 2020.

PhD portfolio

1. PhD training	Year	ECTS
General Courses		
Biostatistical Methods I: Basic Principles (NIHES)	2015	5.7
E-BROK	2015 & 2019	1.7
Biomedical English Writing (MolMed)	2016	2
Biomedical Research Techniques (MolMed)	2016	1.5
'R' statistical package (MolMed)	2016	1.8
Scientific Integrity	2017	0.3
Biomedical English Writing and Communication	2017	3
Masterclass English	2019	2
Presentation Skills Workshops	2019	1.5
Specific courses		
An introduction to the analysis of next-generation sequencing data (NIHES)	2015	1.4
Principles of genetic epidemiology (NIHES)	2015	0.7
Genomics in molecular medicine (NIHES)	2015	1.4
NeuroPathology course The 13th International Conference on Alzheimer's & Parkinson's Diseases	2017	0.3
Course European Confederation of Neuropathological Societies Neurodegeneration	2017	1.5
Introduction to Cytoscape	2018	0.5
Conferences and seminars		
International Parkinson and Movement Disorder Society Conference. Alpha-Synuclein: The Gateway to Parkinsonism	2015	1
International Dementia with Lewy Bodies Conference (poster presentation)	2015	1
Patiënt- en matelzorgersdag Lewy body dementie (organisation and oral presentation)	2015-2018	1
20th International Congress of Parkinson's Disease and Movement Disorders	2016	1.5
Dementie Update	2016	0.3
Mix and Match meeting Alzheimer Nederland (1 year: organisation)	2015-2017	0.5
The 13th International Conference on Alzheimer's & Parkinson's Diseases (poster presentation)	2017	1.5
Symposium: (Over)leven met dementie met Lewy bodies (oral presentation)	2017 & 2019	1
Publieksmiddag dementie (oral presentation)	2019	0.5
Other		
Research meeting, weekly	2015-2019	4
Sub Investigator medication trials	2015-2019	20
Creation of website www.lewy.nl	2015-2017	2
2. Teaching		
Lectures		
Tutor classes Medicine students	2015	1.5
Presentation Sint Franciscus Gasthuis	2016	0.3
Supervising Master's theses		
Five students	2015-2019	7.5
Total		**68.9**

List of publications

Vergouw LJM, Quadri M, van Steenoven I, Geut H, Breedveld GJ, Netherlands Brain Bank, van de Berg WDJ, Rozemuller AJM, Lemstra AW, van Swieten JC, Bonifati V, de Jong FJ. Familial Dementia with Lewy bodies: a comprehensive analysis of genes involved in Parkinson's or Alzheimer's Disease. *Submitted*

Vergouw LJM*, Geut H*, Breedveld G, Kuipers DJS, Quadri M, Rozemuller AJM, van Swieten JC, de Jong FJ, van de Berg WDJ, Bonifati V. Clinical and pathological phenotypes of patients with *LRP10* variants. *Under review*

van Steenoven I*, Koel-Simmelink MJA*, **Vergouw LJM**, Piersma SR, Pham TV, Ferri G-L, Cocco C, Noli B, Worley PF, Xiao M-F, Xu D, Oeckl P, Otto M, van der Flier WM, de Jong FJ, Jimenez CR, Lemstra AW, Teunissen CE. Identification of novel cerebrospinal fluid biomarker candidates for dementia with Lewy bodies: a proteomic approach. *Under review*

Vergouw LJM, Melhem S, Kaat LD, Chiu WZ, Breedveld G, Boon AJW, Quadri M, van Swieten JC, Bonifati V, de Jong FJ. *LRP10* variants in progressive supranuclear palsy. *Accepted, Neurobiology of Aging*

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List of abbreviations

AAD	age at death
AAO	age at onset
AAS	age at sampling
AAV	adeno-associated viral vector
ABCA7	*ATP binding cassette subfamily A member 7* gene
AD	Alzheimer's disease / autosomal dominant
AHNAK	*AHNAK nucleoprotein* gene
ALS	amyotrophic lateral sclerosis
APOE	*apolipoprotein E* gene
APP	*amyloid precursor protein* gene
AR	autosomal reccessive
ARTAG	age-related astrogliopathy
$A\beta_{1\text{-}42}$	amyloid-$\beta_{1\text{-}42}$
B	benign
BNE	Brain Net Europe
BuChE	*butyrylcholinesterase* gene
C9orf72	*chromosome 9 open reading frame 72* gene
CA2	cornu ammonis region 2
CAA	cerebral amyloid angiopathy
CADD	Combined Annotation Dependent Depletion
CERAD	Consortium to Establish a Registry for Alzheimer's disease
CHCHD2	*coiled-coil-helix-coiled-coil-helix domain containing 2* gene
CHMP2B	*charged multivesicular body protein 2B* gene
CI	confidence interval
cingulate	posterior cingulate gyrus
CJD	Creutzfeldt-Jakob's disease
CLTC	*clathrin heavy chain* gene
CNV	copy number variation
CSF	cerebrospinal fluid
CV	coefficient of variation
D	damaging / disease causing
DaT-SPECT	^{123}I[FP-CIT] single photon emission computed tomography
DLB	dementia with Lewy bodies
e.g.	exempli gratia (for example)
EEG	electro-encephalogram

