GENETIC AND CLINICAL PHARMACOLOGY STUDIES IN *GBA1*-ASSOCIATED PARKINSON'S DISEASE

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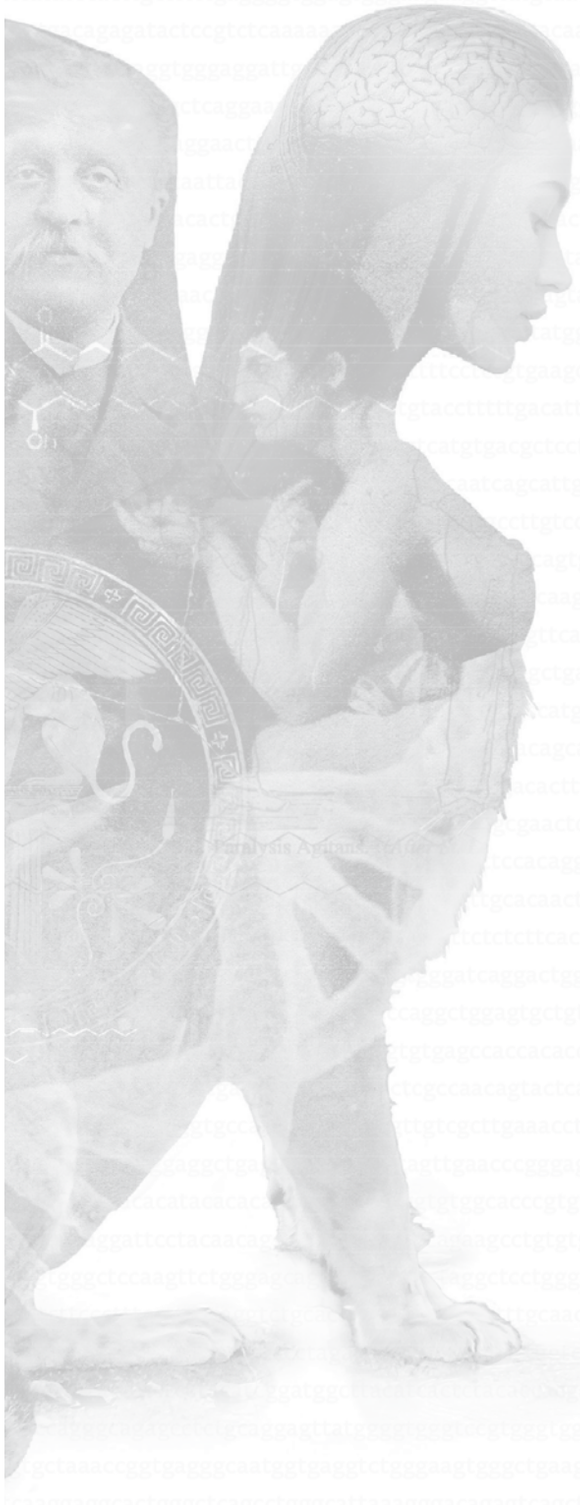

CHAPTER 1

Introduction

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New insights in the pathobiology of Parkinson's disease and possibilities for pharmacotherapy

(Nieuwe inzichten in de pathobiologie van de ziekte van Parkinson en mogelijkheden voor farmacotherapie).

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Over 200 years ago, in 1817, James Parkinson first described the characteristics of the disease that now carries his name in *An Essay on the Shaking Palsy*. [1] In the two centuries following this, our knowledge concerning Parkinson's disease (PD) has increased substantially. The clinical spectrum has been much better characterized, consisting of both motor symptoms and many non-motor-symptoms, like cognitive-, fear-, mood-, sleep-, autonomic- and olfactory dysfunction. The classical concept that this affliction is pathologically distinguished by a progressive loss of the dopaminergic neurons in the substantia nigra pars compacta, has been replaced by a peripheral and central multi-system disorder, associated with cumulative aggregation of the protein alpha-synuclein (α-syn) in axons and synapses, and in cellular inclusion called Lewy Bodies.[2] The role of inflammation in cell loss is both reactive and causative, by both the innate and adaptive immune system.[3] Additionally, epidemiology and genetics contributed to insights how many factors can contribute to the development of this disease. In the Netherlands, the number of people with PD is estimated at 60. 000 [4] with a prevalence of approximately 1% in people above 60 years, up to 2. 5% in people above 80 years. [5] Currently, almost all pharmacotherapy aims to supplement the dopamine deficit, exclusively facilitating symptomatic relief (primarily of motor symptoms), while progression of the underlying disease-process continues. Development of a disease-modifying therapy is of great importance.

Etiology and Genetics

Better understanding of the underlying pathobiology is essential for the development of new drugs. In recent years, the importance of certain impairments in the quality assurance of cellular proteins (proteasome and lysosome) and mitochondria (mitophagy) as well as inflammatory mechanisms in PD pathobiology have become apparent. As a result, independent of primary disease mechanisms, intracellular aggregation of α-syn occurs in the great majority of patients.[6–8] The initiation of these processes and the exact role of α-syn and Lewy Bodies are still under discussion. Animal studies show that overexpression of α-syn alone does not cause neurodegeneration, but only in combination with microbiome changes.[9] The presence of Lewy Bodies has been associated with neurodegeneration in the area in question, but there are also indications that it actually acts neuro-protectively by trapping dysfunctional α-syn.[10] It is crucial to understand the relationship between these different processes in order to better identify potential leads for the development of new drugs.

Regarding the role of genetic factors, in 5-10% of patients PD is explained by dominant or recessive inherited mutations (Table 1), implying that the vast majority of patients have sporadic disease, probably due to an interplay of genetic- and environmental factors. The first genetic causality was found in 1997 in the *SNCA* gene, encoding the protein α-syn. In the following years, hereditary factors involved in PD have been shown to play a role in important processes for the quality control of proteins and mitochondria, whereby dysfunctional mitochondria are cleaned up by the autophagy-lysosomal system.[8,11] For each of these processes, a relationship with α-syn has now been demonstrated. Several genome-wide association studies (GWAS) have shown up to 90 genetic factors (most robust for *SNCA*, *MAPT*, *LRRK2* and *GBA1* (Table 1)) that slightly increase the risk of the disease.[12] Depending on the type of mutation in the *SNCA* and Leucine-rich repeat kinase 2 (*LRRK2*) gene, this plays a role as a dominant inheritance or genetic risk factor in patients with the sporadic form of the disease. Better understanding of the function of these specific genes and elucidation of cellular processes provide insight into the pathobiology of PD, which in turn offers the potential for pharmacotherapeutic intervention. Below, the different genes are grouped according to their role in a particular cellular process and how different disease mechanisms are interrelated. Table 2 provides an update on investigational drugs targeting these mechanisms that were discussed in 2017.

Mitochondria and Quality Control

The mitochondrion is an organelle that allows for energy supply of the cell, and dynamically operates by moving, dividing, and merging constantly, to ensure quality. It interacts with other organelles such as the lysosome and endoplasmic reticulum (ER), where proteins are made. Mitochondria

naturally decline in quality and may then be cleared by mitophagy. New mitochondria arise again through mitochondrial division. The right balance between well-functioning and less well-functioning mitochondria is essential for healthy cell function. Impaired mitophagy leads to an imbalance with mitochondrial dysfunction and neuronal degeneration as a result.[13] *Parkin, PINK1* and *DJ-1* are three genes with autosomal recessive inheritance which all are concerned with the quality control system of the mitochondrion.[8,11] All three genes are characterized by an early-onset presentation with good levodopa response.[11,14] *Parkin* and *PINK1* collaborate in clearing dysfunctional mitochondria. *DJ-1* may protect against mitochondrial damage from oxidative stress and is involved in the *Parkin-PINK1* system, regulating mitochondrial fusion and division.[13] A homozygous mutation in one of these genes disrupts this system to such an extent that the quality of the mitochondria begins to fail. For α-syn it has been shown as well to influence mitochondrial function, because it precipitates in mitochondria, causes oxidative stress and ultimately leads to mitochondrial dysfunction. Conversely, it is known that mitochondria play an important role in axonal transport, including that of α-syn, both anterograde (to the synapse) and retrograde (to the cell body). In case of mitochondrial damage, the equilibrium in transport shifts to retrograde transport, possibly resulting in the accumulation of α-syn in the cell body as a result.[15]

Maternal inheritance is seen in only a small portion within familial PD, which is consistent with mitochondrial inheritance. [16] Acquired mitochondrial damage may also play a role in PD. In the early 1980s, some young drug addicts presented with an acute syndrome almost indistinguishable from PD, after self-injection of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). [6] MPTP inhibits complex 1 in the mitochondrion and limits mitochondrial transport.[15] Post-mortem studies of PD patients have also shown a reduced complex 1 activity in the substantia nigra.[16] A further indication of mitochondrial involvement is based on animal testing, in which parkinsonism is induced after administration of complex 1 inhibiting herbicides and pesticides. The herbicide paraquat, which is now prohibited in the EU, is possibly associated with an increased risk of PD. Due to methodological challenges, this relationship has not yet been proven beyond doubt.[8,17]

Several forms of gene therapy targeting mitochondrial mechanisms, including *parkin* and *PINK1*, are currently in the preclinical stage of development. Clinical trials of gene therapy in PD have so far shown too little effect to get past phase 2 trials.[14] Nilotinib inhibits the non-receptor tyrosine kinase Abelson (c-Abl). In PD, c-Abl is over- active and phosphorylates α-syn and *parkin.* This phosphorylation inhibits the function of *parkin* and the autophagy of α-syn. Nilotinib is used in the treatment of certain forms of leukemia, but lower dosages may improve autophagy of α-syn.[18,19] A 6-month[20] and another 12-month[21] randomized placebo-controlled trial showed no clinical effect (but it's questionable whether this could be expected in this time frame, based on the mechanism). A phase 3 trial will likely follow.

Given the role of oxidative stress in the development of mitochondrial damage, several agents with an antioxidative effect have been investigated, such as coenzyme Q10 and MitoQ. None of these drugs were developed beyond phase 3 trials, due to the lack of a demonstrable effect on the course of the disease.[14] A recent phase 3 trial for inosine, a uric acid precursor that also has antioxidant functions, was terminated early due to lack of effect.[22,23]

Autophagy-lysosomal system

The autophagy-lysosomal system is a system that clears up waste products and dysfunctional organelles through different routes.[11,24] Small waste substances can be directly absorbed by the lysosome, larger substances need help with this and are accompanied by a chaperone. Organelles are first encapsulated (autophagy) and then fuse with the lysosome to be degraded. Several proteins are involved in transport to the lysosome, including *LRRK2* and *VPS35*, which in the case of a mutation can lead to autosomal dominant inheritance of PD.[25,26] Mutations in *LRRK2* explain about 10% of familial PD and mutations in *VPS35* only 0. 1-1% of familial PD. [11] Response to levodopa seems comparable to idiopathic PD for both genes.[26,27] So these are rare mutations, but both emphasize that a disorder in this autophagy-lysosomal system can contribute to the development of PD.

LRRK2 is a complex and large protein, with various enzymatic and interaction domains, among others involved in different transport processes.[7,11] A mutation in *LRRK2* blocks one of the transport systems to the lysosome, namely the chaperone-mediated autophagy system, which also facilitates transport and degradation of alpha-synuclein.[25] Blockade of this transport system leads to α-syn accumulation and -aggregation outside of the lysosome.[25] There is growing evidence of the other functions of *LRRK2*; *LRRK2* also appears to be involved in mitochondrial fusion and transport, and cytoskeletal dynamics, which provide transport within the cell in general.[28]

Research into drugs that target *LRRK2* has been under development for some time, mostly focused on inhibition of the *LRRK2* kinase domain of the enzyme. Because *LRRK2* is a complex molecule with several active domains, it has a greater risk of off-target effects, seen in lung and kidney preclinically, also if the kinase domain is inhibited selectively. There are several agents that penetrate the blood-brain barrier, which are undergoing further preclinical testing.[28] Two investigational *LRRK2* kinase inhibitors (DNL151 and DNL201) finished a first-in-patient study, of which the results are being awaited (ClinTrials: NCT04056689 and NCT03710707).

Vacuolar protein sorting-associated protein 35 (*VPS35*) is part of the *retromer complex*, which recycles proteins from the lysosome to the Golgi system for reuse.[26] This is necessary for adequate functioning of lysosomal membrane proteins and enzymes. In the case of a mutation in *VPS35*, there is a reduced transport of the lysosomal membrane protein Lamp2a, which leads to decreased endocytosis of a *parkin* substrate, inducing apoptosis.[29] Impaired transport of enzymes interferes with lysosome function, which has preclinically been associated with α-syn accumulation.[7] These mechanisms, and potential pharmacotherapeutic targets, are specific for the subgroup of patients with such a mutation. However, recent investigations also suggest that *LRRK2* has a role in the pathogenesis of idiopathic PD.[0] It remains to be determined whether any therapy will be effective for all patients or only those with a specific mutation.

GBA1 associated PD

An enzyme in the lysosome, which is associated with sporadic PD is glucocerebrosidase (GCase), encoded by the *GBA1* gene. A damaging variant in the *GBA1* gene is currently the most common genetic risk factor for developing PD (GBA-PD);[31] a heterozygous mutation in this gene is found in 4-12% of people with sporadic Parkinson's, up to 15% in the Netherlands (**chapter 2**), and up to 20% in Ashkenazi Jewish.[32–34] Sequencing of this gene is methodologically challenging, due to presence of a pseudogene (risk of false-positive results) and due to susceptibility for an allelic imbalance when amplifying the gene (risk of false-negatives). The pseudogene is a highly homologous piece of DNA, which does not get transcribed, next to the functional gene. This pseudogene can contain mutations, which can falsely be attributed to the functional gene. This can be overcome by using a primer set unique to the functional gene. The allelic imbalance means that the two alleles are not amplified equally, which can result in a mutation not being detected adequately. This was in our case resolved by using a different polymerase enzyme (**chapter 3**). GBA-PD is associated with an average of five years earlier age of onset and faster progression of complaints, both motor and cognitive. Response to regular PD medication is similar to idiopathic Parkinson's.[31] It is considered a risk factor because most people with a *GBA1* mutation will not develop PD.[31] The type of mutation determines how much greater the probability is; this is increased by an estimated overall 2- to 7-fold (odds ratios [ORs]).[32–36] Since the absolute risk is still small and there are no therapeutic consequences, experience in genetic couseling for *GBA1* variants in PD is still limited. Despite the on average worse disease course, counseling requires sufficient nuance due to the high inter-individual variability in phenotype of GBA-PD (**chapter 4**). Certain intronic variants may also influence age at onset in a subgroup of patients (**chapter 5**). What is special about this gene is that it was not found by GWAS studies, but by clinical observation. A homozygous mutation in the *GBA1* gene may cause the rare lysosomal storage disease of Gaucher. It can present with hepatosplenomegaly, skeletal and blood disorders and in severe cases neurological abnormalities and early death.[37] In the early 1990s, enzyme replacement

therapy was developed as a breakthrough, which is a very effective treatment of the peripheral stacking of the GCase substrate glucosylceramide (GluCer). In the years that followed, it turned out that, despite this replacement therapy, patients with Gaucher often developed parkinsonism. This was explained by the fact that the therapy does not cross the blood-brain barrier, so that GluCer continues to accumulate in the brain. Based on these findings, *GBA1* seems a promising target for a potential first disease-modifying treatment in PD.

