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Genotype-phenotype association studies and biomarker search in neurologic diseases with special emphasis on the role of sphingolipids and phospholipids

Kumulative Habilitationsschrift

zur Erlangung der Venia Legendi für das Fach Humangenetik

vorgelegt von

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aus

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Apám emlékére

(In memory of my father)

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1. State of the Art

1.1. Genotype-phenotype associations in neurologic diseases — examples

With the arrival of personalized medicine arising in the neurology landscape, genetic testing is playing a greater role in counselling individual patients with neurologic diseases, such as Parkinson´s disease (PD), Charcot-Marie-Tooth disease (CMT), amyotrophic lateral sclerosis (ALS), Alzheimer´s disease (AD), frontotemporal dementia (FTD), or different forms of chorea, such as chorea-acanthocytosis (ChAc).

Studies on the clinical and genetic spectrum are sorely needed, as the demand for a direct-to-consumer testing supports the patients´ interest in determining the genetic contribution to their disease, and we are at the entrance of personalized genomic medicine. There is also increasing evidence that awareness of genotype could guide therapeutic decisions [Garofalo AW, *et al.* 2020; Kelly K, *et al.* 2020]. Furthermore, genetic testing in individuals who are potentially eligible for clinical and therapeutic trials should be considered, as it can improve access and equity. With the advent of whole -exome and whole -genome sequencing (WES and WGS, resp.), it became clear that we have to extend beyond known genes or known disease-associated genetic variants and search for interactions in pathomechanism and associations to the different phenotypes.

In the last couple of years, the applicant of this Habilitation has participated — both as principal investigator (PI), and as collaboration partner — in a number of studies and rare cases on genotype-phenotype associations in a broad spectrum of clinical fields, such as glycogen storage disease [Miltenberger-Miltenyi G, *et al.* 2005], primary pulmonary hypertension [Grunig E, *et al.* 2004; Rindermann M, *et al.* 2003; Janssen B, *et al.* 2002], liver cirrhosis [Machado MV, *et al.* 2014], hypertrophic cardiomyopathy [Brito D, *et al.* 2012], pachydermoperiostosis [Madruga Dias J, *et al.* 2014], and Fabry disease [Azevedo O, *et al.* 2020; Azevedo O, *et al.* 2019; Azevedo O, *et al.* 2020b].

One special interest was the field of neurologic diseases; here, too, the applicant elaborated and participated in works on genotype-phenotype associations, mainly in CMT [Miltenberger-Miltenyi G, *et al.* 2007; Miltenberger-Miltenyi G, *et al.* 2009; Auer-Grumbach M, *et al.* 2008; Rohkamm B, *et al.* 2007; Finsterer J, *et al.* 2006], Rett syndrome [Miltenberger-Miltenyi, *et al.* 2003], dementia [Pires C, *et al.* 2013; Philtjens S, *et al.* 2018; van der Zee J, *et al.* 2013; van der Zee J, *et al.* 2017], ALS [Miltenberger-Miltenyi G, *et al.* 2019; Chester C, *et al.* 2013], PD [Pinho R, *et al.* 2016] and *VPS13A* disease (formerly ChAc, see above) [Niemelä V, *et al.* 2020; Park JS, *et al.* 2022; and in Genereviews: https://www.ncbi.nlm.nih.gov/books/NBK1387/ [Peikert K, *et al.* 2023].

Furthermore, in his latest projects as PI, he investigated the associations of neurologic diseases to lysosomal storage diseases (LSDs), and to associated lipid classes such as sphingolipids and phospholipids [Guedes LC, *et al.* 2017; López de Frutos L, *et al.* 2022; Miltenberger-Miltenyi G, *et al.* 2023]. These translational investigations successfully managed to point out putative pathomechanisms and novel putative biomarkers for neurologic diseases, such as PD and associated LSDs.

One disease group we focused on is the CMT hereditary neuropathy. CMT refers to a group of disorders characterized by a chronic motor and sensory polyneuropathy. They are also known as hereditary motor and sensory neuropathies (HMSN) [Bird TD. 2023]. Until now, over 70 clinical types of CMT were described and more than 80 different genes have been associated with CMT [Bird TD. 2023; Stojkovic T. 2016].

Two works of the applicant on this field are presented in detail below (**3.1.** and **3.2.**, [Miltenberger-Miltenyi G, *et al.* 2007; Miltenberger-Miltenyi G, *et al.* 2009]).

ALS is a rapidly progressive severe neurodegenerative disease, where the adequate and fast clinical and laboratory diagnosis is essential to reduce diagnosis delay. For this, our team elaborated a survey about critical questions for assessing patients with ALS inviting international experts [De Carvalho M, *et al.* 2017]. This work defined a consensual set of clinical data that can be used as a data set for patient registers and for clinical trials.

The most common genetic cause of ALS is the hexanucleotide expansion in the C9orf72 gene (C9orf72exp). C9orf72exp is associated with poor prognosis in ALS; however, the reason for this has not yet been understood [Hardiman O, *et al.* 2017; Knibb JA, *et al.* 2016].

We addressed this issue in one of our projects (**3.3.**, [Miltenberger-Miltenyi G, *et al.* 2019]; see details below) and found that C9orf72exp was associated with faster decline of the respiratory function in ALS patients (as measured by the forced vital capacity [%FVC]), but not with a faster rate of functional decay [Miltenberger-Miltenyi G, *et al.* 2019]. Besides, we found that although FTD was more frequent in the C9orf72exp group of ALS patients, similarly to what was reported elsewhere [Hardiman O, *et al.* 2017; Kiernan MC, *et al.* 2011], the association between C9orf72exp and respiratory progression was independent of the cognitive status of the participants. Patients with FTD had, nonetheless, a faster functional decay, independently of their C9orf72exp status. Our other results on genotype and phenotype in this study were confirmatory, like the associations between shorter survival and both higher age and shorter diagnosis delay [Hardiman O, *et al.* 2017; Knibb JA, *et al.* 2016; Kiernan MC, *et al.* 2011]. Additionally, the positive association that we observed between the respiratory subscore of the ALS respiratory functional rating scale (ALSFRSRrespiratory) and the bulbar and spinal onset forms of the disease indicated that patients with respiratory/axial and generalized onsets have a more compromised respiratory function.

Interestingly, a pathophysiological link between C9orf72exp and the respiratory function has not yet been described. One study found upregulation of the homeobox gene HOXA5, which regulates lung development and function, and of transthyretin in the cerebellum of C9orf72-mutated patients [Finch NA, *et al.* 2017]. This result, together with our findings, suggests that such alterations might have an unequal impact on respiration in ALS.

1.2. Associations between lysosomal storage diseases and neurologic diseases — examples

LSDs are inborn errors of metabolism, leading to accumulation, or storage, of different macromolecules in the late endocytic system [Platt FM. 2014]. LSDs are, in general, monogenic disorders that occur at a collective frequency of 1 in 5,000 live births [Platt FM. 2014]. They are caused by inherited alterations in different genes (localized on both autosomal chromosomes as well as on the X chromosome) that encode lysosomal proteins, most commonly lysosomal enzymes. A subgroup of LSDs involves the lysosomal storage of sphingolipids.

In the recent years, there has been growing evidence of a link between the pathogenesis of neurologic diseases and lysosomal dysfunction. A hallmark is the association observed between PD and Gaucher disease (GD) and —

less known — between PD and FD. Still, a common pathomechanism of PD and GD or PD and FD could not yet be detected.

Bi-allelic pathogenic variants in the *GBA* gene (MIM 606463, Chr1q22), which encodes the lysosomal enzyme glucocerebrosidase (GCase), give rise to the autosomal recessively inherited GD, the most common LSD [Revel-Vilk, S., *et al.* 2020]. The potential molecular link between GD and PD was primarily driven by the report of small series of atypical parkinsonism in patients with GD type 1 [Neudorfer O, *et al.* 1996] and in "non-neuronopathic" GD [Tayebi N, *et al.* 2001; Tayebi N, *et al.* 2003], followed by the observation of increased risk of developing PD in asymptomatic heterozygous carriers of *GBA* gene mutations (GBA-PD), such as the relatives of GD patients [Sidransky E, *et al.* 2012].

Since then, the autophagic-lysosomal system has been reported to play a pivotal role in the aggregation of the PD-associated alpha-synuclein and that loss-of-function of the lysosomal glucocerebrosidase enzyme affects the processing and clearance of alpha-synuclein, further acquainting for the connection between GD and PD [Moors T, *et al.* 2016]. PD patients were reported to not only have lower GCase (especially lower in *GBA* mutation carriers), but also lower cathepsin D and beta–hexosaminidase activity in the cerebrospinal fluid than healthy subjects [Parnetti L, *et al.* 2017].

Today, mutations in *GBA* are recognized as one of the most prevalent genetic factors increasing the risk for sporadic and familial PD and parkinsonism [Cook Shukla L, *et al.* 2023]. The risk for developing PD is higher in GD patients but also in heterozygous *GBA*-mutation carriers than in non-carriers, suggesting common disease pathways for GD and PD. The estimated age-specific risk for PD at 60 and 80 years of age is 4.7% and 9.1% among patients with GD; 1.5% and 7.7% among heterozygotes; and 0.7% and 2.1% among the general population, respectively [Alcalay RN, *et al.* 2014]. However, as GD and PD do not share clinical symptoms, an exact common pathomechanism in these diseases remains mostly unclear and needs further investigations.

Alcalay *et al.* [Alcalay RN, *et al.* 2018], measured both GCase, alpha-GAL A (deficient in FD), acid sphingomyelinase (deficient in Niemann–Pick disease types A and B), acid alpha-glucosidase (deficient in Pompe disease), and galactosylceramidase (deficient in Krabbe disease) in dried blood spots of PD patients and healthy controls. This work showed that PD patients (sporadic and those carriers of the *LRRK2*-G2019S or *GBA* variants) have lower GCase and lower alpha-GAL A enzymatic activity. These results were reinforced by another study where lower levels of alpha-GAL A were found in the leukocytes of sporadic PD patients, when compared to age- and gender-matched healthy controls [Wu G, *et al.* 2008].

Also, in the search for associations between LSDs and PD, Wise *et al.* [Wise AH, *et al.* 2017] performed an online survey to determine the prevalence of PD in FD patients. They identified 4 PD patients among the 90 FD cases, (higher than observed in the general population), further supporting the hypothesis of an increased risk of developing PD in individuals with *GLA* gene mutations.

In one study of our Portuguese Reference Center for Lysosomal Storage Diseases we analyzed the prevalence of PD in a large cohort of 229 Portuguese FD patients, all sharing the same pathogenic *GLA* variant p.F113L, a founder mutation [Azevedo O, *et al.* 2019] in the region of Guimarães, Northern Portugal, which is associated

with the late-onset phenotype of FD. We could report a significantly increased prevalence of PD in our cohort, all presenting high cerebrovascular burden and weak response to levodopa [Gago MF, *et al.* 2020].

1.3. Role of sphingolipids and phospholipids in the molecular pathways and as putative biomarkers in neurologic diseases

Sphingolipids are a class of lipids containing a backbone of sphingoid bases. They are synthetized — among others — in lysosomes and, as such, they are strongly associated with different types of LSDs.

Figure 1 and Table 1 (both imported from the work of Platt, FM. Sphingolipid lysosomal storage disorders. Nature 510, 68–75, 2014 [Platt FM. 2014]) give a simple demonstration of the metabolic pathway of sphingolipid classes as well as of the associated lysosomal enzymes and LSDs.

Fig. 1.

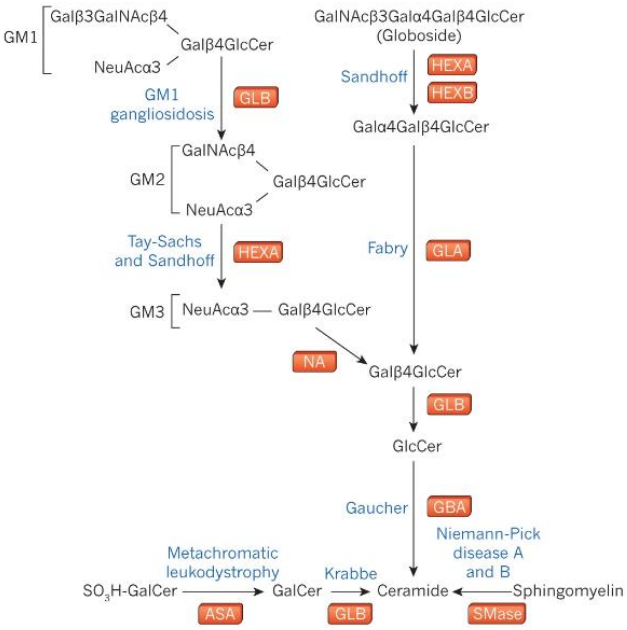


Fig.1: Glycosphingolipid catabolism and associated lysosomal storage diseases. Figure taken from Platt, FM. Sphingolipid lysosomal storage disorders. Nature 510, 68–75 (2014) [Platt FM. 2014]. The enzymes that catalyse the metabolism of one metabolite to another are shown in red, and the diseases that result from defects of these enzymes are shown in blue. ASA, arylsulphatase A; GBA, β-glucocerebrosidase; GLA, α-galactosidase; GLB, β-galactosidase; HEXA, β-hexosaminidase A; HEXB, β-hexosaminidase B; NA, neuraminidase; SMase, acid sphingomyelinase.