EIF4G1	*eukaryotic translation initiation factor 4 gamma 1* gene
ELISA	enzyme-linked immunosorbent assay
ExAC-NFE	Exome Aggregation Consortium - Non Finnish Europeans
EXT2	*exostosin glycosyltransferase 2* gene
FATHMM	Functional Analysis Through Hidden Markov Models
FCGBP	*Fc fragment of IgG binding protein* gene
FDR	false discovery rate
FREM2	*FRAS1 related extracellular matrix protein 2* gene
frontal	frontal gyrus (Brodmann area 10)
FTD	frontotemporal dementia
GATK	Genome Analysis Toolkit
GBA	*glucocerebrosidase* gene
Gcase	glucocerebrosidase
GERP	Genomic Evolutionary Rate Profiling
GGA	golgi associated, gamma adaptin ear containing, ARF binding protein
GIGYF2	*GRB10 interacting GYF protein 2* gene
GlcCer	glucosylceramide
GLSph	glucosylsphingosine
GnomAD	Genome Aggregation Database
GoNL	Genome of the Netherlands
GRCh37	genome reference consortium human 37
GRN	*granulin* gene
GWA	genome wide association
HEG1	*heart development protein with EGF like domains 1* gene
HEX	Healthy Exomes
i.e.	id est (in other words)
IQR	interquartile range
KRT73	*keratin 73* gene
L	low
LB	Lewy body / likely benign
LBD	Lewy body disease
LC	locus coeruleus / liquid chromatography
LFQ	label-free quantification
LN	Lewy neurite
LR	logistic regression
LRP10	*low-density lipoprotein receptor related protein 10* gene
LRRK2	*leucine-rich repeat kinase 2* gene

LRT	Likelihood Ratio Test
M	medium
MAF	minor allele frequency
MAPT	*microtubule-associated protein tau* gene
M-CAP	Mendelian Clinically Applicable Pathogenicity
MCI	mild cognitive impairment
MEC	medical ethics committtee
MLPA	Multiplex Ligation dependent Probe Amplification
MMSE	Mini-Mental State Examination
MR-DWI	magnetic resonance diffusion weighted imaging
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSA	multiple system atrophy
MS/MS	tandem mass spectrometry
MTA	medial temporal lobe atrophy
N	polymorphism / neutral
n.a.	not applicable
NA	not available
ND	not done
NIA-AA	National Instittue on Aging - Alzheimer Association
NPTX2	neuronal pentraxin 2
NPTXR	neuronal pentraxin receptor
P	polymorphism automatic
parietal	inferior parietal lobe
PARK2	*parkin* gene
PCSK1N	ProSAAS
PC	prohormone convertase
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PDYN	proenkephalin-B
PINK1	*PTEN induced putative kinase 1* gene
PolyPhen2 HDIV	Polymorphism Phenotyping version 2 human diversity
PolyPhen2 HVA	Polymorphism Phenotyping version 2 human variation
PP	probably pathogenic
PRKAR1B	*protein kinase cAMP-dependent type I regulatory subunit beta* gene
PRNP	*prion protein* gene
PSEN1	*presenilin 1* gene
PSEN2	*presenilin 2* gene

PSP	progressive supranuclear palsy
PSWC	periodic sharp wave complex
p-tau	tau phosphorylated at threonine 181
RBD	rapid eye movement-sleep behaviour disorder
Ref	reference
REM	rapid eye movement
SCARB2	*scavenger receptor class B member 2* gene
SCG2	secretogranin-2
SD	standard deviation
SE	standard error
SIFT	Sorting Intolerant From Tolerant
SN	substantia nigra
SNCA	*α-synuclein* gene
SNCB	*β-synuclein* gene
SNP	single nucleotide polymorphism
SORL1	*sortilin related receptor 1* gene
SQSTM1	*sequestosome* gene
SRM	selected reaction monitoring
STR	short tandem repeat
SVM	Support Vector Machine
SYNE1	*spectrin repeat containing nuclear envelope protein 1* gene
T	tolerated
temp-occ	temporo-occipital cortex
temporal	medial temporal gyrus
TREM2	*triggering receptor expressed on myeloid cells 2* gene
t-tau	total tau
UN	unknown
var / V	variant
VGF	neurosecretory protein VGF
VPS13C	*vacuolar protein sorting 13 homolog C* gene
VPS35	*vacuolar protein sorting-associated protein 35* gene
WES	whole exome sequencing
WGS	whole genome sequencing
WHO	World Health Organization
wt	wild-type