GBA1

Sufficiently decreased GCase activity leads to accumulation of GluCer in the lysosome, resulting in dysfunction of the autophagy lysosomal system, primarily seen in Gaucher's disease.[24] **Chapter 6** describes how certain glycosphingolipids (like GluCer) may be used as biomarkers in clinical trials and that GluCer is elevated in plasma only in GBA-PD compared to healthy volunteers. In preclinical models, mutations in the *GBA1* gene lead to accumulation of α-syn, but vice versa, induced α-syn overexpression also leads to decreased GCase activity. It is a self-amplifying process, aggravated by age-related degradation of the relevant enzymes.[11,31] Mutations in the *GBA1* gene can also cause the wrong folding of GCase, which can cause trapping of the misfolded enzyme in the endoplasmic reticulum (ER), which needs to be cleared via a dedicated control system. *Parkin* ubiquitinates misfolded protein, so that they are cleaned up by the lysosome or proteasome. Excessive use of this system creates 'ER stress', as a result of which other proteins, such as α-syn, can no longer be properly broken down.[38] Cell studies show GCase depletion also amplifies cell-to-cell transmission of α-syn.[39] Alpha-synuclein exocytosis takes place mainly when this is accumulated in a cell, as a back-up processing mechanism complementary to the lysosome, so that adjacent microglial can clean this up, which in a healthy situation have an increased capacity to phagocytosis and lysosomal degradation.[7]

Since the *GBA1* mechanism in PD came to light, several companies are trying to develop a drug that targets this. Ambroxol, a small molecule chaperone, has been shown in animal studies to cross the blood-brain barrier, activate GCase and lower α-syn.[40] In an open-label trial in GBA-PD, ambroxol was safe and well-tolerated and penetrated CSF.[41] Results of an RCT are being awaited (ClinTrials: NCT02914366). A second agent, venglustat (GZ/SAR402671), is a synthesis inhibitor of the substrate of GCase (GluCer), which in animal studies also leads to a reduction in α-syn and shows an improvement in memory experiments.[42] First-in-patient studies of venglustat showed favorable safety and tolerability, with CSF penetration and reduction of plasma and CSF GluCer,[43] but a 52-week efficacy trial unfortunately did not meet primary endpoints and further development for GBA-PD is stopped.[44,45] In **chapter 7 and 8**, results are discussed of LTI-291, a GCase activator.

Immune response

Activated microglial cells in the substantia nigra have been reported nearly a century ago and cytokine profiles have shown that the innate immune system is involved.[3] Recent studies now also show involvement of the adaptive immune system. Dopaminergic neurons present antigens via MHC-I in response of microglial cell cytokines, activated by α-syn, upon which cytotoxic T cells clear them. In addition, a specific MHC-II subtype is associated with PD and occurs in 30% of patients, in contrast to only 15% of healthy controls. This MHC-II subtype appears to be more sensitive to present α-syn and thus induce an immune response.[3] In a study with 67 PD patients, 40% of the patients had an immune response against an epitope of α-syn. *Parkin* and *PINK1* may regulate antigen presentation of mitochondrial peptides, representing the interface between the lysosomal and mitochondrial mechanisms in the pathogenesis of PD.[3,46]

Immunotherapy is in development, separated into active therapy, by means of vaccinations that induce a humoral and cellular reaction against α-syn aggregates, and passive therapy, by means of antibodies directly targeting α-syn aggregates. Preclinical research shows a decrease in α-syn aggregates and the several first-in-patient studies were safe and efficacy results are being awaited.[47–50] (ClinTrials: NCT04075318) Active and passive immunotherapy is also being investigated for amyloid beta and tau in

Alzheimer's disease and other related neurodegenerative diseases with different phase 1, 2 and 3 clinical trials. Despite promising preclinical results, patient studies in Alzheimer's disease unfortunately did not demonstrate clinical improvement.[50]

Other strategies

Some other developments have taken place based on epidemiological research. Various substances may be protective against developing PD, like the use of dihydropyridine calcium antagonists, beta agonists, nicotine and caffeine. For isradipine, a dihydropyridine calcium channel blocker, a 36-month RCT did not show a clinical neuroprotective effect.[51] An 18-month trial on transdermal nicotine showed a worsening effect compared to placebo.[52] A phase 3 trial on caffeine versus placebo showed no effect on motor symptoms after 18 months of treatment, but a slight worsening of cognition and dyskinesias in the caffeine group.[53] Beta agonists are currently being investigated. (Dutch public trial registry Trialregister: NL8002)

The effect of influencing the microbiome on the course in preclinical models of PD is developing rapidly. The role of the microbiome as a pharmacotherapeutic target in humans will become clearer in the coming years.[9,54]

An elaborate overview of investigational drugs targeting PD in various stages of development was recently published.[55]

Conclusion

As the complexity of mechanisms involved in PD is increasingly unraveled, a growing number of possibilities is uncovered to develop targeted pharmacotherapy. Thus, hope arises for a better perspective for patients with PD. Since the vast majority of patients have a non-familial form of PD, and the pathogenesis here is multifactorial, the question is whether pharmacotherapy will not consist of a multi-target approach, possibly adapted to the dominant mechanisms of an individual. This thesis focusses on the further unravelling of one of these mechanisms: the *GBA1* gene, encoding the lysosomal enzyme GCase. Several questions are addressed: How prevalent are mutations in this gene in the Netherlands and does it affect disease onset (**chapter 2** and **5**)? What methodological challenges accompany the sequencing of this gene (**chapter 3** and **4**)? What biomarkers may be used in clinical trials targeting GCase (**chapter 6**)? And what are the effects of the novel GCase activator LTI-291, when first administered to healthy volunteers (**chapter 7**) and to GBA-PD patients (**chapter 8**)?

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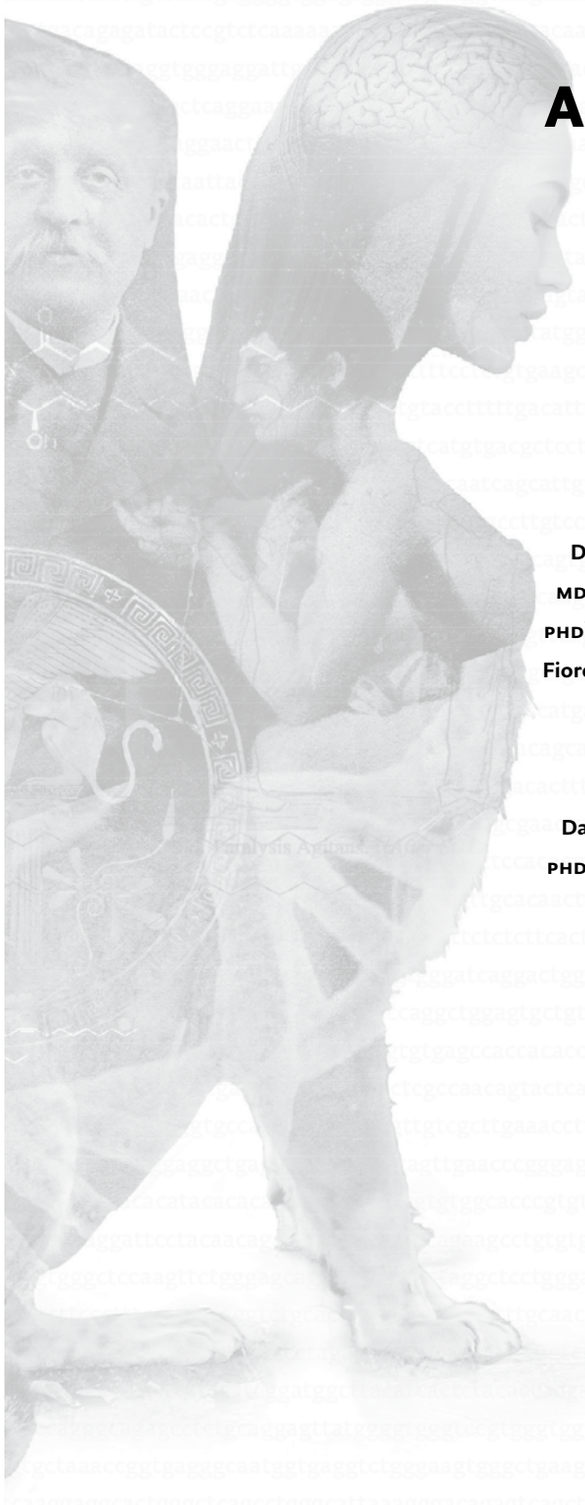

CHAPTER 2

A large-scale full *GBA1* gene screening in Parkinson's disease in the Netherlands

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Abstract

Background The most common genetic risk factor for Parkinson's disease known is a damaging variant in the *GBA1* gene. Rarely the entire *GBA1* gene has been studied in a large cohort from a single population.

Objective Assess the entire *GBA1* gene in Parkinson's disease from a single large population.

Methods The *GBA1* gene was assessed in 3402 Dutch Parkinson's disease patients using next generation sequencing. Frequencies were compared to Dutch controls (n=655). Family history of Parkinson's disease was compared in carriers and non-carriers.

Results 15. 0% of patients had a *GBA1* non-synonymous variant (including missense, frameshift and recombinant alleles), compared to 6. 4% of controls (OR 2. 6;p<0. 001). 18 novel variants were detected. Variants previously associated with Gaucher's disease were identified in 5. 0% of patients compared to 1. 5% of controls (OR 3. 4;p<0. 001). The rarely reported complex allele p. D140H+p. E326K appears to likely be a Dutch founder variant, found in 2. 4% of patients and 0. 9% of controls (OR 2. 7;p=0. 012). The number of first-degree relatives (excluding children) with Parkinson's disease was higher in p. D140H+p. E326K carriers (5. 6%, 21/376) compared to p. E326K carriers (2. 9%, 29/1014) (OR 2. 0;p=0. 022), suggestive of a 'dose-effect' for different *GBA1* variants.

Conclusion Dutch Parkinson's disease patients display one of the largest frequencies of *GBA1* variants reported so far, consisting for a large part of the mild p. E326K variant and the more severe Dutch p. D140H+p. E326K founder allele.

Introduction

The most common genetic risk factor known to date for Parkinson's disease (PD) is a damaging variant in the *GBA* (*GBA1*) gene, encoding the lysosomal glucocerebrosidase enzyme.[1] To avoid confusion with the non-lysosomal genes *GBA2* and *GBA3*, the *GBA* gene is also referred to as *GBA1*. In most populations, 4-12% of PD patients carry a heterozygous *GBA1* variant and in Ashkenazi Jewish PD patients this is approximately 20%.[2,3] The risk of PD in *GBA1* variant carriers is increased by an estimated overall 2-7 fold (Odds Ratios, OR).[2–5] Rare homozygous or compound heterozygous *GBA1* variants can cause the autosomal recessive lysosomal storage disorder Gaucher's disease (GD). Over 400 variants have been reported associated with GD[6,7] and all of these alleles are potential risk factors for developing PD.

Full *GBA1* gene sequencing is essential to unambiguously identify gene variants, considering a long tail of rare variants or even population-specific variants.[3,4,8] Nevertheless, rarely the entire *GBA1* gene has been sequenced in a large cohort from a single population. Here, we report such a large-scale *GBA1* screening performed in the Netherlands, in the framework of a large program aimed to identify patients with *GBA1* variants for a clinical trial targeting the *GBA1* mechanism. We sequenced the *GBA1* entire open reading frame (ORF) in 3402 people with PD living in the Netherlands. Variant frequency was compared to an existing Dutch control cohort (n=655). Family history of PD was assessed in a subset of patients with the most common variants, to compare familial aggregation.

Material and Methods

Participants

PD patients were included in the Netherlands between April-2017 and March-2018, see supplementary data for details. Age at diagnosis of ≤50 years was considered early onset and >50 years was considered late onset PD.

This study was approved by an Independent Ethics Committee. Written informed consent was obtained from all participants according to the Declaration of Helsinki.

An independent Dutch study of 655 patients with abdominal aortic aneurysms was used for comparison (see supplementary), using WES data (average *GBA1* coverage was 101 times). Data regarding the presence of neurological disease were unavailable.

Genotyping

Saliva was obtained from patients using Oragene DNA OG-500 tubes (DNA Genotek). DNA isolation, next generation sequencing (NGS) and data analysis was performed by GenomeScan BV, Leiden, the Netherlands. Primers were selected to unambiguously sequence the functional *GBA1* gene and not the pseudogene, using long-range PCR. In a post-hoc experimental setup using long-read sequencing with the PacBio Sequel system, phasing was assessed in three samples. See supplementary material for methodological details, including validation of a subset using Sanger sequencing.

Historically, *GBA1* variants have been described based on the amino acid position excluding the 39-residue signal sequence at the start (also known as 'allelic nomenclature'). Both the Human Genome Variation Society (HGVS) recommended nomenclature and the allelic nomenclature are given (NCBI Reference Sequence: NM_000157. 3). If an allele contained more than one exonic variant, this is referred to as a complex allele.