In the last years, various studies reported that — besides the associated enzymatic defects — sphingolipids and phospholipids might serve as potential diagnostic and prognostic biomarkers for LSDs such as GD [Revel-Vilk, S, *et al.* 2020; Irún, P, *et al.* 2020; Rolfs A, *et al.* 2013], FD [Simonetta I, *et al.* 2020], and Acid Sphingomyelinase Deficiency (Niemann-Pick disease type A and B) [Chuang, WL, *et al.* 2014].

Table 1.

Disease	Biochemical defect	Gene involved	Protein defect	Major symptoms	CNS pathology
GM2 synthase deficiency	Loss of GM2 and downstream gangliosides	*B4GALNT1*	GM2S	Spastic paraplegia	Yes
Gaucher types 1, 2 and 3	GlcCer storage	*GBA*	Glucocerebrosidase	Hepatosplenomegaly, haematological defects, bone disease and CNS disease in types 2 and 3	In types 2 and 3
Fabry	Gb3 storage	*GLA*	α-galactosidase	Renal, cardiovascular and peripheral pain	Yes (some mild cases of cerebrovasculopathy)
Tay–Sachs	GM2 ganglioside storage	*HEXA*	B-hexosaminidase α subunit	Progressive neurodegeneration	Yes
Sandhoff	GM2 ganglioside storage	*HEXB*	B-hexosaminidase β subunit	Progressive neurodegeneration	Yes
GM1 gangliosidosis	GM1 ganglioside storage	*GLB1*	β-galactosidase	Progressive neurodegeneration	Yes
Niemann–Pick type C	Storage of all GSLs, cholesterol, sphingomyelin and sphingosine	*NPC1* *NPC2*	NPC1 NPC2	Progressive neurodegeneration	Yes

Table 1. Examples of inborn errors of glycosphingolipid metabolism. Table taken and modified from Platt, F. Sphingolipid lysosomal storage disorders. Nature 510, 68–75 (2014) [Platt FM. 2014]. CNS, central nervous system; GlcCer, glucosylceramide; GM2S, GM2 synthase; GM3S, GM3 synthase; GSL, glycosphingolipid; LSD, lysosomal storage disease.

Furthermore, in the recent years, sphingolipids have started to be identified as potential serum biomarkers in various other diseases such as hepatitis related to HBV and HCV infection [Qu F, *et al.* 2014] and Type I diabetes [Meikle PJ, *et al.* 2017].

We reported, for the first time, in rheumatoid arthritis, serum sphingolipid changes compatible with those changes observed previously in the synovial tissue and synovial fluid (project carried out during work at the Ludwig-Maximilians-Universität zu München [Miltenberger-Miltenyi G, *et al.* 2019]).

Before our project **3.5.**, less had been reported about serum phospholipids in GD, PD and in GBA-PD. Still, an important role of phospholipids in the pathomechanism for GD is plausible, as the disease is characterized by infiltration of phospholipid-laden macrophages (Gaucher cells) in the spleen, liver, bone marrow, lungs and the central nervous system [Cassinerio E, *et al.* 2014; Cox TM. 2001].

We carried out two large projects investigating on the role of sphingolipids and phospholipid in the molecular pathway and as putative serum biomarkers in the above-mentioned diseases (**3.4.** and **3.5.**, [Guedes LC, *et al.* 2017; López de Frutos L, *et al.* 2022], see details below).

Besides, we carried out a project on the role of sphingolipids and phospholipids in ChAc (a.k.a. VPS13A disease) (**3.6.** [Miltenberger-Miltenyi G, *et al.* 2023], see details below). ChAc usually begins in the third to fifth decades of life and involves a spectrum of primarily basal ganglia-related neurological and psychiatric symptoms. Acanthocytosis (morphologically altered erythrocytes) is not an obligatory finding. No disease-modifying treatment is available and management is directed at controlling symptoms.

To date, no neuropathological hallmark has been identified in ChAc in contrast to the inclusion bodies and other structures seen in PD and Alzheimer's diseases. Histopathologically, ChAc shows striking neuronal loss affecting the caudate nucleus (CN) and putamen with a robust reactive astroglial response, while the cortex is predominantly spared [Bader B, *et al.* 2008].

Recent studies have demonstrated that VPS13 proteins localize to membrane contact sites and might be responsible for bulk lipid transport between the membranes of various subcellular organelles [Dziurdzik SK, *et al.* 2021; Leonzino M, *et al.* 2021]. In human cells, VPS13A/chorein localizes between the endoplasmic reticulum (ER) and mitochondria, as well as between the ER and lipid droplets [Kumar N, *et al.* 2018]. Mitochondrial binding with a role in lysosomal degradation has also been reported [Muñoz-Braceras S, *et al.* 2019]. In yeast, VPS13 is recruited via adaptor proteins to inter-organellar contact sites, each mediating a distinct function [Bean BDM, *et al.* 2018]. Thus, it is becoming increasingly apparent that impaired inter-organellar lipid mobility is a common element of this novel group of neurodegenerative VPS13 diseases [Yeshaw WM, *et al.* 2019].

Sphingolipid and phospholipid level changes have recently been reported in human brain in Huntington's disease (HD; [Phillips GR, *et al.* 2021]) and PD [Xicoy H, *et al.* 2020], implicating dysfunction of lipid metabolism in their pathophysiology.

Prior to our project **3.6.**, no such data had been demonstrated for ChAc, although the presence of neurologic symptoms and erythrocyte membrane alterations (both of which are also seen in other disorders of lipid metabolism such as abetalipoproteinemia) raises the question of whether these lipids are also altered in this disease. In our project (**3.6.** [Miltenberger-Miltenyi G, *et al.* 2023], see details below), we addressed this scientific question.

2. Aim of the habilitation

The main objective of this cumulative habilitation project is to (a) describe genotype-phenotype associations in various neurologic diseases and to (b) investigate and point out the role of sphingolipids and phospholipids in the pathomechanism and as putative biomarkers for selected neurologic diseases.

I) Describe genotype-phenotype associations in various types of Charcot-Marie-Tooth disease

a) Clinical and electrophysiological features in CMT with mutations in the *NEFL* gene

b) Identification and in-silico analysis of 14 novel *GJB1*, *MPZ* and *PMP22* gene mutations

II) Investigate the association between the *C9orf72* expansion and the disease progression in amyotrophic lateral sclerosis

a) *C9orf72* expansion is associated with accelerated decline of respiratory function and decreased survival in amyotrophic lateral sclerosis

III) Investigate the role of sphingolipids and phospholipids in the pathomechanism and as putative biomarkers in Parkinson´s disease and in chorea-acanthocytosis

a) Serum lipid alterations in GBA-associated Parkinson's disease

b) Serum Phospholipid Profile Changes in Gaucher Disease and Parkinson's Disease

c) Sphingolipid and phospholipid levels are altered in human brain in chorea-acanthocytosis

3. Published manuscripts

3.1. Clinical and electrophysiological features in CMT with mutations in the *NEFL* gene [Miltenberger-Miltenyi G, *et al.* 2007]

Background

The Charcot-Marie-Tooth disease (CMT; MIM 118220), also known as hereditary motor and sensory neuropathy, occurs with an estimated frequency of 1:2500 individuals world-wide [Stojkovic T. 2016]. The main clinical characteristics are slowly progressive distal muscle weakness and wasting first of the lower and later usually also of the upper limbs, sensory loss, and foot deformity. Till the date of our study, only 13 different neurofilament light chain polypeptide gene (*NEFL*) mutations had been identified in 55 patients with CMT from 16 families and were reported to be associated with both axonal and demyelinating variants of CMT.

In this study, we investigated the clinical features of additional 11 patients with CMT and *NEFL* mutations and explored possible genotype-phenotype correlations.

Patients & Methods

Standardized neuromuscular and nerve conduction studies were performed in all patients, and the coding regions of the peripheral myelin protein 22 (*PMP22*), myelin protein zero (*MPZ*), gap junction ß-1 protein (*GJB1*), and *NEFL* genes were analyzed by direct DNA sequencing.

Results

The index patient of family 1 showed a heterozygous single nucleotide exchange (c.278T>C) resulting in a leucine-to-proline amino acid change at codon 93 (p.L93P) in the coil 1a domain of the NEFL protein (**Fig. 3.1.1.**). The leucine-93 residue is highly conserved. All of the affected family members but none of the 5 unaffected family members carried this mutation; which was also absent in 100 controls. In the affected patients of family 2 as well as in the sporadic patient, we found a heterozygous C-to-G nucleotide change in the first coding exon of *NEFL* (c.23C>G) resulting in a proline-to-arginine substitution at codon 8 (p.P8R) located in the head domain of the NEFL protein. The p.P8R change was absent in clinically unaffected individuals of family 2. Besides, it was absent in the unaffected parents of the sporadic case, suggesting that in this patient the mutation arose de novo (**Table 3.1.1.**).

Fig. 3.1.1.

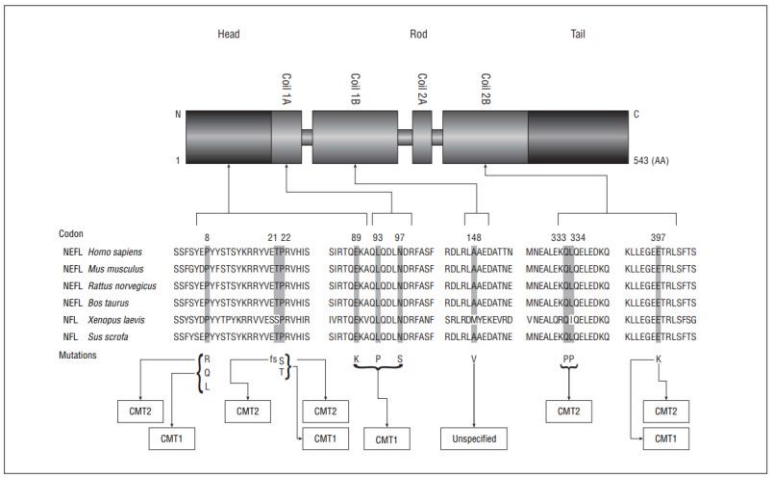


Fig.3.1.2: Compilation, sequence homology, and localization of *NEFL* mutations reported until our paper with respect to protein domains. Mutation sites are indicated in gray. Amino acid changes and associated phenotypes (according to

the original publications) are indicated below the mutation sites. CMT1 indicates Charcot-Marie-Tooth disease, demyelinating variant; CMT2, Charcot-Marie-Tooth disease, axonal variant; N, N terminus; C, C terminus; AA, amino acid.