Genotypes were classified into four categories, based on clinical associations, using the Human Gene Mutation Database:[7]

- Gaucher disease associated 'GD'
- Parkinson's disease associated 'PD'
- Synonymous
- Novel

If a subject had both a known and a novel variant, the genotype was considered novel. See supplementary data for details.

All variants that were 6 nucleotides or closer to a splice site, were assessed with four in silico splicing programs implemented in Alamut (Alamut Visual version 2. 13; see supplementary data).

A two-step cross-validation was performed to assess risk of both false positive and false negative results when using WES (see supplementary).

Family History

All patients with the *GBA1* p. D140H+p. E326K, p. E326K, p. N370S or p. L444P variants and a random subset of patients who did not carry *GBA1* variants as per our methods and variant selection criteria (henceforth referred to as *GBA1* 'wildtype') were given a questionnaire to assess familial aggregation of PD and to assess a possible founder location of the p. D140H+p. E326K complex allele. See supplementary material for details.

Statistical analysis

Fisher's exact test was used for categorical variables and the Mann-Whitney U-test for continuous variables. Significance was flagged at p<0. 05. ORs were calculated with a 95% CI. IMB SPSS Statistics 25 software was used.

Results

In total, 3638 PD patients were included, of which 3402 could be genotyped. Of the remaining 236 samples, no DNA could be extracted or PCR failed. Demographics can be found in Supplementary Table 1. 81% of patients was recruited through referral by a neurologist.

Sequencing

Average coverage was 2703 times (Supplementary figure 1). The subset of samples used in the Sanger sequencing validation were all confirmed (see supplementary data).

GBA1 variants

All *GBA1* exonic and splice site variants are listed in Table 1, including frequency comparison between PD and controls. In short, the total PD cohort had 15. 0% non-synonymous variants (including missense, frameshift and recombinant alleles) versus 6. 4% in controls (OR 2. 6, 95% CI: 1. 9-3. 6, p<0. 001).

For 'GD'-variants observed in patients (5. 0%) versus controls (1. 5%), the OR is 3. 4 (95% CI: 1. 8-6. 5, p<0.001) and for the 'PD'-variants observed in patients (9. 3%) versus controls (4.4%), the OR is 2. 2 (95% CI: 1. 5-3. 3, p<0. 001).

In total, 19 'GD'-variants, 5 'PD'-variants, 12 synonymous variants and 18 novel variants were identified. In one sample with p. D140H+p. E326K, phasing was confirmed using PacBio sequencing. See supplementary data for a further description of variants found. Supplementary Table 3 contains a variant frequency comparison with data from GoNL[9] and GnomAD[10,11] for reference, however methodology in these cohorts was not dedicated to *GBA1* sequencing.

No intronic variants were assessed to have a possible effect on splicing (Supplementary Table 4).

Control cohorts cross-validation

In the control cohort, 42 samples had a non-synonymous *GBA1* variant detected using WES that could be tested with our NGS protocol. Using NGS, four control samples were detected to be false-positive and three samples were partially false-negative (for p. D140H in a p. D140H+E326K complex allele). Conversely, after rerunning 48 GBA-PD samples with WES, one false-negative was detected. See supplementary data for details.

Demographics based on *GBA1* status

Demographics are given in Supplementary Table 1, divided over carriers of a non-synonymous variant or not. A larger portion of carriers had early onset PD (27. 2%) compared to non-carriers (18.2%) (p<0. 001). Conversely, of all subjects with early onset, 20. 1% had a *GBA1* variant, compared to 13. 1% in those with late onset (p<0. 001).

GBA1 variants and familial aggregation of PD

A questionnaire was completed by 180 carriers of p. E326K, 24 carriers of p. N370S, 28 carriers of p. L444P (including 4 complex and 3 recombinant alleles), 73 carriers of p. D140H+p. E326K and 135 *GBA1* wildtypes. Combining all carriers, 3. 6% of all siblings and parents combined had PD, compared to 2. 0% in siblings and parents of non-carriers (OR 1. 8, 95% CI: 1.0-3. 2; p=0.043). None of the children developed PD, probably due to the present younger age, so these were excluded from analysis of first-degree relatives (Supplementary Table 2). Supplementary figure 2 depicts the total number of first-degree relatives (excluding children) per variant type and the percentage of these relatives with PD. A variant 'dose-effect' was seen, see supplementary data for details.

Founder location p. D140H+p. E326K

Supplementary data and figure 3 shows a heat map of descent of grandparents of p. D140H+p. E326K carriers, visually suggesting (no formal statistical testing) the northern Netherlands as a possible founder location for this complex allele.

Discussion

To our knowledge, this study is the largest cohort known to date from a single country that has had full gene *GBA1* sequencing in PD patients. A total of 15. 0% of all patients had non-synonymous *GBA1* variants, which is the highest prevalence reported to date in a non-Ashkenazi Jewish population. The relatively high prevalence of the population specific p. D140H+p. E326K complex allele and the long tale of rare variants, including 18 novel variants, highlight the importance of sequencing the full *GBA1* ORF. Identifying all these variants will strengthen our understanding of the effect of *GBA1* variants and it facilitates recruitment for the upcoming *GBA1*-targeted trials, hopefully resulting in a first disease-modifying drug for PD.[12]

Comparing different countries,[3,4,8,13–26] the p. E326K variant is reported most frequently in the Netherlands (present study) and Scandinavian countries.[20,24] Table 2 compares the most common *GBA1* variants and the p. D140H+p. E326K complex allele in large PD cohorts from single countries that performed full *GBA1* ORF sequencing. Swedish[24] and Russian[15] cohorts

were included despite selective sequencing, because of their size, in order to compare the p. E326K variant. This overview shows the near-exclusive appearance of p. D140H+p. E326K in the Netherlands. The p. D140H+p. E326K complex allele has only sporadically been reported, once in GD,[27,28] sporadically in PD[4,29] and once in Lewy Body Dementia.[30]

Intronic splice site variants have rarely been systematically assessed previously,[17,23] however these do not seem to play a role in GBA-PD pathology in our Dutch cohort.

The importance of adequate genotyping methodology when sequencing *GBA1* was once more confirmed. In the control cohort, the *GBA1* variants were reassessed with NGS, which identified four false-positive p. L444P variants in WES. Also, three p. D140H variants were falsely not identified in three samples that also carried the p. E326K variant. The performance of the hybridization capture panel was lower over the p. D140H region, reflected in a local lower coverage. Combined with a possible allelic imbalance for this specific variant, where the amplification prefers the wildtype allele over the p. D140H allele, this could explain the false-negative output. Therefore, caution is advised when using *GBA1* data generated using a methodology not specifically designed for *GBA1* sequencing (including databases like ExAC or gnomAD).

Because the p. E326K and p. T369M variants do not cause Gaucher's disease, these have long been termed polymorphisms. However, it has been shown in meta-analyses that these variants do confer an increased risk of developing PD (OR 1.99 for p. E326K and OR 1.74 for p. T369M)[31–33] and therefore, despite not causing GD, should not be considered neutral polymorphisms.

Of all participants diagnosed with PD at 50 years of age or earlier, 20. 1% had a *GBA1* variant. When genetic testing is performed in early-onset PD, *GBA1* is not always included. Because of the high prevalence of *GBA1* variants in early-onset PD, it deserves consideration to include this in the screening, although the predictive value of a *GBA1* variant for offspring is still limited.

GBA1 variant carriers have a larger frequency of a positive family history for Parkinson´s disease[4,5,34] compared to non-carriers. In the current study, carriers of p. D140H+p. E326K had significantly more first-degree relatives with PD compared to p. E326K carriers. This implies a 'dose-effect' of variant severity in familial aggregation. However, it did not reach statistical significance for other variant types, likely due to the rarity of these variants.

The current study has some limitations. Since our NGS method used short-read sequencing, phasing of multiple variants could not be determined, unless these were within approximately 500 base pairs of each other. However, for a single p. D140H+p. E326K sample phasing was confirmed using PacBio and p. D140H was never seen without p. E326K. A recombinant gene could be identified if the long-range PCR resulted in two distinct peaks on the Fragment Analyzer. See supplementary data for a further discussion of possible limitations.

In conclusion, this study is a successful example of how to ascertain and genotype a large cohort of patients with PD, within a short timeframe, which is relevant for progressing clinical trials aimed at developing personalized treatments.

The Dutch PD population appears to have a relatively large number of *GBA1* variant carriers, consisting mostly of the mild p. E326K variant and the likely more severe Dutch p. D140H+p. E326K complex allele, with a possible founder-effect in the northern part of the Netherlands. In total, 18 novel *GBA1* variants were detected. *GBA1* variant carriers had a younger age at onset and a higher chance of a positive family history for PD, with a trend towards a 'dose-effect' based on clinical association of the variant.

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Table 1 Listing of all found exonic and splice site variants, including specifications. The sixth column 'allelic name' contains the annotation historically used in Gaucher's disease literature, excluding the 39-amino acid signaling peptide. All genotype frequencies are compared to the AAA control cohort, ors are given with the 95% CI and a p-value. A p-value of <0.05 is given in bold and the rows of these genotypes are filled grey. or could not be calculated if frequency was 0 in either group. If six cases or less were affected in patients and zero in controls, p-value is set to na. The coding (or sense) strand for *GBA1* is the reverse strand of the dna (as opposed to the forward strand). The chromosome position and nucleotide reflect the forward strand, whereas the cdna annotation indicates the variant on the coding strand, which is in this case the reverse strand, and therefore these are complementary. Both intronic splice site variants were predicted not to affect splicing (see supplementary material) and were therefore not included in the overall analysis.

GENOTYPE INFORMATION						COHORTS				
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
HETEROZYGOUS (simple and complex)										
155210876:C	c.26_27del	-	1	p.(Glu9GlyfsTer8)	E-30GFS*8	Novel	0.0 (1)	0 (0)	NA	NA
155210492:G	c.44T>C	-	2	p.(Leu15Ser)	L-24S	Novel	0.0 (1)	0 (0)	NA	NA
155210492:G 155210490:C	c.44T>C c.46A>G	-	2 2	p.[(Leu15Ser;Ser16Gly)]	L-24S+S-23G	Novel Novel	0.0 (1)	0 (0)	NA	NA
155210441:C	c.95A>G	-	2	p.(Gln32Arg)	Q-7R	Novel	0.0 (1)	0 (0)	NA	NA
155209813:T	c.171C>A	-	3	p.(Cys57Ter)	C18*	Novel	0.0 (1)	0 (0)	NA	NA
155209752:A	c.232C>T	rs146774384	3	p.(Arg78Cys)	R39C	Novel	0.0 (1)	0 (0)	NA	NA
155209732:AC	c.251_252insC	-	3	p.(Ser84ArgfsTer15)	S45RFS*15	Novel	0.0 (1)	0 (0)	NA	NA
155208421:A	c.475C>T	rs397515515	5	p.(Arg159Trp)	R120W	GD	0.1 (5)	0 (0)	NA	NA
155208361:G 155206167:T	c.535G>C c.1093G>A	rs147138516 rs2230288	5 8	p.[(Asp179His;Glu365Lys)]	D140H+E326K	GD	2.4 (82)	0.9 (6)	2.7 (1.2-6.1)	**0.012**
155208060:T	c.626G>A	-	6	p.(Arg209His)	R170H	Novel	0.0 (1)	0 (0)	NA	NA

GENOTYPE INFORMATION						COHORTS				
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
155208001:T	c.685G>A	-	6	p.(Ala229Thr)	A190T	GD	0.0 (1)	0 (0)	NA	NA
155207965:T	c.721G>A	rs398123534	6	p.(Gly241Arg)	G202R	GD	0.0 (1)	0 (0)	NA	NA
155207367:T	c.764T>A	rs74500255	7	p.(Phe255Tyr)	F216Y	GD	0.0 (1)	0 (0)	NA	NA
155207266:T	c.865G>A	-	7	p.(Gly289Ser)	G250S	Novel	0.0 (1)	0 (0)	NA	NA
155207249:C	c.882T>G	rs367968666	7	p.(His294Gln)	H255Q	GD	0.1 (2)	0 (0)	NA	NA
155207235:G	c.896T>C	-	7	p.(Ile299Thr)	I260T	GD	0.1 (2)	0 (0)	NA	NA
155206172:G	c.1088T>C	-	8	p.(Leu363Pro)	L324P	GD	0.0 (1)	0.2 (1)	0.2 (0.0-3.1)	0.297
155206170:T	c.1090G>A	rs121908305	8	p.(Gly364Arg)	G325R	GD	0.0 (1)	0 (0)	NA	NA
155206167:T	c.1093G>A	rs2230288	8	p.(Glu365Lys)	E326K	GD	6.3 (213)	2.6 (17)	2.5 (1.5-4.1)	**<.001**
155206158:A	c.1102C>T	rs374306700	8	p.(Arg368Cys)	R329C	GD	0.1 (2)	0 (0)	NA	NA
155206101:C	c.1159T>G	-	8	p.(Trp387Gly)	W348G	GD	0.0 (1)	0 (0)	NA	NA
155206093:G	c.1167G>C	-	8	p.(Gln389His)	Q350H	Novel	0.0 (1)	0.2 (1)	0.2 (0.0-3.1)	0.297
155206037:A	c.1223C>T	rs386626586	8	p.(Thr408Met)	T369M	GD	2.5 (86)	1.8 (12)	1.4 (0.8-2.6)	0.332
155205634:C	c.1226A>G	rs76763715	9	p.(Asn409Ser)	N370S	GD	0.9 (30)	0.3 (2)	2.9 (0.7-12.2)	0.151
155205619:C	c.1241T>G	-	9	p.(Val414Gly)	V375G	Novel	0.0 (1)	0 (0)	NA	NA