Table 1. Clinical and Electrophysiological Features of Patients With Charcot-Marie-Tooth Disease Investigated in This Study

Patient	Age at Study Time, y	Age at Onset, y	Initial Symptoms	Weakness in LL/UL[a]	Atrophy in LL/UL[b]	Vibration[c] LL	Vibration[c] UL	Pain or Temperature in LL/UL	Reflexes in LL/UL	Pes Cavus	Additional Disorders	NCV, m/s (CMAP, mV) for Median/Ulnar
Family 1												
IV:25	63[d]	~15	Gait problems	+++/+	++/+	0/8	6/8	R/N	A/A	Yes	Hypertension	38 (2.9)/40 (3.3)
V:11	55	~15	Clumsiness during sport	+++/–	++/–	0/8	7/8	R/N	A/D	Yes	Psoriasis	35 (2.0)/36 (4.1)
IV:18	70	~14	Gait problems	+++/++	++/++	0/8	7/8	N/N	A/D	Yes		36 (1.2)/29 (1.5)
V:10	46	~8	Gait problems	++/–	+/–	0/8	8/8	N/N	A/A	Yes	Diabetes, hypertension, coronary heart disease	38 (5.0)/40 (2.4)
Family 2												
Patient 1	49	12	Unsteady gait	+++/++	++/++	ND	ND	ND/ND	A/A	Yes	Hyperlordosis	ND
Patient 2	77	15	Gait problems	+++/++	++/++	ND	ND	R/R	A/A	Yes	Cerebellar signs	39 (0.2)/ND
Patient 3	71	12	Unsteady gait	+++/++	++/++	ND	ND	ND/ND	A/A	Yes	Kyphoscoliosis	ND
Patient 4	61	25	Gait problems	+++/++	++/++	ND	ND	R/R	A/A	Yes	Fasciculations	ND
Patient 5	51	13	Unsteady gait	+++/++	++/++	ND	ND	R/R	A/A	Yes	Ataxia, tremor	36 (1.9)/ND
Patient 6	50	ND	Unsteady gait	+++/++	++/++	ND	ND	R/R	A/A	ND	Schizophrenia, ataxia	ND
Patient 33	29	8	Foot deformity	+++/++	++/++	ND	ND	R/R	A/A	Yes		23 (0.5)/ND

Table 3.1.1. Abbreviations: A, absent; CMAP, compound motor action potential; D, diminished; LL, lower limbs; N, normal; NCV, nerve conduction velocity; ND, no data; R, reduced; UL, upper limbs. **a** For weakness in LL, + indicates ankle dorsiflexion less than grade 4 Medical Research Council (MRC); ++, ankle dorsiflexion less than grade 4 MRC and proximal weakness; and +++, ankle dorsiflexion less than grade 4 MRC and wheelchair bound. For weakness in UL, − indicates no weakness; +, intrinsic hand muscle weakness grade 4 MRC; and ++, intrinsic hand muscle weakness less than grade 4 MRC. **b** For atrophy, − indicates no atrophy; +, mild atrophy; and ++, pronounced atrophy. **c** Vibration in the LL was measured at the metacarpophalangeal joint of the hallux; vibration in the UL was measured at the distal interphalangeal joint of the index finger. **d** Patient died in 2004.

Table 2. Clinical and Electrophysiological Features of Patients With Charcot-Marie-Tooth Disease With Mutations in the *NEFL* Gene Described in the Literature

Domain	Mutation	Source	Patients, No.	Families/ Sporadic Patients, No.	Age at Onset, y	Muscle Weakness[a] LL	Muscle Weakness[a] UL	Atrophy[b] LL	Atrophy[b] UL	Sensory Loss[c]	Reflexes	Pes Cavus	NCV, Median, m/s (CMAP, mV)	CMT Variant
Head	p.P8R	Jordanova et al[8] (2003), De Jonghe et al[10] (2001), present study	20	3/2	7-25	+ to +++	ND to +	+	+	+	N, D, A	Yes	23-39 (0.5-4.9)	CMT1, CMT2
Coil 1a	p.P8Q	Jordanova et al[8] (2003)	1	1/0	< 5	+++	+	+	+	+	D	Yes	21 (1.0)	CMT1
	p.P8L	Jordanova et al[8] (2003)	1	0/1	< 2	++	++	+	+	–	A	Yes	13-15 (2.0)	CMT1
	p.T21fs	Leung et al,[17] (2006)	1	0/1	71	+++	ND	+	ND	+	A	Yes	ND	CMT2
	p.P22T	Yoshihara et al[13] (2002)	2	1/0	18-24	+++ to ++++	ND	+	ND	+	ND	ND	29.3-35.7 (0.01-0.74)	CMT1
	p.P22S	Georgiou et al[14] (2002), Fabrizi et al[15] (2004)	12	2/0	< 10 to 36	+ to ++	+ to ++	+	+	+	D, A	Yes	21-54 (0.8-7.4)	CMT1, CMT2
	p.E89K	Jordanova et al[8] (2003)	1	0/1	< 2	+	+	ND	ND	+	A	Yes	27 (3.9)	CMT1
	p.L93P	Present study	4	1/0	~8 to ~15	++ to +++	– to ++	++	+	ND	A	Yes, no	35-38 (1.2-5.0)	CMT2
Coil 1b	p.N97S	Jordanova et al[8] (2003), Yoshihara et al[13] (2002)	2	0/2	< 1 to 15	+ to +++	+ to +++	+	+	ND to +	A	Yes	22-26 (0.52-4.7)	CMT1
	p.A148V	Yoshihara et al[13] (2002)	1	0/1	33	+	+	+	+	+	ND	ND	ND	Unspecified
Coil 2b	pQ333P	Mersiyanova et al[9] (2000)	12	1/0	10-20	ND	ND	+	ND	ND	ND	ND	ND	CMT2
	p.L334P	Choi et al[11] (2004)	1	0/1	ND	ND	ND	ND	ND	ND	ND	ND	ND	CMT2
	p.E397K	Choi et al[11] (2004), Züchner et al[12] (2004)	8	2/0	4-46	+ to ++	–	ND	ND	+	N, D	Yes, no	57-63 (10-14.8)	CMT1, CMT2

Table 3.1.2. Abbreviations: A, absent; CMAP, compound motor action potential; CMT1, Charcot-Marie-Tooth disease, demyelinating variant; CMT2, Charcot-Marie-Tooth disease, axonal variant; D, diminished; LL, lower limbs; N, normal; NCV, nerve conduction velocity; ND, no data; UL, upper limbs. **a** For weakness in LL, + indicates ankle dorsiflexion less than grade 4 Medical Research Council (MRC); ++, ankle dorsiflexion less than grade 4 MRC and proximal weakness; and +++, ankle dorsiflexion less than grade 4 MRC and wheelchair bound. For weakness in UL, − indicates no weakness; +, intrinsic hand muscle weakness grade 4 MRC; and ++, intrinsic hand muscle weakness less than grade 4 MRC. **b** For atrophy, − indicates absent; +, present. **c** For sensory loss, − indicates no; +, yes.

Conclusion

We described the phenotypic features of 11 further patients with CMT from 3 families with *NEFL* mutations, including a novel *NEFL* mutation, p.L93P. Nerve conduction studies suggested that this mutation resulted in an axonopathy with secondary demyelination in all of the affected individuals, which was also seen in family 2 associated with a p.P8R mutation. Indeed, neuropathological studies in 2 cases demonstrated predominantly axonal atrophy in intermediate CMT, but axonal swelling, paranodal abnormalities, and onion bulb formations have also been observed [Zuchner S, *et al.* 2004; Fabrizi GM, *et al.* 2004].

Until our work, 22 patients with a mutation involving the proline residue at codon 8 [Jordanova A, *et al.* 2003; De Jonghe P, *et al.* 2001] had been reported and at least 5 different mutational events could be inferred, suggesting that codon 8 is a hot spot for *NEFL* mutations. Of the 13 patients with this mutation, 1 was classified as having intermediate CMT and the others were classified as having CMT1, with NCVs ranging from 13 to 39 m/s.

Phenotypic variability can be seen with codon 8 mutations not only with NCVs but also with disease onset, which varied between ages 2 and 25 years [Jordanova A, *et al.* 2003; De Jonghe P, *et al.* 2001]. The phenotypic variability found for codon 8 mutations can also be observed for mutations at codon 22 in the head domain of the *NEFL* protein [Fabrizi GM, *et al.* 2004; Yoshihara T, *et al.* 2002; Georgiou DM, *et al.* 2002] or at codon 397 in the coil 2b domain [Fabrizi GM, *et al.* 2004; Choi BO,, *et al.* 2004] (**Fig. 3.1.2.**).

This indicates that no simple genotype-phenotype correlation exists and suggests the existence of modifiers in *NEFL*-related CMT. However, it appears that mutations localizing to the NEFL protein head domain cause more severe motor and sensory NCV slowing than mutations in the coil 2B domain (**Fig. 3.1.2. and Table 3.1.2.**) while the intrafamilial phenotype appears to “run true.”

Remarks

Our publication reached 53 citations (Google Scholar). Furthermore, our publication is cited on Online Mendelian Inheritance in Man (OMIM) catalog on *NEFL* gene at the paragraph “Genotype/Phenotype Correlations” (https://www.omim.org/entry/162280).

3.2. Identification and in-silico analysis of 14 novel *GJB1*, *MPZ* and *PMP22* gene mutations [Miltenberger-Miltenyi, *et al.* 2009]

Background

Among several classifications, CMT can be subdivided according to the nerve electrophysiological criteria into two main groups [Harding AE, *et al.* 1980; Kaku DA, *et al.* 1993]: CMT1, which is characterized by reduced motor nerve conduction velocities (MNCV) of the median nerve (MNCV <38 m/s) and nerve demyelination, and the axonal variant, CMT2, with MNCV being almost normal (MNCV >38 m/s), but with reduced amplitudes of compound motor-evoked potentials. An additional classification of intermediate CMT has been suggested in cases with MNCV between 25 and 45 m/s [Davis CJ, *et al.* 1978]. Regarding the most commonly associated genes, CMT1 is frequently associated with duplications on the chromosome 17p11.2–p12 (CMT1A), which contains the peripheral myelin protein 22 gene (*PMP22*; MIM 601097) [Nelis E, *et al.*

1996]. Mutations in the gap junction b-1 protein located on chromosome Xq13.1 (*GJB1* or Connexin 32, Cx32; MIM 304040) lead to CMT type X1 (CMTX1), and electrophysiology may suggest both demyelinating and/or axonal nerve damage, thus resulting in either a CMT1, CMT2 or in an intermediate phenotype [Szigeti K, *et al.* 2006]. Myelin protein zero (*MPZ* or P0; MIM 159440) mutations are associated with CMT1, but can also cause CMT2 or the Dejerine–Sottas syndrome (DSS) [Shy ME, *et al.* 2004; Marrosu MG, *et al.* 1998]. The *PMP22* mutations can cause CMT1, DSS, congenital hypomyelination (CHN) and, if inactivated, can also cause hereditary neuropathy with a liability to pressure palsies (HNPP) [Nelis E, *et al.* 1999].

Patients & Methods

We examined 250 unrelated Austrian CMT patients for the CMT1A duplication 17p11.2–p12. Subsequently, a total of 171 individuals without duplication were screened for mutations in the *GJB1*, *MPZ* and *PMP22* genes. We reported on the mutation frequency distribution in this population. The pathogenicity of novel and published mutations was also evaluated using the bioinformatics program PANTHER [Mi H, *et al.* 2007].

Results

We found 79 patients with *PMP22* duplication (31.6%). The coding regions of the *PMP22, MPZ* and *GJB1* genes were analyzed by direct sequencing in the remaining patients: 28 patients carried different mutations, 14 of which were novel (**Table 3.2.1**.). We scored the pathogenicity of novel missense variants by segregation studies and by their exclusion in control samples. Our comprehensive literature study found that up to 60% of the reported variants in these genes had not been evaluated regarding their pathogenicity, thus we used the PANTHER bioinformatics tool to score novel and published missense variants. The PANTHER program scored known polymorphisms as such, but scored around 82–88% only of the published and novel mutations as most likely deleterious. Variants associated with axonal CMT were less likely to be classified as deleterious, and the *PMP22* p.S72L mutation repeatedly associated with severe CMT, was classified as a polymorphism using default parameters.

Table 3.2.1.

ID	Sex	Family history	Age of onset (years)	Age at examination (years)	mNCV/ CMAP (m/s/mV)	sNCV/ SNAP (m/s/μV)	UL weakness	LL weakness	Tendon reflexes	Sensory impairment	Pes cavus	Additional findings	Gene	Nucleotide position	aa change
1	M	S[a]	5–6	41	48/1.9	37/7.2	+	+++	N	ND	+++	No	*GJB1*	c.11C>A	p.T4K
2	M	F (4)	7–8	28	ND	ND	+++	+++	A	ND	+++	Cerebellar signs in one affected individual	*GJB1*	c.94A>G	p.R32G
3	F	F (3)	6	31	40/3.6	NR	+	+	D	ND	+	No	*GJB1*	c.155delT	p.I52TfsX31
4	F	F (2)	5–6	50	31/0.1	NR	++	+++	A	ND	++	No	*GJB1*	c.278T>A	p.M93K
5	M	F (8)	ND	68	ND	ND	+++	+++		ND	+++	No	*GJB1*	c.437A>C	p.E146A
6	M	F (2)	Newborn	18	34/14	38/5	–	+++	D/A	Yes	+++	No	*GJB1*	c.592T>G	p.S198A
7	F	F (4)	16	46	35/1.6	37/1	+	+++	A	Yes	ND	No	*GJB1*	c.829_904 del14	p.S277GfsX128
8	F	F	2	9	4/0.8	NR	++	++	A	Yes	+	No	*MPZ*	c.89T>G	p.I30S
9	F	S[a]	10	37	53/ND	58/ND	ND	ND	ND	ND	ND	No	*MPZ*	c.98A>T	p.Y33F
10	F	F	5–6	38	16/6	27/9	+	+	A/D	Yes	++	No	*MPZ*	c.148T>G	p.C50G
11	M	F (5)	Childhood	27	29/2	33/ND	–	++	D	Yes	+++	No	*MPZ*	c.298C>T	p.Q100X
12	M	S *de novo*	1	1.5	11/ND	NR	++	++	D	No	no	Macro-cephalia	*MPZ*	c.553delC	p.R185AfsX66
13	M	F (3)	43	45	34/12	36/17	–	–	D	Yes	+++	No	*MPZ*	c.670G>T	p.D224Y
14	F	F	10	52	39/7.0	38/10	–	+	N/A	Yes	++	Gait ataxia	*PMP22*	c.332T>C	p.M111T

Table 3.2.1. Clinical and genetic findings of CMT patients with novel mutations in the *GJB1, MPZ* and *PMP22* genes. Sex: F, female; M, male; Family history: S, sporadic; F, familial; if F is the number of affected relatives with the same mutation. a Parents not available for examination; mNCV, motor nerve conduction velocities of median nerve; sNCV, sensory nerve conduction velocities of median nerve; CMAP, compound motor action potential of abductor pollicis brevis muscle; SNAP, sensory nerve action potential of median nerve; ND, no data; NR, no response; UL, upper limbs; LL, lower limbs; +, mild; ++, moderate; +++, severe; -, absent; N, normal; A, absent; D, diminished; aa, amino acid.

Conclusion

In summary, our data suggested that this in silico analysis tool could be useful for assessing the functional impact of DNA variants. The CMT1Adup, *GJB1, MPZ* and *PMP22* mutation frequencies were in the range of those described in other CMT patient collectives with different ethnical backgrounds.

Remarks

Our work was one of the first approaches to use in-silico-tools for the evaluation of genetic variants regarding pathogenicity. The work has been cited 19 times since publication and led to further collaborations in compatible themes [Auer-Grumbach M, *et al.* 2008; Rohkamm B, *et al.* 2007; Finsterer J, *et al.* 2006].

3.3. *C9orf72* expansion is associated with accelerated decline of respiratory function and decreased survival in amyotrophic lateral sclerosis [Miltenberger-Miltenyi G, *et al.* 2019]

Background

ALS is a devastating neurodegenerative disorder with short survival, mainly due to respiratory failure [Hardiman O, *et al.* 2017; Knibb JA, *et al.* 2016]. A pathological repeat expansion in the *C9orf72* gene is observed in about 10% of the European ALS population and is associated with a worse prognosis [Knibb JA, *et al.* 2016; Kiernan MC, *et al.* 2011]. Still, the exact function of this gene is unknown. To understand the role of the *C9orf72* expansion in disease prognosis, we tested the impact of this mutation on the respiratory function in ALS.

Patients & Methods

We studied 372 consecutive patients with ALS (according to the revised El Escorial and Awaji criteria). Patients were categorized regarding the absence (C9orf72-0) or presence (C9orf72exp) of the *C9orf72* expansion. Diagnosis delay, region of onset, age at diagnosis, gender and comorbid FTD were included as independent variables during regression analysis.

First, we directly compared the mutated and non-mutated groups using Wilcoxon rank-sum and Pearson's $\chi 2$ tests. Subsequently, we tested the influence of the aforementioned independent variables on the ALS Functional Rating Scale-Revised (ALSFRS-R) global and respiratory scores (ALSFRS-Rglobal and ALSFR-Rrespiratory) and on the predicted value of the forced vital capacity (%FVC) — dependent variables — using multiple regressions.

To analyse the progression of ALSFRS-Rglobal, ALSFRS-Rrespiratory and %FVC, we used the baseline and two subsequent assessments. We performed three multiple regressions that controlled for (i) the time spans between assessments, (ii) the independent variables and (iii) the interactions between the time spans and the presence of the *C9orf72* expansion (**Table 3.3.1.A–C**). We corrected the regression analysis for multiple comparisons, using Bonferroni correction.

Results

Baseline assessment: From the 372 patients, 340 were classified as C9orf72-0 and 32 as C9orf72exp. Only nine C9orf72-0 patients did not tolerate riluzole. Direct comparison of the demographic and clinical data

did not yield significant differences between groups, except for FTD that was more frequent in the mutated group (p<0.001). The regression analysis disclosed that C9orf72exp did not influence the dependent variables (all p>0.2). However, diagnosis delay was negatively associated with ALSFRS-Rglobal (p<0.05/3), and ALSFRS-Rrespiratory was positively associated with both spinal (p<0.001/3) and bulbar (p<0.01/3) onset forms, compared with other onset forms.

Longitudinal assessment: Data from 242 C9orf72-0 and 26 C9orf72exp patients were available for the two ALSFRS-R regressions, and from 111 C9orf72-0 and 13 C9orf72exp patients for the %FVC regression. During disease progression, ALSFRS-Rglobal was negatively associated with the presence of FTD (p<0.01/3), but not with the mutation (p>0.8; **Table 3.3.1.A**). Similarly, ALSFRS-Rrespiratory was not significantly influenced by the mutation (p>0.3), although it was negatively associated with age (p<0.05/3), and positively with diagnosis delay (p<0.05/3) and the bulbar (p<0.01/3) and spinal (p<0.001/3) onset forms versus other onset forms (**Table 3.3.1.B**).

In contrast, the regression on %FVC showed a significantly faster decline in the C9orf72-mutated group (p<0.01/3; **Table 3.3.1.C**; **Figure 3.3.1.A**; mean %FVC/month decline rates: −2.62 and −1.00, between the first and third assessments, and −4.81 and −1.66, between the second and third assessments, for C9orf72exp and C9orf72-0 patients, respectively).

Survival analysis: Data were available from 344 patients (313 C9orf72-0, 31 C9orf72exp). The C9orf72 expansion was independently associated with reduced survival (p=0.002; **Figure 3.3.1.B**). Moreover, higher age at diagnosis and shorter diagnosis delay predicted shorter survival (both p<0.001).

Table 3.3.1.

Table 1 Longitudinal assessment of disease progression for amyotrophic lateral sclerosis patients with and without the *C9orf72* repeat expansion across three data points

Dependent variable	Intercept	Diagnosis delay	Bulbar onset	Spinal onset	Age at diagnosis	Gender	FTD	C9orf72	Time after baseline	C9orf72 * time after baseline
(A) $ALSFRSR_{global}$ n_0=242; n_1=26	0.94***	−0.05	−0.04	−0.03	−0.09	0	−0.06**	0	−0.24***	0.03
(B) $ALSFRSR_{respiratory}$ n_0=242; n_1=26	0.91***	0.12*	0.08**	0.12***	−0.11*	0.02	−0.01	0.02	−0.20***	0.08
(C) %FVC n_0=111; n_1=13	0.75***	0.04	−0.15	−0.08	−0.08	0.04	0.06	0.08	−0.18***	**−0.40****

Table 3.3.1. Longitudinal assessment of disease progression for amyotrophic lateral sclerosis patients with and without the C9orf72 repeat expansion across three data points. Effects of the independent variables on the dependent variables are expressed by the corresponding regression coefficients, with significant, positive (negative, resp.) coefficients meaning that higher values of the independent variables are associated with higher (lower, resp.) values of the dependent variable. ALSFRS-R measurements were separated on average by 3.5 and 2.9 months, and the %FVC measurements on average by 4.7 and 4.4 months, for the non-mutated and mutated groups, respectively. Estimated coefficients for the multiple regressions using the (A) global score on the ALSFRS-R (ALSFRS-Rglobal); (B) respiratory subscore on the ALSFRS-R (ALSFRS-Rrespiratory) and (C) predicted forced vital capacity (%FVC) as the dependent variables, and the variables indicated in the columns as the independent variables. All variables were scaled so that their maximum absolute value was equal to 1. Patients' gender was coded with 0 (male) or 1 (female). The faster decline of the %FVC values in the C9orf72-mutated group is shown in bold. Sample sizes of the non-mutated (n0) and mutated (n1) groups used in each regression are indicated. *, ** and *** indicate p values <0.05/3, 0.01/3 and 0.001/3, respectively. ALSFRS-R, ALS Functional Rating Scale-Revised; %FVC, forced vital capacity, FTD, frontotemporal dementia.

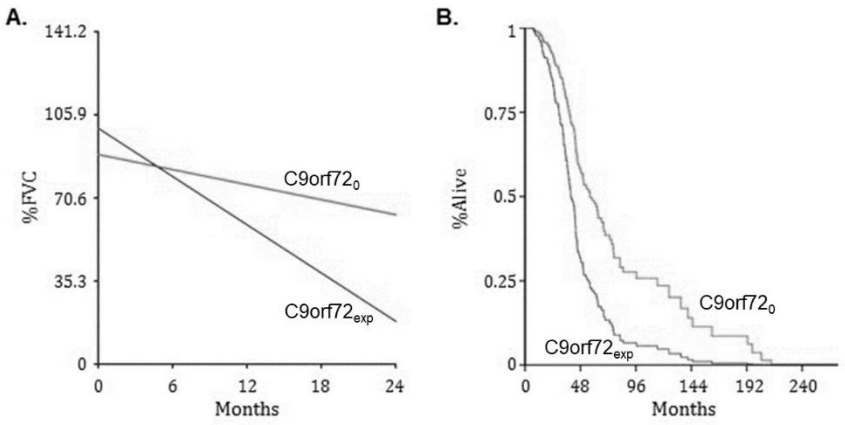


Fig.3.3.1: ALS patients with the C9orf72 repeat expansion (mutated) present a faster decrease of the predicted forced vital capacity (%FVC), and reduced survival, than the non-mutated patients. (A) Estimated %FVC values for the non-mutated (C9orf72-0; n=111) and mutated (C9orf72exp; n=13) groups, during the first 2 years after baseline evaluation, when controlling for diagnosis delay, region of onset, age at diagnosis, gender, and frontotemporal dementia, via multiple regression (see "Methods"; table 1C). (B) Estimated cumulative survival curves for the non-mutated (C9orf72-0; n=313) and mutated (C9orf72exp; n=31) groups, when controlling for the aforementioned variables, via Cox regression (see "Methods").

Conclusion

Taken together, we demonstrated that the *C9orf72* expansion is independently associated with accelerated respiratory dysfunction and shorter survival in ALS.

Remarks

Our paper was cited 16 times (Google Scholar). Notably, our result that the *C9orf72* repeat expansion is associated with accelerated respiratory function decline, was subsequently confirmed in a study carried out by a European reference center for ALS, involving 630 ALS patients [Rooney J, *et al.* 2019].

3.4. Serum lipid alterations in GBA-associated Parkinson's disease [Guedes LC, *et al.* 2017]

Background

Mutations in the *GBA* gene, encoding for the lysosomal enzyme glucocerebrosidase, are associated with Gaucher disease. Alterations in plasma sphingolipids have been reported in Gaucher, and similarly in brain extracts in Lewy body disease. As *GBA* mutations are prevalent risk factors for Parkinson's disease and overlap of molecular pathways are presumable, here we assessed the lipid profiles in Parkinson's patients with and without *GBA* mutations.

Patients & Methods

415 patients diagnosed with sporadic and familial PD were recruited from the registries of the movement disorders outpatient clinic of Hospital de Santa Maria, University of Lisbon. Standardized case report forms (CRF) were used and data were collected about the medical history of patients and their families. The generally used Hoehn and Yahr scale (HY) was applied for staging the disease.

DNA samples of the 415 PD patients were screened for mutations. The *LRRK2* gene was analyzed previously for the most common pathogenic mutations by sequencing exons 31 and 41, which include the mutations

p.Arg1441His and p.Gly2019Ser, respectively [Ferreira JJ, *et al.* 2007]. The *GBA* gene was screened by PCR following direct bidirectional sequencing of all exons and intron-exon boundaries. Primers were gene specific to separate from the pseudogene.

For the chitotriosidase enzymatic assay and the lipidomics array we selected 64 samples out of the 415 PD patients, divided in two genetic groups: Group 1 (n = 29): all patients, who carried mutations in the *GBA* gene that were described as pathogenic for GD and/or associated with PD vs. Group 2 (n = 35) non-*GBA* mutation carriers.

We measured the chitotriosidase activity, using a 4-MU based substrate, as described elsewhere [Pinto R, *et al.* 2004]. Statistical evaluation of the results was carried out – balanced groups - using one-way ANOVA testing.