GENOTYPE INFORMATION						COHORTS				
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
155205605:A	c.1255G>T	-	9	p.(Asp419Tyr)	D380Y	GD	0.0 (1)	0 (0)	NA	NA
155205581:T	c.1279G>A	rs149171124	9	p.(Glu427Lys)	E388K	GD	0.1 (3)	0 (0)	NA	NA
155205568:C	c.1292A>G	-	9	p.(Asn431Ser)	N392S	GD	0.0 (1)	0 (0)	NA	NA
155205518:G	c.1342G>C	rs1064651	9	p.(Asp448His)	D409H	GD	0.0 (1)	0 (0)	NA	NA
155205043:G	c.1448T>C	rs421016	10	p.(Leu483Pro)	L444P	GD	0.6 (21)	0 (0)	NA	-
155205016:A 155205017:G	c.[1475A>T; 1474G>C]	-	10 10	p.(Asp492Leu)	D453L (D453V+D453H)	Novel	0.1 (4)	0 (0)	NA	NA
155204996:T	c.1495G>A	-	10	p.(Val499Met)	V460M	GD	0.0 (1)	0 (0)	NA	NA
155204986:G	c.1505G>C	-	10	p.(Arg502Pro)	R463P	GD	0.1 (2)	0.2 (1)	0.4 (0.0-4.2)	0.410
155204829:A	c.1568C>T	-	11	p.(Ser523Leu)	S484L	Novel	0.0 (1)	0 (0)	NA	NA
155204818:T	c.1579T>A	-	11	p.(Ser527Thr)	S488T	GD	0.0 (1)	0 (0)	NA	NA
155204811:C	c.1586A>G	-	11	p.(His529Arg)	H490R	Novel	0.0 (1)	0 (0)	NA	NA
LIKELY RECOMBINANT ALLELES										
155207210:A, 155207203:C, 155205008:G,	c.924C>T, c.931A>G, c.1483G>C,	- - -	7 7 9	p.(Leu307=), p.(Ser310Gly), p.(Ala495Pro),	L268=, S271G, D409H	Novel	0.0 (1)	0 (0)	NA	NA
			9 10 10 10		D409H, L444P, A456P, V460= (A.K.A. Rec*TL*)	GD	0.0 (1)	0 (0)	NA	NA

GENOTYPE INFORMATION						COHORTS				
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
			10 10 10		L444P, A456P, V460= (A.K.A. Rec*NciI*)	GD	0.1 (4)	0 (0)	NA	NA
HOMOZYGOUS OR COMPOUND HETEROZYGOUS(variant details in listing above)										
				p.[(Leu363Pro)];[(Thr408Met)]	L324P/T369M	GD/GD	0.0 (1)	0 (0)	NA	NA
				p.[(Asp179His;Glu365Lys)]; [(Thr408Met)]	D140H+E326K/ T369M	GD/GD	0.0 (1)	0 (0)	NA	NA
				p.[(Asp179His;Glu365Lys)]; [(Glu365Lys)]	D140H+E326K/ E326K	GD/GD	0.0 (1)	0 (0)	NA	NA
				p.[(Glu365Lys)];[(Thr408Met)]	E326K/T369M	GD/GD	0.1 (4)	0 (0)	NA	NA
				p.[(Glu365Lys)];[(Glu365Lys)]	E326K/E326K	GD/GD	0.2 (6)	0 (0)	NA	NA
				p.[(Thr408Met)];[(Thr408Met)]	T369M/T369M	GD/GD	0.0 (1)	0 (0)	NA	NA
UNCERTAIN PHASING(variant details in listing above)										
155210424:T, ...	c.112T>A, ...	-, ...	2, ...	p.(Ser38Thr)(;)(Thr408Met)	S-1T, T369M	Novel, GD	0.0 (1)	0 (0)	NA	NA
				p.(Gln32Arg)(;)(Asn409Ser)	Q-7R, N370S	Novel, GD	0.0 (1)	0 (0)	NA	NA
				p.[(Asp179His;Glu365Lys)](;) (Val498=)	D140H+E326K, V459=	GD, Syn	0.0 (1)	0 (0)	NA	NA
..., 155204793:T	..., c.1604G>A	..., rs80356773	..., 11	p.[(Asp179His;Glu365Lys)](;) (Arg535His)	D140H+E326K, R496H	GD, GD	0.0 (1)	0 (0)	NA	NA
				p.(Arg209His)(;)(Glu365Lys)	R170H, E326K	Novel, GD	0.0 (1)	0 (0)	NA	NA
				p.[(Glu365Lys)];[(Thr408Met)] (;)(Leu483Pro)	E326K/T369M, L444P	GD/GD, GD	0.0 (1)	0 (0)	NA	NA

GENOTYPE INFORMATION							COHORTS			
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
..., 155205574:T	..., c.1286G>A	..., -	..., 9	p.(Glu365Lys)(;)(Gly429Glu)	E326K, G390E	GD, Novel	0.0 (1)	0.2 (1)	0.2 (0.0-3.1)	0.297
				p.(Glu365Lys)(;)(Val498=)	E326K, V459=	GD, Syn	0.0 (1)	0 (0)	NA	NA
				p.(Glu365Lys)(;)(Val499=)	E326K, V460=	GD, Syn	0.0 (1)	0 (0)	NA	NA
				p.(Thr408Met)(;)(Asp492Leu)	T369M, D453L	GD, Novel	0.0 (1)	0 (0)	NA	NA
				p.(Thr408Met)(;)(Leu483Pro)	T369M, L444P	GD, GD	0.1 (3)	0 (0)	NA	NA
				p.(Asn409Ser)(;)(Leu483Pro)	N370S, L444P	GD, GD	0.0 (1)	0 (0)	NA	NA
SYNONYMOUS										
155209816:A	c.168C>T	rs145773486	3	p.(Val56=)	V17=	Syn	0 (0)	0.2 (1)	NA	0.161
155209684:T	c.300G>A	-	3	p.(Thr100=)	T61=	Syn	0.0 (1)	0 (0)	NA	NA
155208422:A	c.474C>T	rs147411159	5	p.(Ile158=)	I119=	Syn	0.1 (5)	0 (0)	NA	NA
155208389:T	c.507C>A	-	5	p.(Ile169=)	I130=	Syn	0.0 (1)	0 (0)	NA	NA
155208350:T	c.546G>A	-	5	p.(Gln182=)	Q143=	Syn	0.0 (1)	0 (0)	NA	NA
155207990:T	c.696G>A	rs375731497	6	p.(Gly232=)	G193=	Syn	0.0 (1)	0.2 (1)	0.2 (0.0-3.1)	0.297
155207984:A	c.702G>T	-	6	p.(Gly234=)	G195=	Syn	0.0 (1)	0 (0)	NA	NA
155206111:A	c.1149C>T	-	8	p.(Gly383=)	G344=	Syn	0.0 (1)	0 (0)	NA	NA
155206036:T	c.1224G>A	rs138498426	8	p.(Thr408=)	T369=	Syn	0.1 (2)	0 (0)	NA	NA
155205018:A	c.1473C>T	rs149257166	10	p.(Pro491=)	P452=	Syn	0.0 (1)	0 (0)	NA	NA

GENOTYPE INFORMATION							COHORTS			
Position Chr 1 (GRCh37/hg19)	cDNA NM_000157.3	rsID	Exon	Protein NP_000148.2	Allelic name	Clinical Association	PD patients % (n) (n=3402)	Control % (n)(n=655)	OR (95% CI)	P-value
155204997:A	c.1494C>T	rs371779859	10	p.(Val498=)	V459=	Syn	0.1 (3)	0 (0)	NA	NA
155204994:G	c.1497G>C	rs1135675	10	p.(Val499=)	V460=	Syn	0.0 (1)	0 (0)	NA	NA
SPLICE SITE (distance of 6 nucleotides or less)										
155207374:T	c.762-5G>A	-	Intr.	-	-	Novel	0.0 (1)	0 (0)	NA	NA
155206264:A	c.1000-4G>T	-	Intr.	-	-	Novel	0 (0)	0.2 (1)	NA	0.161
Exonic variants (details above) fulfilling splice site criteria (variant (distance)) (see supplementary Table 4 for splicing prediction): p.E-30Gfs*8 (1), p.S-1T (4), p.F216Y (3), p.T369= (1), p.T369M (2), p.N370S (2), p.R463P (1)										
GROUPED COMPARISONS										
All Novel genotypes							0.7 (23)	0.3 (2)	1.5 (0.4-4.9)	0.788
All PD genotypes (p.E326K, p.T369M, p.E388K, p.S488T, p.N392S)							9.3 (317)	4.4 (29)	2.2 (1.5-3.3)	<.001
All GD genotypes							5.0 (170)	1.5 (10)	3.4 (1.8-6.5)	<.001
Total non-synonymous							15.0 (510)	6.4 (42)	2.6 (1.9-3.6)	<.001

GD=Gaucher's disease, PD=Parkinson's disease, syn=synonymous, NA=not applicable, Intr.=intronic.

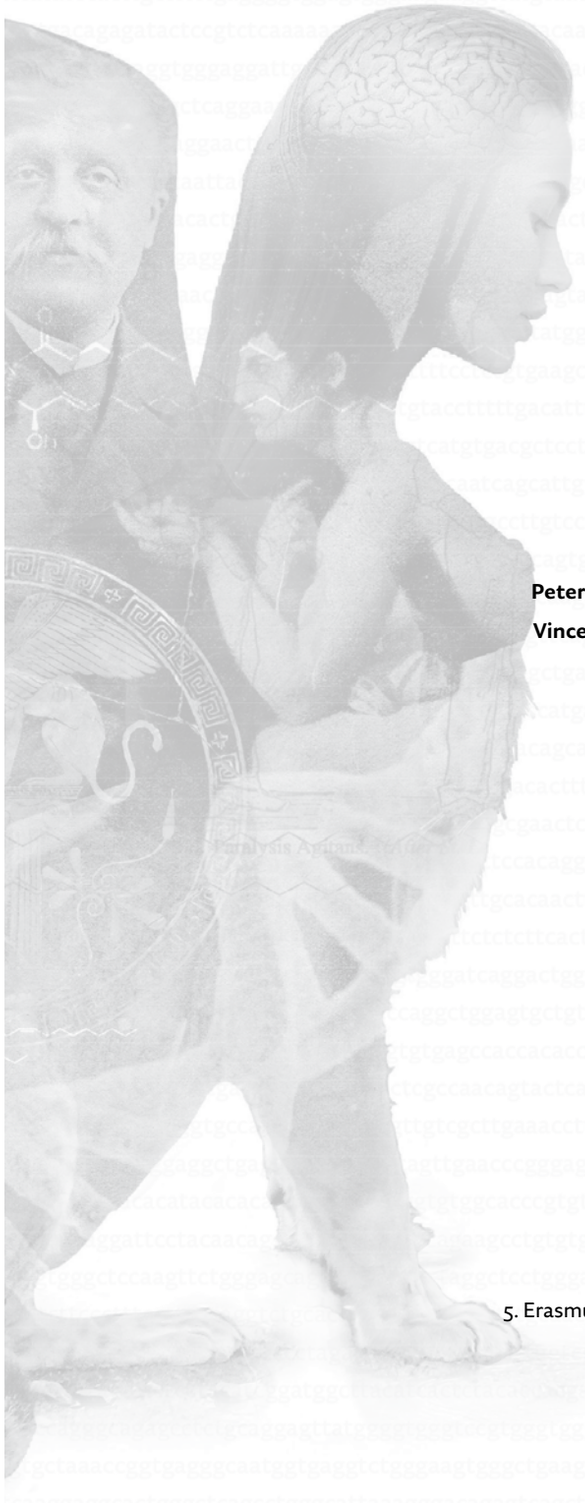
Table 2 International comparison of Parkinson's disease cohorts that performed full *GBA1* gene sequencing, sorted based on total % of *GBA1* variant carriers. All variant frequencies are given in percentages. Sweden and Russia performed selective sequencing. France is a European study, with 89% of subjects from France. North Africa is primarily Algeria, but also Morocco, Tunisia and Libya.

	PD N=	*GBA1* %	E326K	T369M	N370S	L444P	D140H + E326K	Other
Ashkenazi Jewish (3)	735	18.0	1.6	0	11.8	0.3	0	4.2
This cohort (NL)	**3402**	**15.0**	**6.7**	**2.5**	**0.9**	**0.6**	**2.5**	**1.8**
France (4)	1130	12.5	4.2	1.5	2.9	1	0.1	2.7
Colombia (8)	131	12.2	1.5	0	2.3	2.3	0	6.1
Norway (20)	442	12.0	6.6	3.6	0.2	1.4	0	0.5
Spain (17)	532	11.7	3	0.9	0.9	2.4	0	4.3
US (22)	1369	11.6	5	2.2	1.3	1.2	0.1	1.9
UK (21)	1893	11.1	4.5	1.8	0.6	1.6	0.1	2.4
Eastern Canada (16)	225	11.1	1.8	4.9	0.9	1.8	0	1.8
Belgium (26)	266	9.8	4.1	1.1	1.1	1.5	0.4	1.5
Japan (23)	534	9.4	0	0	0	4.1	0	5.2
New Zealand (25)	229	9.2	4.8	3.1	0.4	0	0.4	0.9
Sweden (24)	1625	8.3	5.8	N/A	0.4	2.2	N/A	N/A
Peru (8)	471	7.2	1.1	0.6	0.2	2.8	0	1.8
Russia (15)	762	6.6	2.4	2.5	0.5	1.1	N/A	N/A
Greece (18)	172	6.4	0.6	0	0	1.2	0	4.7
Portugal (13)	230	6.1	0.9	0.9	2.2	1.3	0	0.9
Korea (14)	277	6.1	0	0	0	0.7	0	5.4
North Africa (19)	194	4.6	0.5	1.0	1.0	1.5	0	0.5

SCAN ME

SUPPLEMENTARY MATERIAL

H2ST1/ H2ST2 / H2ST3 / H2ST4 / H2SF1 / H2SF2 / H2SF3 / H2SF4



CHAPTER 3

False negatives in *GBA1* sequencing due to polymerase dependent allelic imbalance

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Abstract

A variant in the *GBA1* gene is one of the most common genetic risk factors to develop Parkinson's disease (PD). Here the serendipitous finding is reported of a polymerase dependent allelic imbalance when using next generation sequencing, potentially resulting in false-negative results when the allele frequency falls below the variant calling threshold (by default commonly at 30%).

The full *GBA1* gene was sequenced using next generation sequencing on saliva derived DNA from PD patients. Four polymerase chain reaction conditions were varied in twelve samples, to investigate the effect on allelic imbalance:

1. the primers (n=4);
2. the polymerase enzymes (n=2);
3. the primer annealing temperature (T_a) specified for the used polymerase;
4. the amount of DNA input.