Serum lipid extracts were prepared by modifying published lipid extraction protocols [Chan RB, *et al.* 2012; Shaner RL, *et al.* 2009]. For the total lipid extract, each lipid extract sample was prepared from 100 microl of serum. For the total lipid extract, glycerophospholipids and sphingolipids were separated with normal-phase HPLC as described [Shaner RL, *et al.* 2009]. In total, we measured 40 lipid subclasses. Quantification of endogenous lipid species was accomplished using multiple reaction monitoring (MRM) transitions that were developed in earlier studies [Chan RB, *et al.* 2012; Shaner RL, *et al.* 2009].

Results

29/415 patients (6.9%) carried 8 different *GBA* mutations associated with Gaucher or Parkinson's, including one novel mutation (**Table 3.4.1.**). Chitotriosidase activity was similar across the genetic groups (**Table 3.4.2.**). The levels of some key lipids were altered in *GBA* mutation carriers vs. non-carriers: Monohexosylceramide, Ceramide and Sphingomyelin were elevated; while Phosphatidic acid (PA), Phosphatidylethanolamine (PE), Plasmalogen phosphatidylethanolamine (PEp) and Acyl Phosphatidylglycerol (AcylPG) were decreased (**Fig. 3.4.1.**).

Table 3.4.1: *GBA* variants identified in the PD patient group. Note: het: heterozygous, hom: homozygous, CH: compound heterozygous.

***GBA* mutations identified in the PD patient group**. Note: het: heterozygous, hom: homozygous, CH: compound heterozygous.

Nucleotide change	Amino acid change	Common Variant Name	State	Frequency
Mutations pathogenic for GD				
c.149_150insGTAT	p.Tyr50*		Het	1
c.681T > G	p.Asn227Lys		Hom	1
c.1226A > G	p.Asn409Ser	N370S	Het	7
c.1246G > A	p.Gly416Ser	G377S	Het	2
c.1448T > C	p.Leu483Pro	L444P	Het, CH (with p.Lys13Arg and with p.Glu365Lys, resp.)	5
Mutations increasing the risk for PD				
c.38A > G	p.Lys13Arg		Het, CH (with p.Leu483Pro)	2
c.1093G > A	p.Glu365Lys	E326K	Het, CH (with p.Leu483Pro)	7
c.1223C > T	p.Thr408Met		Het	6
c.1279G > A	p.Glu427Lys	E388K	Het	1

Table 3.4.2. Main clinical characteristics (A) and disease characteristics (B) of the 64 PD patients. Note: (**A**) statistical comparisons were performed between the study groups: Chi Square Test was used for categorical variables, and the Kruskal-Wallis test for continuous variables. The significance level assumed was 0.05; (**B**) p-values were obtained using one-way ANOVAs.

A.

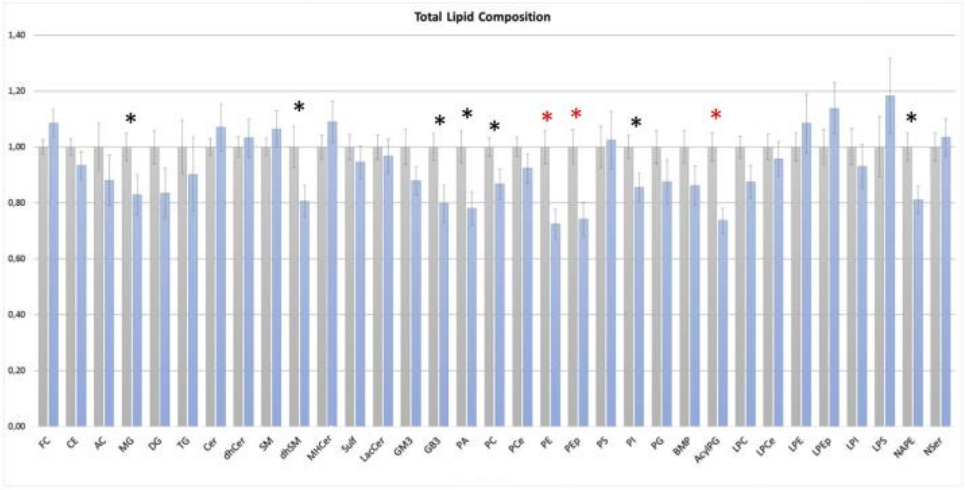
Mutation		***GBA***		***LRRK2***		**iPD control**		
Gender		**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**p-value**
	n	16	13	9	9	10	7	0,869
	%	55,2	44,8	50	50	58,8	41,2	
Ethnicity		**Caucasian**	**Black**	**Caucasian**	**Black**	**Caucasian**	**Black**	
	n	27	2	18	0	17	0	0,288
	%	93,1	6,9	100	0	100	0	
Family History		**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	
	n	9	20	11	7	4	13	0,045
	%	31	69	61,1	38,9	23,5	76,5	
Motor Fluctuations at the time of blood colection		**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	
	n	18	11	17	1	12	5	0,048
	%	62,1	37,9	94,4	5,6	70,6	29,4	
Dyskinesias at the time of blood colection		**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	
	n	14	15	12	6	7	10	0,286
	%	48,3	51,7	66,7	33,3	41,2	58,8	

B.

Mutation	***GBA***		***LRRK2***		**iPD control**		**p-value**
	Average	**Std Dev**	**Average**	**Std Dev**	**Average**	**Std Dev**	
Age at disease onset	55,7	10,7	53,3	10,6	63,9	9,2	0,008
Age at blood collection	66	8,2	63,7	9,3	75,9	8,1	<0,001
Disease duration at blood collection	10,3	5,8	10,4	7,1	11,9	6,5	0,666
Hoehn and Yahr score at blood collection	2,4	0,7	2,6	0,9	2,8	1,0	0,398
Chitotriosidase activity (nmol\h\ml)	73,27	44,8	81,61	64,03	116,89	98,91	0,110

Fig. 3.4.1.

A.



B.

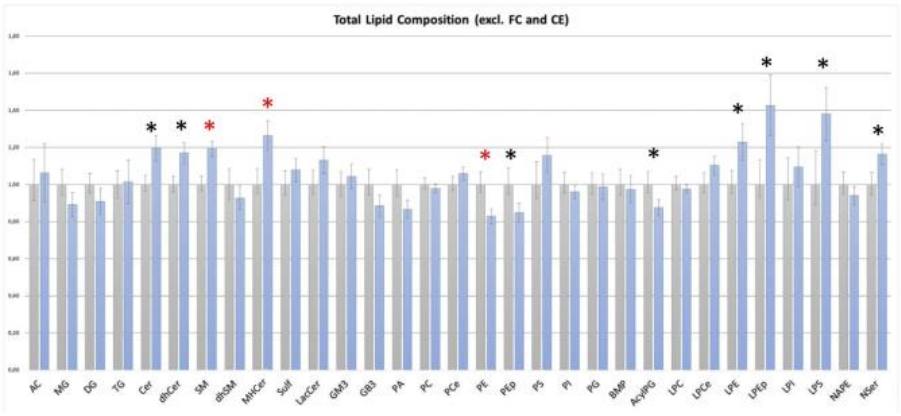


Fig. 3.4.1. Comparative lipid profile of serum of the *GBA* variant carrier (n = 29) vs. non-GBA mutation carrier (n = 35) PD patients. A: Total Lipid Composition; B: Total Lipid Composition excluding FC and CE. Note: The individual lipid subclasses of *GBA* mutation carrier group of patients was expressed as relative to control (non-mutated) group level. (FC) Free Cholesterol, (CE) Cholesterol Ester, (AC) Acyl Carnitine, (MG) Monoacylglycerol, (DG) Diacylglycerol, (TG) Triacylglycerol, (Cer) Ceramide, (dhCer) Dihydroceramide, (SM) Sphingomyelin, (dhSM) Dihydrosphingomyelin, (Sulf) Sulfatide, (MHCer) Monohexosylceramide (galactosylceramide þ glucosylceramide), (LacCer) Lactosylceramide, (GM3) Monosialodihexosylganglioside, (GB3) Globotriaosylceramide, (PA) Phosphatidic acid, (PC) Phosphatylcholine, (PCe) Ether phosphatidylcholine, (PE) Phosphatidylethanolamine, (PEp) Plasmalogen phosphatidylethanolamine, (PS) Phosphatidylserine, (PI) Phosphatidylinositol, (PG) Phosphatidylglycerol, (BMP) Bis(monoacylglycero)phosphate, (AcylPG) Acyl Phosphatidylglycerol, (LPC) Lysophosphatidylcholine, (LPCe) Ether lysophosphatidylcholine, (LPE) Lysophosphatidylethanolamine, (LPEp) Plasmogen Lysophosphatidylethanolamine, (LPI) Lysophosphatidylinositol, (LPS) Lysophosphatidylserine, (NAPE) N-Acyl Phosphatidylethanolamine, (NSer) N-Acyl Serine; grey bars: non mutation carriers; blue bars: GBA mutation carriers; black asterisk: $p < 0.05$ when compared with non-carrier group; red asterisk: $p < 0.05$ after FDR correction.

Conclusion

Our study explored lipids as potential blood biomarkers in *GBA*-mediated PD. The study included only a limited number of patients and the results demonstrate cross-sectional findings, thus further studies including larger sample numbers and a longitudinal follow-up might help to confirm these results. Notably, sphingolipid levels were not observed to correlate with Levodopa equivalent dose [Mielke MM, *et al.* 2013].

Still, the significant changes of lipid levels in our study, together with the findings of other studies [Mielke MM, *et al.* 2013; Clark LN, *et al.* 2015], point out that these molecules have probably a crucial role in PD. In fact, our results revealed additional information to previous findings on brain tissue [Clark LN, *et al.* 2015; Gegg ME, *et al.* 2015] and on blood of non-carrier PD patients [Mielke MM, *et al.* 2013]. We showed that various lipids have altered levels in the serum in *GBA* mutation carriers. As biomarkers, these lipids might help to detect individuals at elevated risk for the developing the disease.

Remarks

Our work has reached 69 citations until now (Google Scholar).

3.5. Serum Phospholipid Profile Changes in Gaucher Disease and Parkinson's Disease [López de Frutos L, *et al.* 2022]

(Project carried out during works at the Ludwig-Maximilians-Universität zu München)

Background

Alterations in the levels of serum sphingolipids and phospholipids have been reported in GD and in PD, suggesting a potential role of these lipids as biomarkers. This project's objective was to detect novel associations and novel candidate biomarkers in the largest Spanish cohort of Gaucher and Parkinson diseases of the Iberian Peninsula.

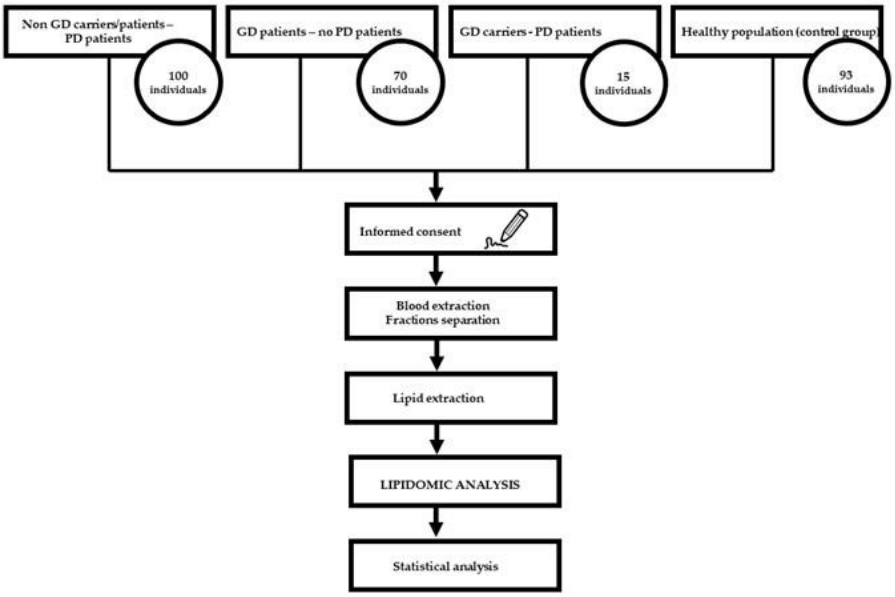
Patients & Methods

We collected clinical, demographic, and laboratory data from 278 participants in total: 100 sporadic PD patients (variants in GBA1 were excluded), 70 GD patients (GD1 and GD3 patients with confirmed bi-allelic *GBA* pathogenic variants), 15 heterozygous *GBA*-variant carriers who developed PD (GBA-PD), and 93 healthy controls (**Fig. 3.5.1.**).

GCase activity was measured in 58 GD, 4 GBA-PD patients, and 6 controls but not in PD patients as in this group the blood samples were not enough to obtain leukocytes (**Table 3.5.1.**). Data on therapy in the GD group with ERT (imiglucerase or velaglucerase alfa) or with SRT (eliglustat or miglustat) was available in 69 patients (42 treated and 27 non-treated). The Hoehn and Yahr (H&Y) scale was collected from 46 PD and 5 GBA-PD patients. Data on PD therapy with dopamine agonists was obtained from 82 PD and from 10 GBA-PD patients, while therapy with other antiparkinsonian drugs was collected from 44 PD and 6 GBA-PD patients.

Levels of phosphatidylcholine (PC), lyso-phosphatidylcholine (LPC), plasmalogenphosphatidylcholine (PLC), phosphatidyl-ethanolamine (PE), lyso-phosphatidylethanolamine (LPE), plasmalogen-phosphatidyl-ethanolamine (PPE), phosphatidylserine (PS), lyso-phosphatidylserine (LPS), phosphatidylinositol (PI), and phosphatidylglycerol (PG) were measured using high-performance liquid chromatography–mass spectrometry (HLPC-MS). Lipid levels were quantified in two distinct manners: (method #1) by summing the levels of all species (considering all non-detectable values to be null); and (method #2) by summing the levels of the species that had less than 30% non-detectable. Study workflow is shown in **Fig. 3.5.1.**.

Fig.3.5.1. Study workflow



Results

There was no evidence towards a difference regarding age of onset of PD symptoms ($p = 0.23$), disease duration ($p = 0.946$), or the H&Y score ($p = 0.581$) between the groups (Table 3.5.1). A difference appeared between groups in the mean age of blood collection ($p < 0.001$), with GBA-PD as the youngest group.

Table 3.5.1

Table 1. Clinical, demographic and therapy data of the patient and control groups.

	PD	GBA-PD	GD	Controls	*p*-Value
N (total = 278)	100	15	70	93	-
Demographic and Clinical Data:					
Male (%)	60 (60%)	7 (50%)	27 (38.6%)	36 (39.4%)	X2 (3) = 11.34; $p = 0.01$
Mean age of symptom onset (years) (± std) PD (n = 100) GBA-PD (n = 11)	59.97 (±10.09)	56 (±12.88)	-	-	t (109) = 1.2041, $p = 0.23$
Mean age of blood collection (years) (±std)	68.0 (±8.62)	58.3 (±13.99)	67.5 (±7.27)	63.0 (±15.49)	F (3, 274) = 5.75, $p = 0.001$
Mean Disease Duration (years) (±std) PD (n = 100) GBA-PD (n = 11)	8.04 (±5.90)	7.91 (±8.05)	-	-	t (109) = 0.067, $p = 0.946$
Mean GCase activity (nmol/mg/h) (±std) GD (n = 58) GBA-PD (n = 4) controls (n = 6)	-	5.38 (±1.95)	0.98 (±0.68)	6.15 (±1.66)	F (2,65) = 127.5, $p < 0.001$
Mean Hoehn and Yahr score at blood collection (±std) PD (n = 46) GBA-PD (n = 5)	3.26 (±1.02)	3.00 (±0.71)	-	-	t (49) = 0.55, $p = 0.581$
Patients treated with Antiparkinsonians (%)	44 (44%)	6 (40%)	-	-	X2 (1) = 0.0001; $p = 0.9903$
Patients treated with Dopamine Agonist (%)	82 (82%)	10 (66.7%)			X2 (1) = 1.0781; $p = 0.299$
Patients treated with imiglucerase (%)			12 (17%)		
Patients treated with velaglucerase alfa (%)			16 (23%)		
Patients treated with eliglustat (%)			6 (9%)		
Patients treated with miglustat (%)			8 (12%)		

PD Parkinson's disease, GBA-PD GBA mutation carrier Parkinson's, GD Gaucher disease.

Absolute lipid levels are shown in **Fig. 3.5.2.**. One-way ANOVAs showed significantly different levels of a range of phospholipid classes between groups (PC: $p < 10^{-4}$, LPC: $p < 10^{-8}$, PE: $p = 0.002$, LPE: $p < 10^{-3}$, P-PE: $p = 0.001$, LPS: $p = 0.008$, PG: $p < 10^{-4}$). Post-hoc tests revealed increased PC, PE and LPE in the GD and PD groups vs. controls, decreased LPC in the GD, PD and GBA-PD groups vs. controls, as well as in PD vs. GD, increased P-PE in PD vs. controls and increased LPS and PG in GD vs. controls and PD.

When including age at sample collection and sex as variables, we found elevated serum PC levels in both the GD and PD patients when compared with controls ($p < 0.001$ and $p = 0.005$, respectively; **Table 3.5.2.A**).

In contrast, decreased LPC levels were present in these two groups ($p = 0.001$ and $p < 0.001$, respectively) and in the GBA-PD patients ($p = 0.002$) vs. controls (**Table 3.5.2.A**).

When comparing the three patient groups, the PC and LPC changes had higher magnitudes in the GD than in the PD group ($p = 0.036$ and $p = 0.008$, respectively; **Table 3.5.2.B**). The lipid levels were not influenced by the age at blood collection or sex (all $p > 0.05$).

Furthermore, the GD patients showed increased PE ($p = 0.001$), LPE ($p = 0.0001$), and P-PE ($p = 0.019$; **Table 3.5.2.A**) levels, when compared with controls. Elevation of LPE ($p = 0.012$) and P-PE ($p = 0.0004$; **Table 3.5.2.A**) was also observed in the PD group versus controls. Additionally, the GD patients showed increased levels of LPS and PG ($p = 0.001$ and $p < 0.001$, respectively; **Table 3.5.2.A**). These results were vastly replicable when assessing lipid levels according to the aforementioned 30% cut-off rate (method #2) and also when analyzing the relative lipid levels.

Dopamine agonist treatment in the PD group showed a positive association with the increase in the PC/LPC ratio and with P-PC levels ($p = 0.0278$ and $p = 0.014$, respectively). On the other hand, the H&Y scale or the

disease duration did not seem to influence the lipid levels (**Table 3.5.2.C**). All findings were replicable when analyzing relative lipid levels.

In the GD group, we found that velaglucerase alfa had a positive association with the increased PE and LPE levels ($p = 0.030$ and $p = 0.002$, respectively; **Table 3.5.2.D**). On the contrary, miglustat treatment normaized the elevated PC/LPC ratio ($p = 0.034$; **Table 3.5.2.D**). The latter finding remained significant when assessing lipid levels according to the aforementioned 30% cut-off rate (method #2) and also when analyzing the relative lipid levels (**Table 3.5.2.D**).

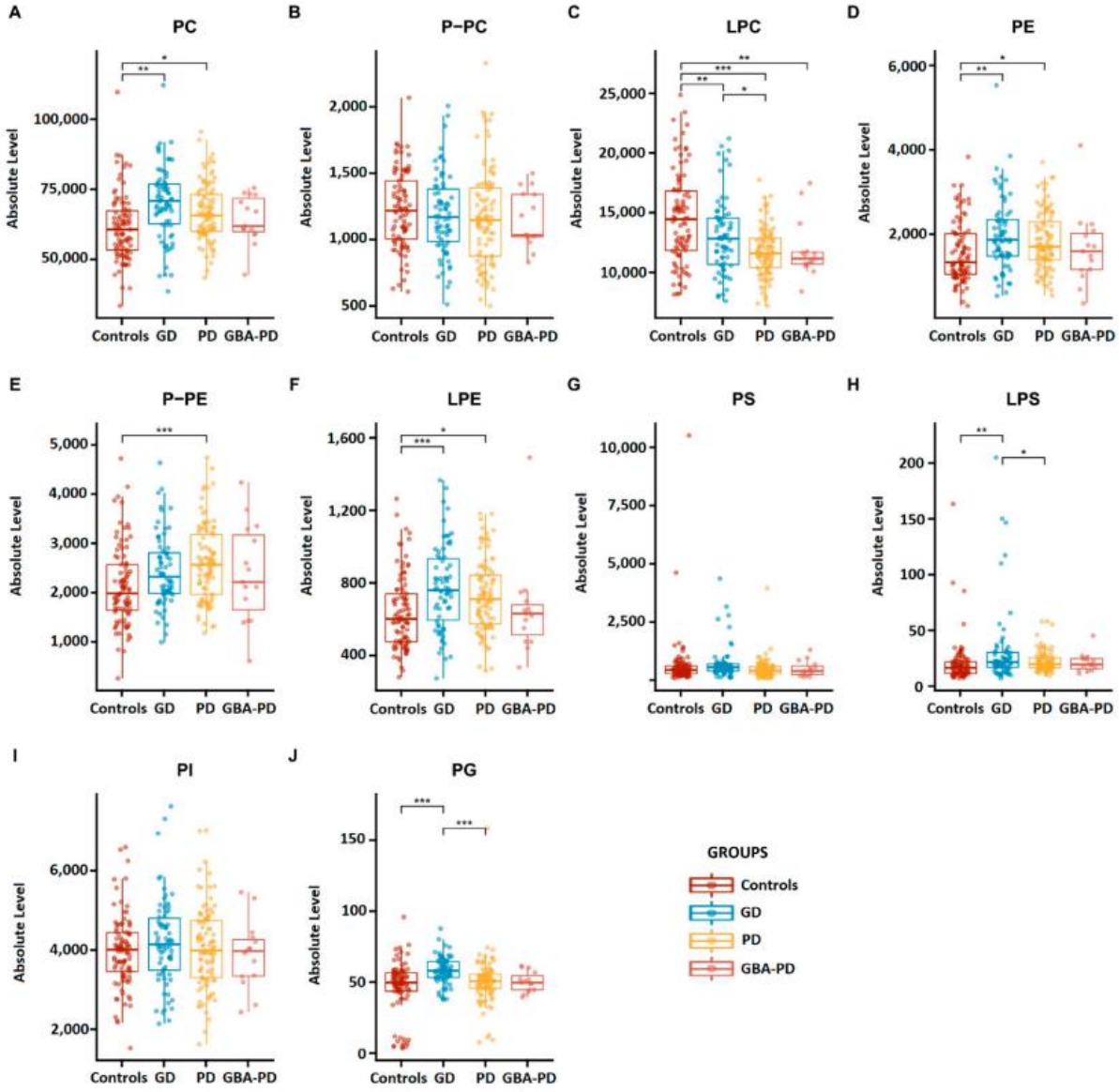


Fig. 3.5.2: Absolute phospholipid levels plotted by group (A–J).

PD Parkinson's disease, GBA-PD *GBA* mutation carrier Parkinson's, GD Gaucher disease, lipid nomenclature as in the text. Lipid data are expressed as normalized intensities relative to exactly measured internal standards and constitute relative abundances per ml plasma. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Table 3.5.2.