Initially, 1295 samples were sequenced using Q5 High-Fidelity DNA Polymerase. 112 samples (8. 6%) had an exonic variant and an additional 104 samples (8. 0%) had an exonic variant that did not pass the variant frequency calling threshold of 30%. After changing the polymerase to TaKaRa LA Taq DNA Polymerase Hot-Start Version: RR042B, all samples had an allele frequency passing the calling threshold. Allele frequency was unaffected by a change in primer, annealing temperature or amount of DNA input.

Sequencing of the *GBA1* gene using next generation sequencing might be susceptible to a polymerase specific allelic imbalance, which can result in a large amount of false-negative results. This was resolved in our case by changing the polymerase. Regions displaying low variant calling frequencies in *GBA1* sequencing output in previous and future studies might warrant additional scrutiny.

Introduction

Variants in the *GBA1* gene are, apart from the GWAS risk loci, the most common risk factor known to date to develop Parkinson's disease (PD).[1,2] Sequencing of the *GBA1* gene is known to be challenging, due to the highly homologous nearby pseudogene *GBAP1*.[3,4] *GBAP1* is not transcribed, but is in close proximity to *GBA1* and the exonic region of *GBAP1* shares 96% sequence homology with the coding region of the *GBA1* gene. False positive results are a well-known complication if highly homologous pseudogenes are not accounted for during sequencing. This can be overcome by using primers specific for the functional *GBA1* gene, long range amplification of the entire gene and by masking the pseudogene during alignment.[4]

We recently performed a large-scale screening of the *GBA1* gene in 3638 patients with Parkinson's disease from the Netherlands, based on a next generation sequencing (NGS) protocol.[5] The pseudogene was accounted for by use of NGS with long-range polymerase chain reaction (PCR) and a primer-set unique to the *GBA1* gene.

Here we report the serendipitous finding of an initially significant number of false negative results in our study, which could be readily solved by changing the polymerase enzyme. The corrected results were used for the previously published manuscript.[5] We noted that a *GBA1* variant that was previously detected in a patient in another study,[6] could not be confirmed by our sequencing method. Upon further investigation, the previous finding turned out to be a true positive result, while in our NGS method the variant was present, but it did not pass the default variant calling filter (heterozygous variant detected in more than 30% of the reads). A heterozygous allele should have a variant calling frequency of approximately 50% for both variants and a homozygous allele should have a variant calling frequency of approximately 100%, with very little noise using modern techniques.[7] Upon experimental lowering of this variant calling filter to 2%, the total *GBA1* variant hit-rate almost doubled, primarily driven by the relatively common NM_000157. 3:c. 1093G>A;p. (Glu365Lys) (allelic name E326K) variant.

This paper describes how changing the polymerase enzyme normalized all variant frequencies, thereby uncovering the false negative results, by

using a structured assessment of different primers, PCR primer annealing temperatures (T_a), amounts of DNA input and two different polymerases.

Results

Genotyping

Initially, 1295 samples were sequenced using Q5 High-Fidelity DNA Polymerase. 112 samples (8. 6%) had an exonic variant with a variant frequency higher than 30%. An additional 104 samples (8. 0%) had an exonic variant with a variant frequency lower than 30%, see Figure 1A.
The pattern of normal and abnormal variant frequencies is depicted per exonic variant in Figure 1 (A and B, bottom row). Some variants were only detected at a normal or abnormally low read frequency (e.g. c. 1093G>A;p. (Glu365Lys) (E326K) and NM_000157. 3:c. 1448T>C;p. (Leu483Pro) (L444P)), some variants were only detected normally or abnormally high (e.g. NM_000157. 3:c. 1223C>T;p. (Thr408Met) (T369M) and NM_000157. 3:c. 1226A>G;p. (Asn409Ser) (N370S)) and some variants could be either low, normal or high. If a sample had multiple variants, the imbalance was consistent over variants.

Based on intronic variants, including known benign variants, many samples without exonic variants were also imbalanced. Due to uncertainty of sequencing in GC-rich and repeat regions, some intronic variants with a low frequency could be sequencing or mapping errors as opposed to imbalanced amplification. Therefore, allelic balance could not be assessed for all samples without an exonic variant (data not shown). An overview of all intronic and exonic variant frequencies of all samples sequenced by Q5 polymerase is given in Supplementary figure 1.

Assessment of PCR conditions

PCR yield of human control DNA per T_a for Q5 and TaKaRa using all four primer sets can be seen in Figure 2. PCR yield using TaKaRa was generally higher than using Q5. A T_a of 62°C for Q5 and 63°C for TaKaRa was chosen.

The PCR product increased with increasing DNA input (4 ng, 20 ng, 100 ng) of the human control samples. Using the PD samples, primer set 2 had the lowest yield. Samples using primer set 2, control samples with 4 ng DNA input and negative controls were omitted from library preparation and sequencing. Variant frequencies per polymerase and per primer set of control samples with varying DNA input can be seen in Table 1 and variant frequencies of the PD samples can be seen in Table 2. Difference in DNA input did not affect the variant frequencies, based on control samples with 20 ng or 100 ng input. Choice of primers did not affect the variant frequencies, based on three different primer sets unique to the *GBA1* gene. Samples amplified using TaKaRa polymerase showed balanced variant frequencies, including the initially imbalanced samples using Q5 polymerase. PD sample 1 had a low yield after PCR using primer set 1 and TaKaRa polymerase and PD sample 9 had a low yield after PCR using primer set 3 and Q5 polymerase, therefore these two samples could not reliably be analyzed.

Confirmation of genotype

All samples with an exonic *GBA1* variant based on Q5 polymerase (variant frequency ranges: 2. 0%-23. 7% (n=58), 39. 6%-57. 4% (n=144), 81. 4%-94. 5% (n=11) and 99. 8%-100% (n=3)), were confirmed using TaKaRa polymerase (variant frequency ranges: 44. 8%-57. 4% (n=213) and 99. 4%-99. 8% (n=3)), see Figure 1 (A and B). All samples with a variant frequency between 80% and 95% using Q5 polymerase turned out to be heterozygous using TaKaRa polymerase, so in these samples there was an allelic imbalance in favor of the allele containing the *GBA1* variant over the reference while using Q5 polymerase.

Discussion

This paper describes the serendipitous finding of a polymerase dependent allelic imbalance when sequencing the *GBA1* gene, resulting in a high number of false negative results. In our cohort, the variant hit rate increased by 93% after changing the polymerase, primarily driven by the relatively common c. 1093G>A;p. (Glu365Lys) (E326K) variant and the [c. 535G>C];[c. 1093G>A];

p. [(Asp179His);(Glu365Lys)] (D140H+E326K) complex allele (a likely Dutch founder allele). This artifact was initially disguised by the commonly used variant calling filter set at a frequency of 30%. Our previous publication[5] was based on correct data after this artifact was detected and corrected. Considering this finding, we strongly advise to further explore the lower frequency regions of *GBA1* sequencing output in previous and future cohorts. Similar allelic imbalance in sequencing studies can potentially have a major impact on the prevalence of *GBA1* variants reported in other populations.

Preferential engagement of the polymerase to a specific allele can have multiple causes, like differences in GC content between alleles, heterozygous variants in the primer region, methylation status or altered folding.[8–11] A different variant in the primer annealing site was excluded as a cause due to equivalent results when using different primer sets. No specific intron or exon variant could be detected that differentiated between balanced and imbalanced samples. At this point, it remains unclear what causes this imbalance. Similarly, it is unclear how this translates to other polymerases or whether this could be resolved using modified PCR conditions. Concentration of the Q5 polymerase enzyme could not be varied, because this is provided in a mastermix solution.

Methylation status can alter the DNA secondary structure and melting properties.[10] Amplification is not prevented by methylation, but it is rather driven preferentially toward the unmethylated allele when the methylation status of the two alleles is distinct.[11] Similarly, altered DNA strand folding, like hairpin and G-Quadruplex structures, can also interfere with PCR.[12,13]. Coadjuvants to improve DNA denaturation, and performing PCR amplification in a KCl free buffer have been suggested to circumvent these problems.[11,12] Histone modifications are known to be involved in epigenetic modification,[9] but these are typically removed during DNA purification, so an effect on PCR seems unlikely.

A previous assessment of allele dropout showed a majority to be caused by nonreproducible PCR failures rather than sequence variants,[14] but this seems unlikely in the current report, considering the widespread and reproducible imbalances reported.

Most exonic variants, if abnormal in read frequency, displayed a decrease in frequency to below 30%. Some exonic variants however, like c. 1223C>T;p. (Thr408Met) (T369M) and c. 1226A>G;p. (Asn409Ser) (N370S), only showed normal or abnormally high read frequencies. Samples with a variant frequency up to 94. 5% turned out to be heterozygous (correct frequency ~50%) after changing to TaKaRa polymerase. This shows that also marginally abnormal results should be interpreted with caution.

Most exonic variants that occurred in an imbalanced frequency in certain samples, were also seen in a normal frequency in other samples (using Q5 polymerase). This implies that the exonic variant is not exclusively responsible for the imbalance. Conversely, exonic variants that were only seen in a normal frequency, are not precluded from a potential imbalance, because these variants were less prevalent and may therefore have only been detected with balanced frequencies by chance. Using the TaKaRa polymerase, the allelic imbalance was eliminated for all exonic variants and most intronic variants, but the imbalance could still be seen for some remaining intronic variants (Supplementary figure 2 and 3). These intronic variants mostly were in high-repeat intronic regions, or in other regions with a relatively low coverage, considered technical noise. In samples containing these imbalanced intronic variants, other intronic (and sometimes exonic) variants did have balanced frequencies, strengthening the notion that the imbalanced intronic variants were a technical artifact.

These findings could also explain discrepancies between multiple reports from the same or nearby populations. Both in the United Kingdom and in Spain, the c. 1093G>A;p. (Glu365Lys) (E326K) variant was initially not found,[15,16] but in later cohorts it was reported.[17–19]

Considering the ongoing drug development targeting the *GBA1* pathway, more and more people with Parkinson's disease will be screened for *GBA1* variants. Both for counseling purposes and for adequate enrollment in upcoming clinical trials, a reliable sequencing method is essential.

Material and Methods

Participants

PD patients were included in the Netherlands as described previously.[5] This study was approved by an Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek), Assen, The Netherlands. Written informed consent was obtained from all participants according to the Declaration of Helsinki.

Genotyping

Saliva was obtained from patients using Oragene DNA OG-500 tubes (DNA Genotek). DNA isolation, next generation sequencing (NGS) and data analysis was performed by GenomeScan B.V., Leiden, the Netherlands, as described previously [5]. The *GBA1* gene was unambiguously amplified using primers unique to the functional gene; these primers were used previously (Mata et al. 2016). Initially, the Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Inc.) was used. After the imbalanced variant frequencies were detected, a structured assessment of various PCR conditions was conducted.

DNA was isolated according to standardized procedures using the QIAsymphony DSP DNA Midi Kit (Qiagen). The DNA concentration of the samples was determined using Picogreen (Invitrogen) measurement prior to amplification. Afte PCR, the long-range PCR product was fragmented using the Bioruptor Pico (Diagenode) to an average size of 300 - 500 bp before sequencing on an Illumina sequencer. Library preparation was performed using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs E7370S/L). End repair/A-tailing, ligation of sequencing adapters and PCR amplification was performed according to the procedure described in the NEBNext Ultra DNA Library Prep kit instruction manual. The quality and yield of the library preparation was determined by Fragment Analyzer analysis. Clustering and DNA sequencing (paired-end 150 bp) using the Illumina cBot and HiSeq 4000 was performed according to the manufacturer's protocols. Image analysis, base calling and quality check was performed with the Illumina data analysis pipeline RTA v2. 7. 7 and Bcl2fastq v2. 17.

Data analysis was performed using a standardized in-house pipeline developed by GenomeScan B.V., based on the Genome Analysis Toolkit's (GATK) best practice recommendations,[20] including instructions for raw data quality control, adapter trimming, quality filtering, alignment of short reads, and frequency calculation. During the alignment step (using Burrows-Wheeler Aligner v0. 7. 4) to the human reference (hg19), the *GBAP1* gene was masked due to the high homology with *GBA1*. By masking *GBAP1*, mapping quality of *GBA1* reads increased, especially at the 3'-prime of the gene, where the homology is the highest.

GBA1 variants are described based on the amino acid position excluding the 39-residue signal sequence at the start (also known as 'allelic nomenclature'), which is used historically in *GBA1* research (format: E326K). The recommended nomenclature by the Human Genome Variation Society (HGVS) is also given (format: p. (Glu365Lys)) and further variant details can be found in our previous publication[5]. The used NCBI Reference Sequence is NM_000157. 3, NP_000148. 2, assembly GRCh37.

Assessment of PCR conditions

To investigate the cause of the variant frequency imbalance, four PCR conditions were varied: 1) the primers (n=4); 2) the polymerase enzymes (n=2); 3) the primer annealing temperature (T_a) specified for the used polymerase; and 4) the amount of DNA input.

Twelve samples with a *GBA1* variant of varying variant frequencies were further analyzed for this purpose: two homozygous samples, four heterozygous samples (with balanced variant frequencies) and six samples with an abnormal variant frequency, see Table 3. Additionally, commercial human DNA and negative controls were used.

The four primers investigated can be found in Table 4. Primer set 1 was used throughout the original genotyping project. The two polymerases used were Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs Inc.) and TaKaRa LA Taq DNA Polymerase Hot-Start Version: RR042B. Q5 DNA

Polymerase is composed of a novel polymerase that is fused to the processivity-enhancing Sso7d DNA binding domain.[21] TaKaRa LA Taq DNA Polymerase combines Taq DNA polymerase and a DNA-proofreading polymerase, with 3'→5' exonuclease activity.[22]

First, the optimal primer annealing temperature (T_a) was assessed for both polymerases, using all four primer sets and 100ng commercial human DNA. A standard three-step PCR cycle was performed according to the instructions of the supplier. For the Q5 polymerase, a T_a of 58-66°C with increments of 2°C was investigated and for the TaKaRa polymerase, a T_a of 60-68°C with increments of 2°C was investigated. Concentrations of the PCR products were measured using Picogreen (Invitrogen). The size of the PCR products was determined by Fragment Analyzer analysis.

Using the determined T_a per polymerase, the four primers were assessed using the twelve DNA samples from PD patients, commercial human control DNA and a No Template Control. The commercial human DNA was used to vary the DNA input, using 4 ng, 20 ng and 100 ng. For all PD samples, 100 ng was used. See Table 5 for an overview.

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Table 1 Variant frequencies based on DNA input of 20ng and 100ng of commercial human control samples. 4ng was not sequenced due to a low yield.

		TaKaRa			Q5		
	Benign SNP	Primer 1	Primer 3	Primer4	Primer 1	Primer 3	Primer4
Control 100ng	RS762488	49.1	48.2	47.2	33.5	32.1	28.1
Control 20ng	RS762488	46.3	52.2	44.4	35.7	32.8	30.1
Control 100ng	RS2075569	49.8	50.2	48.8	32.1	30.9	27.3
Control 20ng	RS2075569	47.8	47.9	46.2	33.9	31.9	28.7

Table 2 Variant frequencies of GBA-PD samples based on three different primer sets unique for the functional *GBA1* gene and two different polymerase enzymes. See Table 1 for HGVS nomenclature.

		TaKaRa			Q5		
Sample	Variant	Primer 1	Primer 3	Primer4	Primer 1	Primer 3	Primer4
1	E326K	Low yield*	99.9	99.8	100	100	100
2	E326K	99.8	99.9	99.8	99.9	100	100
3	E326K	51.2	53.2	51.7	47.7	48.3	50.4
4	E326K	48.6	50.4	50.5	47.5	49.4	51.4
5	E326K	48.1	47.6	47.1	33.4	32.3	31
6	T369M; L324P	50.7; 47.1	48.1; 50.3	50.7; 47.8	50; 47.7	49.5; 49	50.8; 48
7	T369M	48.7	50.8	49.7	63.3	64.5	68.3
8	L444P	44.4	42.4	43.9	27.8	28.3	23.6
9	E326K	48.4	49.8	48.4	34.2	Low yield*	29.8
10	E326K	49	47	48.7	27	28.6	23.6
11	E326K	48.5	48.3	49.9	24.8	23	18.8
12	N370S	49.5	53.1	53.7	51.5	53.5	52.9

**Two samples had a relatively low yield after PCR and could not reliably be sequenced.*

Table 3 Selection of twelve PD samples with varying *GBA1* variant frequencies. Samples 5, 8, 9, 10 and 11 have an abnormally low variant frequency and sample 7 has a frequency too high for a normal heterozygous and too low for a normal homozygous variant. Sample 6 is compound heterozygous (T369M on one allele and L324P on the other allele). Both the HGVS nomenclature is given and the *GBA1* allelic name, which excludes the 39-amino acid signaling peptide, both using accession NP_000148.2.

Sample	*GBA1* (HGVS)	*GBA1* (ALLELIC NAME)	Variant frequency
1	p.(Glu365Lys)	E326K	100
2	p.(Glu365Lys)	E326K	97.8
3	p.(Glu365Lys)	E326K	47.4
4	p.(Glu365Lys)	E326K	50.9
5	p.(Glu365Lys)	E326K	9.3
6	p.([Leu363Pro];[Thr408Met])	T369M; L324P	50.5; 47.5
7	p.(Thr408Met)	T369M	87.4
8	p.(Leu483Pro)	L444P	9.6
9	p.(Glu365Lys)	E326K	10.6
10	p.(Glu365Lys)	E326K	5.2
11	p.(Glu365Lys)	E326K	3.7
12	p.(Asn409Ser)	N370S	50.9

Table 4 Four different *GBA1* primer sets that will lead to amplification of the functional gene and not the pseudogene GBAP1. Genomic position based on Hg19.

GBA1 primer set	Name	Sequence (5'->3')	Start genomic position
1 *(Mata et al. 2016)*	GBA_P1_F	GTTGTCACCCATACATGCCC	chr1: 155204150
	GBA_P1_R	CTCTCATGCATTCCAGAGGC	chr1: 155211199
	Product length	7050	
2 *(Jeong et al. 2011)* Lysosomal disorder caused by mutations in the gene for the β-glucocerebrosidase, GBA	GBA_P2_F	TCCTAAAGTTGTCACCCATACATG	chr1: 155202292
	GBA_P2_R	CCAACCTTTCTTCCTTCTTCTCAA	chr1: 155211206
	Product length	8915	
3 *(Liu et al. 2016)* Harvard Medical School and Brigham & Women's Hospital, Cambridge, MA.	GBA_P3_F	CGACTTTACAAACCTCCCTG	chr1: 155203972
	GBA_P3_R	CCAGATCCTATCTGTGCTGG	chr1: 155211737
	Product length	7766	
4 Designed by GenomeScan using NCBI primer blast	GBA_P4_F	AGAAGTTGATCCCGGTGCTG	chr1: 155202617
	GBA_P4_R	ATGATGAAACAAGGGACGCTGC	chr1: 155211676
	Product length	9060	

Table 5 Analysis setup to investigate the effect of four primers sets and of DNA input on variant frequencies. This setup was performed once using Q5 polymerase and once using TaKaRa polymerase. Samples one to twelve were previously assessed to have a *GBA1* variant using Q5 polymerase, some with abnormal variant frequencies, see table 1. Negative control samples contained no DNA.

DNA input (ng)	Primer set 1	Primer set 2	Primer set 3	Primer set 4
100	Sample1	Sample1	Sample1	Sample1
100	Sample2	Sample2	Sample2	Sample2
100	Sample3	Sample3	Sample3	Sample3
100	Sample4	Sample4	Sample4	Sample4
100	Sample5	Sample5	Sample5	Sample5
100	Sample6	Sample6	Sample6	Sample6
100	Sample7	Sample7	Sample7	Sample7
100	Sample8	Sample8	Sample8	Sample8
100	Sample9	Sample9	Sample9	Sample9
100	Sample10	Sample10	Sample10	Sample10
100	Sample11	Sample11	Sample11	Sample11
100	Sample12	Sample12	Sample12	Sample12
100	Human Control	Human Control	Human Control	Human Control
20	Human Control	Human Control	Human Control	Human Control
4	Human Control	Human Control	Human Control	Human Control
0	Negative control	Negative control	Negative control	Negative control

Figure 1 Comparison of exonic variant frequencies using (A) Q5 polymerase or using (B) TaKaRa polymerase. These are the first 216 samples with a suspected *GBA1* exonic variant, initially sequenced using the Q5 polymerase and later repeated with TaKaRa polymerase. The top row shows histograms combining all exonic variants. The bottom row shows dot plots with variant frequencies per specific exonic variant. In normal circumstances, one would only expect a variant frequency around 50% and 100%. 44 samples contained two exonic variants (primarly p.[Asp179His;Glu365Lys] (D140H+E326K)), one sample contained three variants and one sample four. Frequencies below 30% are generally filtered out; the filter was customly set to 2% here. Variant details can be found in our previous publication.[5]

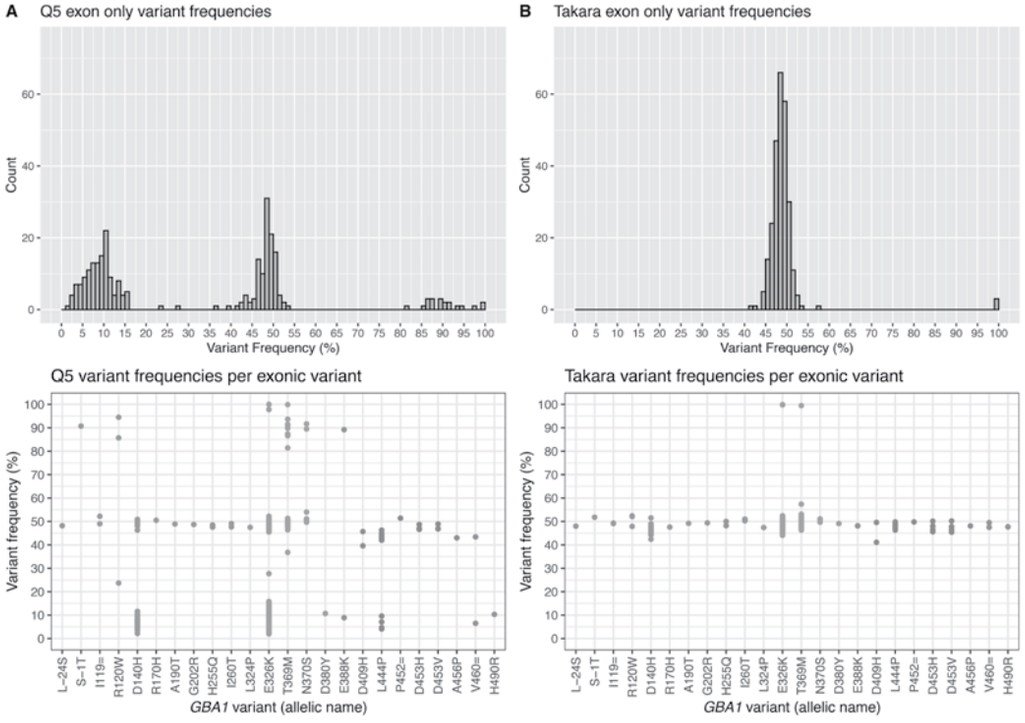
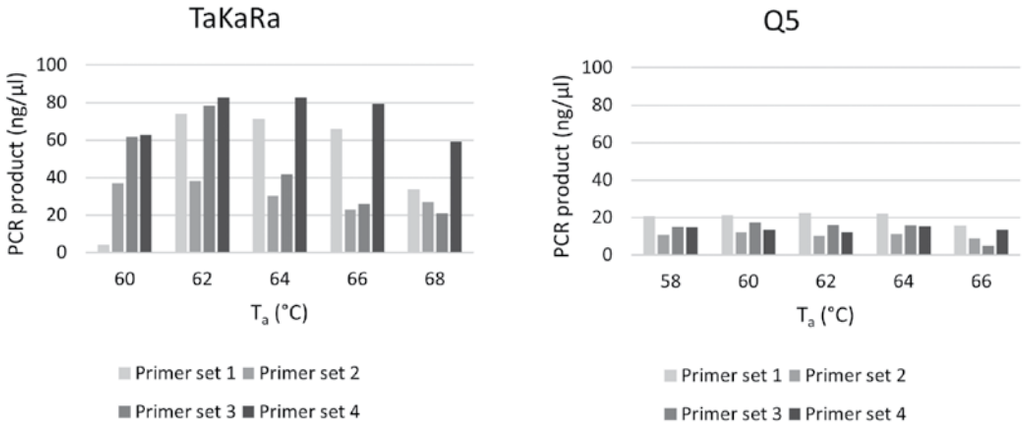
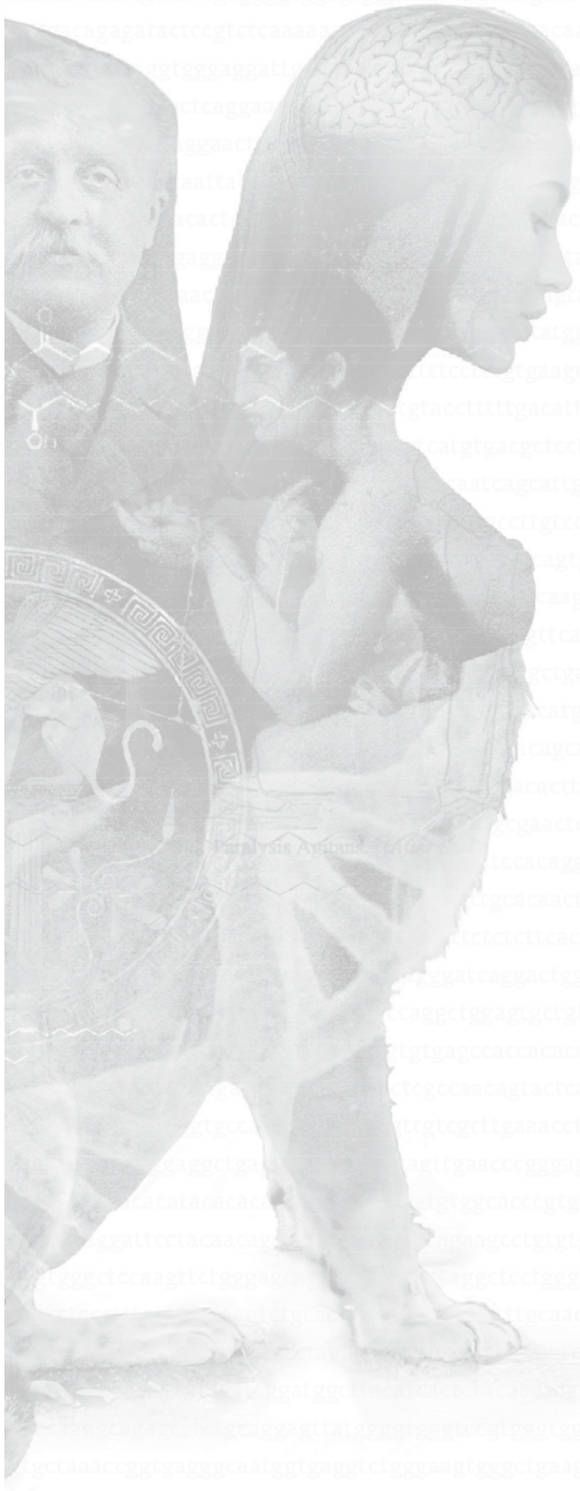


Figure 2 Varying T_a and the respective amount of PCR product, using commercial human DNA (100ng) with four different primer sets, split for the TaKaRa and Q5 polymerase. T_a=primer annealing temperature; PCR=polymerase chain reaction.