Phospholipids	PC	LPC	PC/LPC	P-PC	PE	LPE	PE/LPE	P-PE	PS	LPS	PS/LPS	PI	PG
Between-group analyses													
A. Patient groups vs. control group													
Intercept	0.532***	0.560***	4.408***	0.435***	0.149***	0.363***	0.467***	0.389***	0.068*	0.099***	0.611***	0.460***	0.289***
PD (n=100)	**0.043****	**-0.114*****	**1.268*****	-0.036	0.035	**0.052***	0.009	**0.086*****	-0.018	0.005	**-0.209*****	0.009	0.023
GBA-PD (n=15)	0.025	**-0.105****	**1.085*****	-0.026	0.026	0.012	0.032	0.056	-0.016	0.003	-0.166	-0.005	0.022
GD (n=70)	**0.076*****	**-0.063****	**1.131*****	-0.021	**0.070****	**0.088*****	0.030	**0.061***	0.009	**0.056****	-0.092	0.025	**0.073*****
Sex	0.011	0.002	0.019	0.02	0.029	0.018	0.039	0.03	0.012	0.019	0.030	-0.009	0.012
Age of Collection	0.018	0.033	0.128	0.116	**0.169*****	0.081	**0.250***	0.068	-0.017	-0.01	0.016	0.089	-0.0003
B. Between patient groups													
PD vs GD	**-0.033***	**-0.051****	0.183	-0.015	-0.035	-0.036	-0.021	0.025	**-0.027***	**-0.051****	-0.117	-0.016	**-0.05*****
PD vs GBA-PD	0.018	-0.009	0.137	-0.01	0.009	0.04	-0.023	0.03	0.002	0.002	-0.043	0.014	0.001
GD vs GBA-PD	0.051	0.042	0.046	0.005	0.044	0.062	-0.002	0.005	0.025	0.053	0.074	0.03	0.051
Within-group analyses													
C. Associations with PD disease severity, medication and disease duration (*n* = 46; PD patients)													
Intercept	0.752***	0.733***	4.032***	0.203***	0.338*	0.732***	0.358	0.693**	0.132	0.136*	1.196*	1.006***	0.509**
Hoehn and Yahr scale	-0.092	-0.087	0.212	0.073	-0.071	-0.094	-0.047	0.087	-0.029	0.009	-0.311	-0.162	-0.128
Dopamine Agonist	0.044	-0.027	**0.870***	**0.139***	0.019	-0.025	0.077	0.113	0.02	0.008	0.073	-0.005	0.083
Anti-Parkinsonian	-0.025	-0.05	0.437	0.0009	-0.033	-0.068	0.027	-0.016	0.015	-0.026	0.186	-0.031	0.069
Sex	**-0.045***	-0.014	-0.373	0.024	-0.045	-0.043	-0.057	-0.031	-0.002	-0.006	0.013	**-0.114****	-0.004
Age of Collection	-0.133	-0.169	0.679	0.19	0.089	-0.13	0.364	-0.29	-0.11	-0.031	-0.874	**-0.363***	-0.206
Disease Duration	-0.06	-0.097	0.830	-0.176	-0.006	-0.082	0.258	-0.185	-0.022	0.004	-0.153	-0.089	-0.172
D. Associations with GD therapy [on therapy (n = 42) vs untreated (n=27) GD patients]													
Intercept	0.516***	0.451*	6.221***	0.509**	-0.072	0.270	0.165	0.342	-0.021	0.199	-0.520	0.343	0.602***
Imiglucerase (n=12)	0.020	-0.057	0.418	0.034	-0.035	0.026	-0.0688	-0.047	0.031	-0.008	0.012	-0.018	0.028
Velaglucerase alfa (n=16)	0.064	0.012	0.165	0.097	**0.094***	**0.154****	-0.039	0.007	0.034	0.033	-0.030	0.029	0.056
Eliglustat (n=6)	0.094	-0.008	0.443	**0.138***	0.053	0.050	0.018	0.119	-0.026	-0.072	0.431	0.097	**0.147****
Miglustat (n=8)	-0.032	0.099	**-1.133***	-0.033	-0.072	0.019	-0.172	-0.039	-0.036	0.031	-0.406	-0.024	-0.050
Sex	0.035	**0.079***	-0.323	-0.016	**0.098****	0.062	0.095	0.087*	0.035	0.040	0.307	0.060	0.022
Age of Collection	0.094	0.164	-0.611	0.068	**0.497***	0.290	0.631	0.199	0.219	-0.085	2.266	0.229	0.035

Table 3.5.2. Serum lipid level alterations in the Iberian GD and PD cohort. (A). Patient groups compared with the control group. Results obtained, when controlling for sex and age at blood collection. Patients' sex coded as 0 (female) or 1 (male). (B). Patient groups compared with each other. (C). Association of PD disease severity, therapy and disease duration with lipid level changes. (D). Association of GD therapy with lipid level changes. (A–D). Results are presented as analyzed by method #1 (see "Quantification of the levels of lipid groups and their species"). Lipid nomenclature as in the text. Effects of the independent variables on the normalized lipid levels (dependent variables) are expressed by the corresponding estimated regression coefficients, with positive (negative, respectively) coefficients meaning that higher values of the independent variables are associated with higher (lower, respectively) values of the lipid levels. All variables were scaled so that their maximum absolute value was equal to 1. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Coefficients with P-values below <0.05/3 (i.e., surviving Bonferroni correction) are shown in bold.

Conclusion

Similarly to our study, PC/LPC ratio increase was also observed in other studies of PD [Miletić Vukajlović J, *et al.* 2020], but—to our knowledge—not yet reported in GD and GBA-PD. The altered PC/LPC ratio suggests a potential role of the lecithin-cholesterol acyltransferase and/or phospholipase A2 (Lp-PLA2) enzymes, catalysts of the PC-LPC metabolism [Law SH, *et al.* 2019], in GD and PD pathomechanism. In fact, higher serum Lp-PLA2 levels were reported in PD patients when compared to controls [Lin J, *et al.* 2015].

In PD patients, we found a positive association of dopamine agonists with the increased PC/LPC and P-PC levels. Comparing this finding with other studies, our results suggest that dopamine might activate PC-related cellular cascades, which may be altered in PD or affected by PD medication (for details see original paper and associated references).

Regarding the GD patients, similar to our observations in the serum, increased levels of PC, PI and PG have been reported in fibroblast extracts of GD1 and GD2 patients [Fuller M, *et al.* 2008]. Moreover, a study of Meilke *et al.* found differences in the levels of some PC and PI species in the serum of GD patients vs. controls [Meikle PJ, *et al.* 2008]. In this study, GD patients under ERT treatment showed similar increase in the phospholipid species, as those without ERT, suggesting that ERT has no preventive effect on the phospholipid changes in GD.

On the contrary, we found in these patients a normalizing effect of miglustat on the PC and LPC changes, and this finding was replicable with all methods. One of the hypothesized effects of miglustat is restoring lipid trafficking, although this mechanism is still not completely understood. Miglustat appeared to have beneficial effects on plasma lipid, lipoprotein, and C-reactive protein concentrations in therapy-naïve GD1 patients, resulting in an improved atherogenic lipid profile [Puzo J, *et al.* 2010].

3.6. Sphingolipid and phospholipid levels are altered in human brain in chorea-acanthocytosis [Miltenberger-Miltenyi G, *et al.* 2023]

(This work was carried out in collaboration with the Ludwig-Maximilians-Universität zu München)

Background

Chorea-acanthocytosis (ChAc) is a rare neurodegenerative disease resulting from biallelic mutations in the vacuolar protein sorting-associated protein 13A (*VPS13A*) gene [Peikert K, *et al.* 2023; Dobson-Stone C, *et al.* 2002], a member of the VPS13 gene family [Velayos-Baeza A, *et al.* 2004]. In line with recent developments in the nomenclature of genetically defined diseases, "VPS13A disease" is proposed as a synonym

[Walker RH, *et al.* 2021]. Over 140 *VPS13A* mutations have been reported; most resulting in complete loss of the VPS13A/chorein protein [Dobson-Stone C, *et al.* 2004]. Mutations in other VPS13 family members have been reported in Cohen syndrome (VPS13B) [Kolehmainen J, *et al.* 2003], parkinsonism (PD23) and Lewy body disease (LBD; VPS13C) [Lesage S, *et al.* 2016; Smolders S, *et al.* 2021], and in variable neurological syndromes with ataxia (VPS13D) [Meijer IA. 2005].

As chorein is implicated in lipid transport at intracellular membrane contact sites (see Section: State of the Art), here we aimed to determine sphingolipid and phospholipid level changes in ChAc patients vs. controls.

Patients & Methods

We analyzed 593 lipid species in the caudate nucleus (CN), putamen, and dorsolateral prefrontal cortex (DLPFC) from post-mortem tissues of four ChAc and six non-ChAc patients (see Supplementary Methods in original paper).

Lipidomics profiling was performed using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MSMS). The analyzed lipid groups are presented in **Fig. 3.6.1.**.

The levels of each lipid species in each region (CN, putamen, and DLPFC) were normalized to mol %.

Our lipidomic assay quantified the levels of 593 lipid species in three brain regions (DLPFC, CN and putamen) from each of 10 subjects (6 control and 4 ChAc). In addition, ChAc status (Dx), age at each subject's death (Age at Death) were included as variables.

To demonstrate lipid level expressions, we used heatmaps after standardizing lipid levels separately for each species across all 30 observations.

We fitted models with the statsmodels Python package. Goodness-off it was confirmed using the Jarque-Bera test and normal Q-Q plots of residuals. For each lipid group, we formulated our inference on ChAc-associated lipid changes using estimates of three regression parameters of the Dx:Region term, which quantify the change of lipid level in ChAc vs. control in each of the DLPFC, CN and putamen regions.

We also tested the corresponding three null hypotheses of no change in a given lipid group's level in ChAc vs. control and performed multiple hypothesis test correction with both the Bonferroni and the Benjamini-Hochberg procedures.

Results

There was no evidence indicating a difference in age at death between the ChAc (50 yrs, range 40-61) versus the non-ChAc (55.3 yrs, range 50-59) groups ($p = 0.25$). Nonetheless, we included age at death as an explanatory variable in our linear models, which, for some lipid groups, marginally improved model fit (not shown), suggesting age-related lipid changes in these cases.

We jointly analyzed lipid level changes across all species in a given lipid group, which afforded us enhanced statistical power despite the scarcity of the present data. This approach required standardization of lipid levels for each species, which was also essential for interpretable heatmaps (**Fig. 3.6.1.**). The heatmap for the entire data revealed no wide-spread differences between control and ChAc samples. In contrast, a few local heatmaps restricted to single lipid groups showed increased lipid levels in ChAc specifically in the CN

and putamen but not in the DLPFC. Consistently with these findings, data-driven clustering of samples showed moderate tendency to segregate control and ChAc samples.

We fitted a linear mixed model onto the standardized lipid levels for each lipid group to infer brain region-specific lipid changes in ChAc relative to control. Bis(monoacylglycero)phosphate (BMP), Sulfatide (Sulf), Lysophosphatidylserine (LPS) and Phosphatylcholine ether (PCe) levels were increased significantly (at 5% FDR, Benjamini-Hochberg procedure) in both the CN and putamen, but not in the DLPFC, of ChAc patients vs. controls (**Fig. 3.6.2.**). PS and MG were increased only in the CN, while NAPS only in the putamen in ChAc (**Fig. 3.6.2.**). The only significant lipid level decrease was observed for NSer in the CN and DLPFC, although in both regions the change was modest (**Fig. 3.6.2.**). Inspecting the lipid levels in individual subjects showed that these lipid changes were not primarily driven by outlier ChAc or control samples (**Fig. 3.6.3.**).

We obtained concordant although, as expected, weaker results when fitting the same linear mixed models to an aggregated "group-level" data set, whereby for each lipid group the lipid level was summed across all corresponding species.

We also investigated associations between lipid sidechain length and ChAc, but did not find evidence for such association.

Furthermore, the results were not affected by the post-mortem intervals.

In summary, we found strong statistical evidence for increased levels of several lipid groups in the striatum of ChAc subjects.

Fig. 3.6.1.

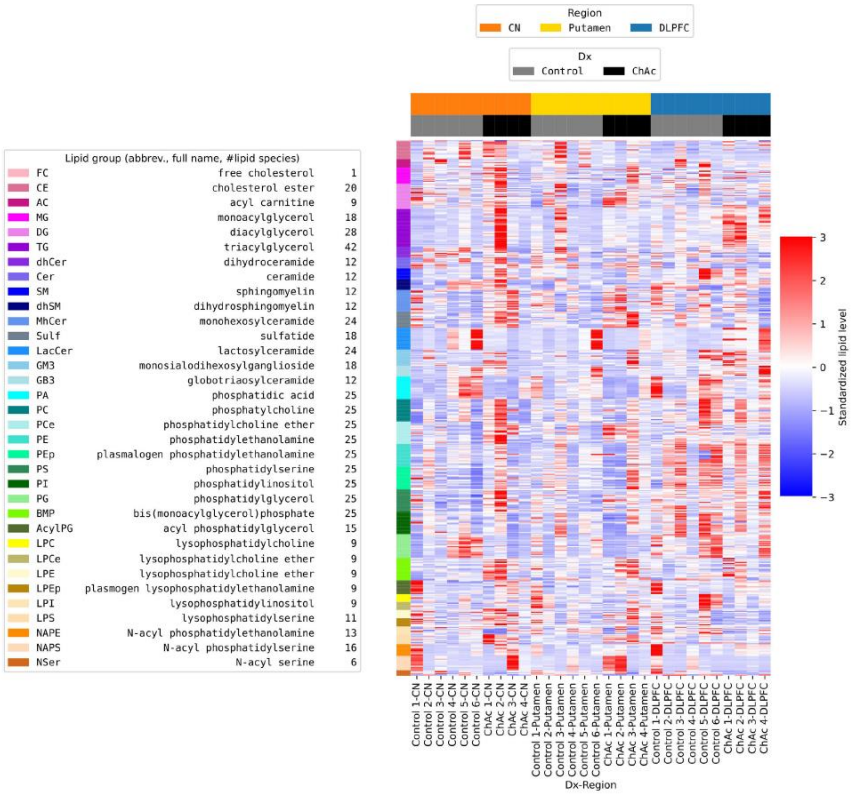
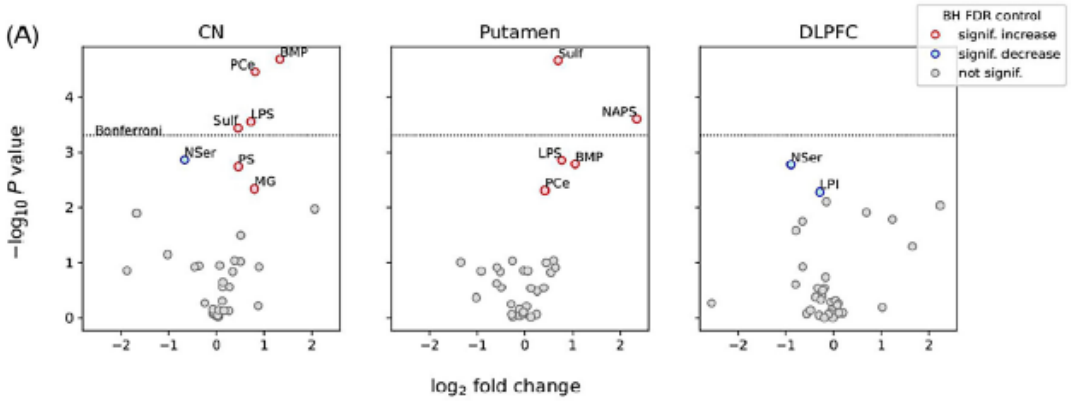