CHAPTER 4

Experience in genetic counseling for *GBA1* variants in Parkinson's disease

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SUPPLEMENTARY MATERIAL

H3SF1 / H3SF2 / H3SF3

SCAN ME

Apart from the GWAS risk loci, variants in the *GBA1* gene are the most common risk factor known to date to develop Parkinson's disease (PD).[1,2] Genetic testing and - counseling of *GBA1* variants is not yet part of common clinical practice, but the need for this will likely increase, since research into this topic has increased considerably over the past two decades and genetic testing will become more common. Several studies show that PD patients have a very positive attitude towards genetic testing.[3–5]

Genetic counseling is offered to support patients in clarifying gaps of knowledge regarding PD genetics as well as the risks, benefits, and limitations of genetic testing and to support them in their decision making process.[6] We use a whole exome sequencing panel of genes associated with movement disorders in familial PD and/or complex PD and/or PD with an early onset, less stricter than formulated in the European guidelines.[7,8] In monogenetic Parkinson disease, with variants in *SNCA*, *PRKN* or *PINK1*, it is relatively straightforward to clarify the inheritance pattern, inform relatives about their risk and discuss the options of predictive- and reproductive testing. If a variant is found associated with reduced penetrance like the founder mutation p. G2019S-mutation in *LRRK2* and especially if the variants are associated with mild differential effects on the risk and expression of PD, like heterozygous variants in *GBA1*, this is more difficult for the patient and relatives to handle and raises a need for genetic counseling tailored to the nature of the variant. *GBA1* encodes the lysosomal enzyme glucocerebrosidase, and is considered one of the most promising potential targets for the development of a disease-modifying drug for PD.[6] In light of these developments, a growing number of patients with PD are being screened for *GBA1* variants.

We recently performed a large-scale full *GBA1* gene screening in 3402 people with PD in the Netherlands.[9] In most populations, 4-12% of PD patients carry a heterozygous *GBA1* variant and in Ashkenazi Jewish PD patients this is approximately 20%.[2,10] In our Dutch cohort, a remarkably high prevalence of 15. 5% exonic or splice site variants was found. Subsequently, 528 patients with PD carrying a variant in the *GBA1* gene were counseled. In this viewpoint we wish to provide some background on *GBA1* in PD and share our experience in counseling of people with PD about the risks of a *GBA1* variant.

The *GBA1* gene is primarily known by the lysosomal storage disorder Gaucher's disease (GD), caused by a bi-allelic damaging variant in this gene. Important to note is that over 400 variants in the *GBA1* gene have been reported to be able to cause GD.[11,12] Some variants have been associated with a more severe phenotype of GD (e.g. L444P (p. Leu483Pro) is associated with the severe type 2-3 GD and N370S (p. Asn409Ser) is associated with the mild type 1 GD), but generally there is a weak genotype-phenotype correlation.[13] Having a heterozygous damaging variant will not cause GD, but it may increase the risk of developing PD. Several variants have been associated with an increased risk in PD, that in homozygous state will not cause GD (like E326K (p. Glu365Lys) and T369M (p. Thr408Met)).[14,15] Within PD, indications of a *GBA1* variant 'dose-effect' on age at onset, motor and non-motor symptoms have been described.[9,16,17].

Carriers of *GBA1* variants have an increased risk to develop PD (GBA-PD) with an earlier onset and possibly a faster motor and non-motor disease progression.[17–22] However, for counseling purposes it is important to acknowledge the existence of large variation in genotype-phenotype correlations and therefore the low predictability for an individual patient. For example in our cohort the mean (range) of age at diagnosis in non-carriers was 60.6 (27-92) years as compared to 56.9 (25-84) years in carriers of *GBA1* variants.

Motor impairment scores are generally worse in GBA-PD compared to idiopathic PD (iPD), but the structurally large standard deviations make an individualized prediction impossible.[17,18,21] Similarly for cognitive decline, this is generally worse in GBA-PD compared to iPD. A meta-analysis shows an OR of 2. 40 (95% CI 1.71-3.38) for developing PD dementia in *GBA1* variant carriers compared to iPD.[22] Nevertheless, between patient variability is again high, making it impossible to individually predict cognitive decline.[20,21,23]

The risk of PD in those who carry a *GBA1* variant is increased by an estimated overall 2-7-fold. Heterozygous and homozygous (potential GD) carriers have similar ORs.[24] Higher ORs have been reported for specific variants, but these are usually based on studies with a small number of carriers.[2,10,25,26] To our knowledge, no extended families have been reported with PD in multiple relatives with a *GBA1* variant as a possible high-penetrance (monogenic) causative factor, making any larger estimated risks unlikely.

Penetrance of *GBA1* is age-dependent and estimated to be between 1-14% at 60 years of age and 10-30% at approximately 80 years of age.[24,27–29] The higher end of these ranges is reported in subjects with familial PD and therefore possibly an overestimation, due to additional genetic burden in these familial cases.[30] The lower end of these ranges is based on parents of GD patients, which are obligate *GBA1* variant carriers, but do not necessarily carry any other genetic risk factors for PD other than *GBA1*.[24,29] A recent study in unselected PD patients (so both patients with and without a positive PD family history) showed an intermediate penetrance of 10. 0% at 60 years and 19. 4% at 80 years.[28] Penetrance was higher in carriers compared to noncarriers, but no statistically significant difference was found between carriers of mild (e.g. N370S) and severe (e.g. L444P) GD-associated variants.[24,27,28] All in all, most people with a homozygous or heterozygous variant will never develop PD.[24,31,32]

To account for the 'dose effect' of different *GBA1* variants, three categories were defined for counseling PD patients:

1. 'Low risk variants', if the allele has been reported in PD, but not as GD-causing
2. 'Moderate risk variants', if the allele has been reported in at least a single GD case, either in a homozygous state or in a compound heterozygous state with other GD-associated variants
3. 'Unknown variants', if a variant was not reported before.

A further 'dose-effect' within all variants previously reported in GD (here 'moderate risk variants') seems plausible, but sample sizes are generally very small for these (over 400!) different variants and therefore these cannot currently be differentiated reliably for personalized counseling.

When counseling a *GBA1* variant, it is important to provide a relevant context. For example, for a 'moderate risk variant' case: 'Of people of 60 years and older, approximately 1% will develop PD. With a *GBA1* variant, there would be an approximate 2-7% risk of developing PD at this age. This also means there is a 93-98% chance of *not* having developed PD at this age'. The age-specific incidence rate of PD of course increases beyond the age of 60 years.[33] *GBA1* can therefore be seen as a modifier of the PD risk, or risk factor in PD, and play a role in the complex disease etiology as such.

Considering the low absolute increase in risk of developing PD, the inability to predict disease progression, and the current lack of therapeutic consequences, we deemed it appropriate to primarily counsel the PD patients by phone and provide similar written information by mail. Patients had the opportunity to request a meeting in person. Only sporadically a patient returned a phone call for additional questions.

A transcript was created for the three *GBA1* categories (supplementary box 2A,B,C). Prior to presenting the transcript, it is advisable to give a brief simplified explanation of genetic principles (supplementary box 1). The primary concern of carriers in our study was often related to the consequences for their children. There is of course a 50% chance of inheriting the *GBA1* variant, but it is important to stress that the risks attributed to *GBA1* are very small so that presymptomatic testing for the *GBA1* variant is, in our view, not justified.

So far, the clinical relevance of having a *GBA1* variant is very limited for an individual. However, a study on deep brain stimulation (DBS) is worth mentioning, in which at 7.5 years after DBS, 6 out of 10 (60%) *GBA1* variant carriers had severe cognitive impairment, compared to 1 out of 16 (6%) in non-carriers.[34] This finding needs validation in a larger cohort, but this could be relevant for DBS decision-making. Furthermore, the prospect of possibly being eligible for a clinical trial based on carrying a *GBA1* variant, may be relevant for an individual as well.

Perhaps, when genotype-phenotype correlations will have been elucidated further in future larger cohorts, a variant-specific counseling can be tailored further.

In conclusion, the increasing amount of genetic testing being performed in Parkinson's disease creates an exciting time in which hopefully important steps are being made towards a personalized disease-modifying treatment. Accompanying this development, we should not forget to adequately inform patients about these findings and their clinical context, and to bring nuance when appropriate.

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CHAPTER 5

Intronic haplotypes in *GBA* modify age at diagnosis of Parkinson's: replication in a sub-group

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SUPPLEMENTARY MATERIAL

H4sT1

SCAN ME

Introduction

In Schierding et al. we identified non-coding variants within *GBA* that were associated with age of PD onset and diagnosis1. Toffoli et al. (this issue) failed to replicate our findings using data from the RAPSODI study and AMP-PD cohort. Here we provide evidence that supports our original findings and discuss the hypothesis that differing diagnostic criteria and/or data conglomeration is a potential basis for the replication failure of Toffoli et al.

Methods

The cohort and methods for PCR amplification and sequencing the *GBA* gene, and not the the pseudogene *GBA*P1, were previously described.[2] For this analysis, patients were classified according to referring neurologist (Figure 1).

Results

Haplotyping analysis of the Netherlands cohort of 1242 patients lacking *GBA* exonic variants did not replicate our findings (Fig. 1A, All). However, stratification by referral source identified a significant association (p=0. 0022) between the *GBA1* intronic haplotype and age at diagnosis (AAD) in individuals who were referred to the study by tertiary centre-based neurologists (Fig. 1A, Tertiary). The difference between the median age-of-diagnosis for the AA and BB *GBA1* intronic haplotypes was 10 years with weak evidence for a dosage effect (Fig 1B). This finding was consistent with our original observation of a dosage effect and 3. 4 year median difference in age-of-diagnosis observed between the 208 deeply phenotyped PD patients (AA vs BB) in the NZBRI cohort, who were diagnosed by a single clinician at a movement disorders clinic.[1,3]

We observed the identification of a significant haplotype-AAD relationship within the tertiary diagnosed patients and not those from the other categories (peripheral, mix and other(mix); Fig 1). This observation may suggest that populations of patients who are at tertiary clinics are distinct from other populations. There are at least two non-exclusive explanations for this. Firstly, it could reflect a scenario in which the diagnostic process for PD, and consequently AAD, varies between cohorts. If so, amalgamating patients diagnosed using differing diagnostic processes into a cohort is likely to obscure potential haplotype-AAD associations. As such, the observation that the RAPSODI study and the multiple cohorts that comprise AMP-PD use differing diagnostic criteria is a concern (Supplementary Table 1). Data conglomeration issues like these are a recognized confounder for genomic studies due to variability in the phenotyping4. Alternatively, it could be argued that some sub-types (e.g. early-onset, or high familial burden) of PD patients are preferentially referred to and examined by tertiary neurologists. This could lead to the tertiary cohort having specific characteristics that are associated with the observed genetic trend.

Alternative explanations for our observations also include:

1. the samples sizes of the NZBRI and Netherlands PD (tertiary) are not sufficiently large and the association is a false positive;
2. founder effects are present in both the NZBRI and Netherlands PD cohorts.

Finally, it is possible that the haplotype-AAD association was not detected in the AMP-PD because the accurate mapping of short-sequencing reads to *GBA* in AMP-PD is confounded by reads from the highly similar *GBA*P1 pseudogene. By contrast, the NZBRI and Netherlands cohorts underwent targeting sequencing of *GBA*.

Large cohorts with harmonised clinical, genomic and transcriptomic datasets are critical resources for the breakthrough discoveries required to substantially advance our understanding of disease, its different trajectories and the identification of potential therapeutic targets. However, as this study has indicated, potential variation in phenotyping, either within a cohort or between cohorts, has the capacity to diminish evidence of possibly important findings.

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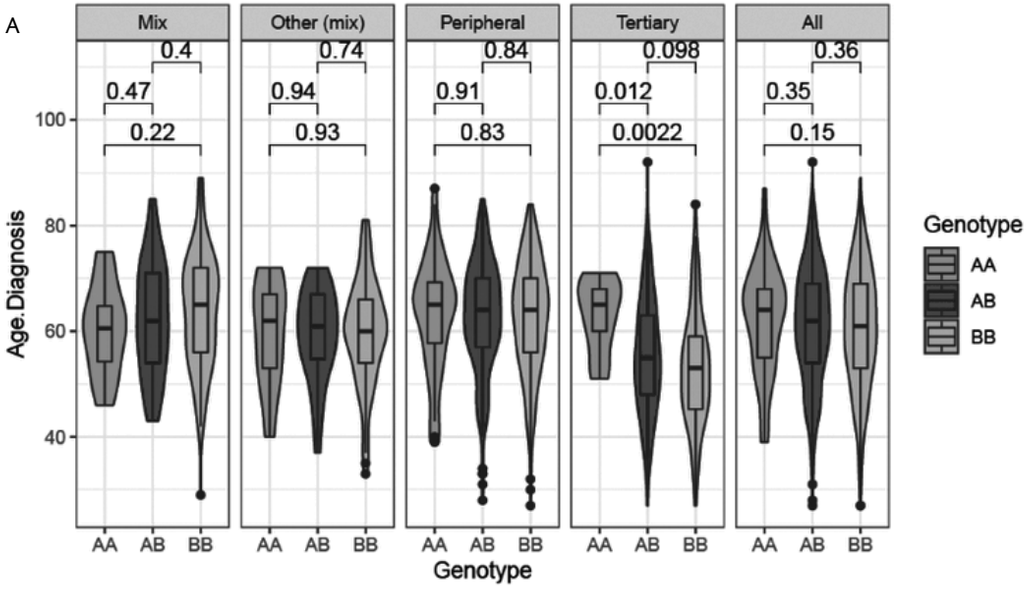
Figure 1 Figure 1. Conglomeration of wild-type (no exonic mutations) *GBA1* sequencing data across diagnostic cohorts obscures the relationship between intronic *GBA* haplotype and age of onset. A) Violin plot illustrating the association of *GBA1* intronic haplotype (AA – homozygous Ref allele (T/T T/T G/G), AB -heterozygous, BB – homozygous Alt allele (G/G C/C A/A)) with age of diagnosis. Patients were classified according to where their neurologist was based. Mix, referral was by a combination of both university and non-university based neurologists from the northern Netherlands; Peripheral, referral by a neurologist in a non-university center; Tertiary, referral by a neurologist located in a tertiary university center; Other(mix), self-referral to the study based on a neurologist diagnosis from a combination of university and non-university centers; ALL, all patients in the study. Statistical significance was tested using the Students t-test and results plotted using R-Shiny. B) Summary data for each category in A).