Fig.3.6.1: Heatmaps depicting standardized lipid levels in the brain samples of non-ChAc (controls) and ChAc patients. X axes: samples from three brain regions from each of the 10 subjects (6 control and 4 ChAc). Y axes: 593 lipid species

of the 34 lipid groups. For each species, lipid level was standardized across all 30 samples so that, for each species, the average level is 0 and the standard deviation is 1.

Fig. 3.6.2.



(B)

Lipid group	ChAc vs controls in CN	ChAc vs controls in putamen	ChAc vs controls in DLPFC
FC	−0.09	0.34	−0.45
CE	0.12	−0.65	0.18
AC	−0.12	−0.14	−0.46
MG	0.79	0.46	−0.10
DG	0.09	0.56	0.07
TG	1.09	−0.05	1.02
Cer	0.23	0.04	−0.08
dhCer	−0.14	−0.21	−0.26
SM	0.85	−0.03	−0.32
dhSM	0.36	0.13	0.20
MhCer	0.77	0.66	−0.34
Sulf	1.28	1.52	−0.05
LacCer	−0.79	−0.42	1.05
GM3	0.37	0.36	0.62
GB3	−0.22	−0.42	1.12
PA	−0.94	−0.77	−0.81
PC	0.71	0.08	0.00
PCe	1.33	0.91	−0.05
PE	−0.02	−0.34	0.08
PEp	0.36	0.09	−0.35
PS	0.89	0.30	0.16
PI	−0.24	−0.47	−0.53
PG	−1.00	−0.67	−0.43
BMP	1.22	0.90	0.72
AcylPG	0.09	0.11	−0.13
LPC	−0.15	−0.64	−0.98
LPCe	0.44	−0.06	−0.85
LPE	0.66	0.62	−0.42
LPEp	0.62	0.45	−0.49
LPI	−0.54	−0.54	−0.98
LPS	1.39	1.22	0.00
NAPE	0.22	0.03	−0.22
NAPS	0.54	1.28	−0.21
NSer	−1.05	−0.48	−1.03

Fig. 3.6.2. Lipid-level alterations in the brain extracts of ChAc, compared to non-ChAc, patients. (A) The X axis of the volcano plots shows the log-transformed-fold change in the expression of lipid groups in ChAc relative to non-ChAc patients. The shared Y axis of the volcano plots shows the minus log-transformed p-values for the null hypothesis of no lipid level change in ChAc relative to controls in a given brain region. P-values were obtained from linear mixed models controlling for brain region, age at death, and subject-to-subject variability (see Regression model in Supplementary Methods for details). Labelled red/blue symbols mark lipid groups with significantly increased/decreased levels in ChAc, respectively, after 5% FDR control with the Benjamini-Hochberg procedure (BH FDR, in the legend). The horizontal dotted line shows the threshold for the more conservative Bonferroni procedure. (B) Estimated regression coefficients reporting on the change in lipid level in ChAc relative to control in the CN, putamen and DLPFC. Because of data standardization the unit is one standard deviation across all 30 samples. Color coding as in (A).

Fig. 3.6.3.

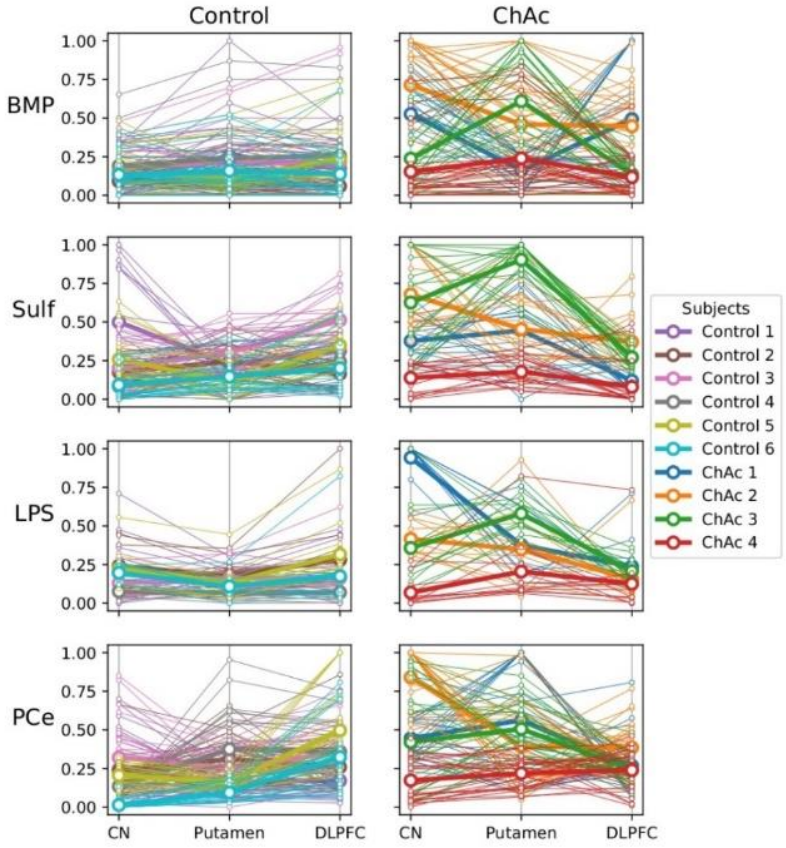


Fig. 3.6.3. Lipid groups with significantly increased levels in the CN and putamen of individual control and ChAc patients. Empty circles show actual lipid levels; the lines connecting circles for individual subjects are only included to enhance visual interpretation. Small circles and thin lines indicate normalized levels of the individual lipid species. Large circles and thick lines show the mean levels across species for each lipid group and subject.

Conclusion

We found significantly elevated levels of several sphingolipids and phospholipids in the striatum of ChAc patients when compared with non-ChAc controls indicating that these lipids may play an important role in ChAC pathophysiology. Our data are supported by the remarkable consistency of our findings across regions for BMP, Sulf, LPS, and PCe.

Elevated BMP levels were reported in the substantia nigra of PD patients [Xicoy H, *et al.* 2020] and BMP isoforms are seen as candidate biomarkers of LRRK2 activity, potentially useful for clinical trials of LRRK2-targeted therapies [Alcalay RN, *et al.* 2020]. Relatedly, depletion of PD23- associated VPS13C, a lipid transfer protein localized at contact sites between the ER and late endosomes/lysosomes resulted in abnormal accumulation of BMP isoforms in lysosomes in HeLa cells [Hancock-Cerutti W, *et al.* 2022].

Sulf was significantly increased in the CN and in the putamen of the ChAc patients, similar to that recently reported in PD and LBD [Beger AW, *et al.* 2022].

In the brains of Vps13aKO mice, neuronal loss and neuroinflammation, with increased microglial density and increased activation of NF-kB p65 in both cortex and basal ganglia was reported [García-García E, *et al.* 2021; Peikert K, *et al.* 2021]. This is compatible with the increased LPS levels in the CN and in the putamen

of the ChAc patients, as LPS is involved in microglial inflammatory responses and gliosis, both parts of the ChAc pathophysiology [Chan RB, *et al.* 2012].

Finally, the increased PCe in the putamen of ChAc patients is consistent with findings in VPS13CKO HeLa cells, that showed reduced PCe in the lysosomes, but an overall increase in the total cell lipidome, suggesting intact biosynthesis with defective intracellular trafficking to the lysosome [Hancock-Cerutti W, *et al.* 2022].

In summary, we presented the first evidence of altered sphingolipid and phospholipid levels in ChAc patients. Our observations are congruent with recent findings in cellular and animal models, and implicate defects of lipid processing in VPS13A disease pathophysiology.

4. Conclusions and outlook

Studies on genotype-phenotype associations are currently one of the most challenging tasks for the professionals (clinicians, clinical geneticists, psychologists, students, basic researchers) involved in the field of neurologic diseases requiring on the clinical level a long-term observation of the patients and on the genetic level the identification of modifying factors influencing the impact of the genetic variants. Novel results can be achieved more effectively by (a) interdisciplinary and (b) international collaborations.

Even more since the appearance of modern sequencing technologies, such as next generation sequencing (NGS, gene panels, whole exome/genome sequencing), the role of clinical geneticists underwent a slight change and – beside the daily patient counseling - less bench-work and more interpretation of individual genetic findings has become main priority. These tasks are in continuous increase in number as a consequence of the permanent modernization of sequencing methods and rapidly increasing amount of genetic data.

At the same time, the landscape of possible biomarker candidates in various diseases, such as PD or ChAc is relatively narrow. The identification of such biomarkers with applicability to early diagnosis and treatment stratification is therefore a relevant, unmet medical need.

Studies on these fields are the main interest of the applicant of this Habilitation.

As such, within this Habilitation project, the applicant could answer several of the open questions regarding the genotype-phenotype associations and the role of sphingolipids and phospholipids in various neurologic diseases, thus contribute to a better understanding of the pathomechanism and to putative biomarker search in neurologic conditions.

The applicant works since 2000 in the field of Human Genetics. Besides his position as medical doctor and university teacher, he was head of a molecular genetic diagnostic lab during 7 years (Faculty of Medicine, University of Lisbon). Thus, he gained experience in different laboratory techniques (sequencing, Western blot, lipidomics, etc.).

His research works are usually translational projects, creating a bridge between classical clinicians [Azevedo O, *et al.* 2019; Azevedo O, *et al.* 2020; De Carvalho M, *et al.* 2017], basic scientists [Pinho R, *et al.* 2016; Park JS, *et al.* 2022] and bioinformatic experts [Guedes LC, *et al.* 2017; López de Frutos L, *et al.* 2022; Miltenberger-Miltenyi G, *et al.* 2023], all that is needed in today´s human genetics, especially in the times of next generation sequencing.

Within the above-mentioned projects led by the applicant and with the projects in which he collaborated with other labs, he supervised the daily work of technicians, students, and young physicians.

Furthermore, with his projects he set up national and international collaborations to Germany, the UK, US, Australia, Spain and Austria. Four major works [Peikert K, *et al.* 2023; López de Frutos L, *et al.* 2022; Miltenberger-Miltenyi G, *et al.* 2019; Miltenberger-Miltenyi G, *et al.* 2023] were carried out during his works at Ludwig-Maximilians-Universität zu München. Further papers from works at Ludwig-Maximilians-Universität zu München will follow.

The above-mentioned projects gave a solid basis to continue with similar studies on genotype-phenotype associations as well as with further projects on sphingolipids and phospholipids in neurologic (or other) diseases.

Currently, the applicant of this Habilitation is principal investigator in a project on sphingolipid changes in dementia and a further project on genotype-phenotype association studies in Fabry disease. Also, further projects about LSDs, neurologic diseases, sphingolipids and phospholipids are currently under his construction.

Taken together, the applicant aims to continue with scientific projects, and he aims to continue and strengthen collaborations with the Ludwig-Maximilians-Universität zu München.

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7. Own publications quoted in the Habilitationsschrift

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