A



B

			Age at diagnosis	
Hospital type	Genotype	Number	Mean +/- SD	Median
Mix	AA	12	60.3 ± 8.7	60.5
Mix	AB	77	62.4 ± 10.6	62
Mix	BB	93	63.8 ± 11	65
Other (mix)	AA	9	59.4 ± 10.4	62
Other (mix)	AB	36	59.2 ± 8.8	61
Other (mix)	BB	81	59.8 ± 10.1	60
Peripheral	AA	48	62.9 ± 11	65
Peripheral	AB	281	62.7 ± 10.6	64
Peripheral	BB	358	62.6 ± 10.6	64
Tertiary	AA	9	63 ± 7	65
Tertiary	AB	96	55.4 ± 11.3	55
Tertiary	BB	142	52.9 ± 10.8	53
All	AA	78	62.1 ± 10.1	64
All	AB	490	61.0 ± 11.0	62
All	BB	674	60.4 ± 11.3	61

SUPPLEMENTARY MATERIAL

H5ST1

SCAN ME

CHAPTER 6

Preparing for *GBA1*-targeting Parkinson's disease trials: a biomarker study in patients with *GBA1*-Parkinson's disease and healthy controls

To be submitted.

Target journal: Molecular Neurodegeneration

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Abstract

Background A mutation in the *GBA1* gene is the most common genetic risk factor of Parkinson's disease (GBA-PD). *GBA1* encodes the lysosomal enzyme glucocerebrosidase (GCase), which hydrolyzes glucosylceramide (GluCer). GluCer is a basic form of a glycosphingolipid (GSL), and member of a vast network of different complex GSLs. Substrates and products of GCase are potential biomarkers for development of compounds targeting GBA-PD. Here, we compared the variability of various GSLs in plasma, peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) across GBA-PD, idiopathic nonGBA PD (iPD), and healthy volunteers (HVs).

Methods Data of five studies were combined. Within-day and between-day variability was assessed, and means were compared of GluCer (various isoforms), LacCer (various isoforms), GluSph, GalSph, GCase activity (using fluorescent 4-methylumbeliferryl-β-glucoside) and GCase protein (using enzyme-linked immunosorbent assay) in plasma, PBMCs and CSF if available, in GBA-PD, iPD and HVs. GSLs were measured using validated LC-MS/MS methods. Leukocyte subtypes were isolated separately to assess cell type differences for GluCer, LacCer and GluSph in HVs. Principal component analysis was used to explore global patterns in GSLs, clinical characteristics (Movement Disorders Society-unified Parkinson's disease rating scale part 3, mini mental state exam, *GBA1* mutation type), and participant status (GBA-PD, iPD, HVs).

Results Within-subject between-day variability for various molecules ranged from 5. 8% to 44. 5%, generally lower in plasma than in PBMCs. GluCer levels in plasma were higher in GBA-PD compared to both iPD and HVs. GSLs in the different matrices (plasma, PBMCs, CSF) did not correlate. Both LacCer (various isoforms) and GluSph were at least 20 times as abundant in granulocytes compared to both monocytes and lymphocytes. Absolute levels of various GSL isoforms differ greatly, up to a factor 140, also within cell type. *GBA1* mutation types could not be differentiated based on GSL data.

Conclusion GluCer can stably be measured over days both in plasma and PBMCs, and may be used as biomarkers in future clinical trials targeting GBA-PD. GluSph and LacCer are stable in plasma, but are strongly affected by leukocyte subtypes in PBMCs. GBA-PD could be differentiated from iPD and HVs, primarily based on GluCer levels in plasma.

Introduction

Parkinson's disease (PD; MIM: 168600) is the second most common multifactorial neurodegenerative disorder that results from complex interactions between environmental and (epi)genetic risk factors.[1] A disease-modifying treatment is still lacking. Through ongoing elucidation of the underlying pathophysiological process, new potential drug targets are being explored.[2,3] One such target is the lysosomal enzyme glucocerebrosidase (GCase; EC 3. 2. 1. 45), encoded by the *GBA1* gene (MIM: 606463). Apart from the common variants in GWAS loci, a variant in the *GBA1* gene is the most common genetic risk factor known to date to develop Parkinson's disease.[4,5] Approximately 4-15% of PD patients carry a heterozygous *GBA1* variant and in Ashkenazi Jewish PD patients the frequency is approximately 20%.[6–8]

Knowledge of the involved pathways will contribute to the identification of drug targets and potential biomarkers to evaluate target engagement of new drugs in early phases of development. GCase metabolizes its substrate glucosylceramide (GluCer) by hydrolysis into glucose and ceramide (Cer). This happens at the luminal lysosomal membrane, with assistance of the membrane bound activator protein saposin C.[9] GluCer is the most basic form of a glycosphingolipid (GSL), which constitutes a vast network of different complex GSLs, reviewed in great detail elsewhere.[10] In short, GSLs are a class of lipids, essential for e.g. membrane functioning and cell signaling, found throughout the body, including the brain. A GSL is built up of three moieties: a saccharide, a fatty acid chain and a sphingosine chain. Sphingosine and a fatty acid chain together form ceramide. The first upstream GSL to GluCer, by addition of a second saccharide, is lactosylceramide (LacCer). Conversely, GSLs are degraded by stepwise removal of sugar groups (each by

a different enzyme) and the eventual hydrolysis by GCase. In case of GCase deficiency, the enzyme acid ceramidase can convert GluCer into glucosylsphingosine (GluSph), by removal of the fatty acid chain.[12]

It is not fully understood how the intra- or extracellular levels of these molecules and their ratio are affected in people with Parkinson's disease with (GBA-PD) or without (idiopathic nonGBA PD; for convenience now referred to as iPD) a mutation in the *GBA1* gene, compared to healthy people without PD.

Across different brain regions, studies have found that GCase activity is decreased in GBA-PD and to a lesser extent in iPD.[13–17] Further, in GBA-PD and iPD GCase protein levels were found to be decreased to normal.[13] There is limited evidence for a decrease in mRNA in iPD.[14] There is conflicting evidence regarding accumulation of GluCer, LacCer and GluSph in various brain regions.[15,16,18,19]

Whereas brain material provides the most direct measurement of the pathologically affected tissue, for obvious reasons this cannot be collected during clinical trials. Data on peripherally collected material is limited. GCase activity is decreased in dried blood spots, in monocytes (but not lymphocytes) and in CSF of both GBA-PD and iPD compared to controls.[20–22] GCase protein was unchanged in monocytes and lymphocytes of GBA-PD and iPD compared to controls.[22] GluCer and LacCer were increased in plasma of iPD compared to controls,[23] but this finding was not replicated in serum.[16] In cerebrospinal fluid (CSF), LacCer (total) was increased in iPD compared to controls, but GluCer could not be measured.[16]

As drugs are currently being developed that aim to restore GCase activity as a disease progression modifying strategy in PD, there is a need for further elucidation of these *GBA1* related molecules in peripherally collected materials, as potential target engagement biomarkers in the clinical trials of these compounds. This report describes the combined data of five studies, evaluating GCase activity, GCase protein, GluCer (multiple isoforms), LacCer (multiple isoforms) and GluSph, in varying matrices (plasma, peripheral blood mononuclear cells (PBMCs) and/or CSF) in GBA-PD, iPD and healthy volunteers (HVs).

Materials and methods

Data of five studies were combined:

1. A biomarker variability study in GBA-PD (n=8), iPD (n=8) and HVs (n=8)
2. Baseline biomarker data of a phase I single dose clinical trial in HVs (n=40)
3. Baseline biomarker data of a phase I multiple dose clinical trial in HVs (n=39)
4. Baseline biomarker data of a phase 2a multiple dose clinical trial in GBA-PD (n=40)
5. Single blood draw to compare biomarkers between immune cell subsets in HVs (n=6)

Study 1 was used to assess the variability (within- and between-subject, within- and between-day) of GCase activity, GCase protein, GluCer, GluSph and galactosylsphingosine (GalSph). Studies 2 (measured GluCer), 3 (measured GluCer, GluSph and LacCer) and 4 (measured GluCer, GluSph and LacCer) were used to expand groups for a comparison between GBA-PD and HVs. Study 5 was used to compare GluCer, GluSph and LacCer between immune cell subsets. At every blood draw, plasma and PBMCs were collected for separate analysis. In studies 3 and 4 CSF was also collected. An overview of measurements per study can be found in Supplementary table 1.

Subjects

For study 1, three groups were enrolled. First, healthy volunteers, 18 to 70 years of age (of which 50% female and of which 50% ≥55 years of age). Second and third, people with iPD and GBA-PD, respectively. For both PD groups, diagnosis was confirmed by a neurologist and Hoehn & Yahr stage was 1 up to 4. All subjects underwent five blood draws on day 1 (at approximately 11:00h, 13:00h, 15:00h, 17:00h and 19:00h) and a single blood draw on day 5 and on day 8 (both at approximately 13:00h), to assess variability. Only HVs remained fasted from the preceding midnight until after the first blood draw on day 1, to assess a potential food effect.

Studies 2, 3 and 4 were randomized placebo-controlled trials.[24,25] Only data of study subjects on placebo were used for the current report (i. e. baseline data of all subjects and data of subjects randomized to placebo).

For study 2, healthy men and women of non-childbearing potential, 18 to 65 years of age, were enrolled for a clinical trial.[24] Subjects underwent four blood draws on day 1 and a single blood draw on day 2.

For study 3, healthy middle-aged or elderly men and women of non-childbearing potential, 50 to 75 years of age, were enrolled for a clinical trial.[24] Subjects underwent a single blood draw on day 1, day 2, day 8 and day 15. Day 1 and 15 contained a single CSF sample.

For study 4, men and women of non-childbearing potential with confirmed GBA-PD, Hoehn & Yahr stage 1 up to 4, ≥18 years of age, were enrolled for a clinical trial.[25] Subjects underwent a blood draw on day 1, twice on day 2, day 8, day 15 and day 29. Day 1 and 29 contained a single CSF sample.

For study 5, healthy volunteers, ≥18 years of age, were enrolled for a single blood draw.

Plasma, PBMCs and CSF collection

Biomarkers were measured in K_2EDTA plasma, PBMCs and CSF. PBMCs were isolated from venous blood using cell preparation tubes (CPTs) containing sodium heparin (Becton Dickinson, NJ, USA) according to manufacturer's instructions. In short, CPTs were centrifuged at 1800xg for 30 minutes at room temperature. PBMCs were collected and washed twice with phosphate-buffered saline (PBS) containing 10% heat inactivated human serum (both Gibco, Thermo Scientific, Waltham, MJ, USA). Cells were snap-frozen at $1*10^7$ cells mL^{-1} in PBS with 0. 1% Bovine Serum Albumin (BSA) buffer and stored at -80°C until analysis. Cells for GluSph analysis were frozen in glass tubes instead of a cryopreservation tube. In studies 3 and 4, for CSF collection, a Pencan® 25G atraumatic needle was used. The first mL CSF was discarded to prevent contamination with blood, after which 4mL was collected in a 15mL Falcon® tube. CSF was transferred to a glass tube and 0. 2% BSA with ascorbic acid was added. This was centrifuged at 2000xg for 3 minutes at room temperature. Supernatant was transferred to glass tubes, snap frozen and stored at -80°C until shipment for analysis. Time from collection to freezing did not exceed 60 minutes.

GCase activity

GCase activity was measured in study 1 only. Analysis was performed by Lysosomal Therapeutics, Inc. (Boston, USA). Activity was measured in PBMCs using the fluorescent artificial substrate 4-methylumbeliferryl-β-glucoside (4-MUG). 50 µL of $1*10^7$ PBMCs / mL was used per analysis. Measurements were performed in duplicate and the average was taken. Technical variability was 45%.

GCase protein

GCase protein was measured in study 1 only. Analysis was performed by Ardena Bioanalytical Laboratory (ABL) (Assen, the Netherlands). The commercial ELISA kit Glucosidase Beta Acid from Cloud Clone Corp. was used. Calibration was 31.3–2000 pg/mL. 350 µL of $1*10^7$ PBMCs / mL was suspended 50 times prior to analysis. Output was reported in pg GCase protein / $1*10^7$ PBMCs, based on a duplicate measurement. Technical assay variability was 15%.

GluCer, LacCer, GluSph and GalSph

GluCer was measured in all studies, LacCer and GluSph were measured in studies 3, 4 and 5, and GalSph was measured in study 1 only. GluCer and LacCer were measured in plasma, PBMCs and CSF if available. GluSph was measured in plasma and PBMCs only. GalSph was measured in plasma only. All GSLs were measured using validated LC-MS/MS methods, by ABL. Technical assay variability was 15% and all data are based on single measurements, which were only repeated if predefined quality criteria were not met. Assay ranges can be found in Supplementary table 2.

Leukocyte subtypes

PBMCs constitute of monocytes and lymphocytes, but inherent to isolation methods, some degree of granulocyte contamination is always present,[26] which can vary between blood draws. In study 5, monocytes, lymphocytes and granulocytes were isolated separately. Magnetic cell separation was the method used for all leukocyte subtype isolations. Whole blood from HVs was collected in tubes containing specific anticoagulants. Negative selection assay was optimized for high purity of isolated cells. The negative selection assay consisted of two depletion cycles comprising directly labelling of undesired cell subtypes. Immuno-magnetic cell separation assays comprised the magnetic labelling step and the magnetic capture. Lymphocytes isolation was done by Direct Human Total Lymphocyte Isolation Kit, monocytes isolation by Direct Human Monocyte Isolation Kit, and granulocytes isolation by Direct Human Pan-Granulocyte Isolation Kit according to manufacturer's instructions (StemCell technologies). To verify the purity of the isolated cells, leukocyte subtypes were labelled with specific antibodies; namely CD45, CD3 and CD19 for lymphocytes, CD45 and CD14 for monocytes and CD45, CD66b, CD123 and CD16 for granulocytes; and evaluated by flow cytometry. A density of $8*10^6$ cells mL^{-1} in PBS containing 0.1% BSA was collected in 2× plastic tubes and 2× glass tubes for each leukocyte subtype. Samples were snap frozen and stored at -80°C until shipment for analysis. Time from blood collection to freezing did not exceed 180 minutes.

Genotyping

The *GBA1* gene was sequenced in all subjects, except for the HVs in study 1 and 5, using saliva derived DNA and methods described previously.[8] In short, next generation sequencing was performed, using long-range polymerase chain reaction and a primer set unique to the functional *GBA1* gene, thereby preventing amplification of the nearby pseudogene. For study 4, all *GBA1* genotypes were confirmed in whole blood.

In study 1 and 4, only *GBA1* variants previously reported in PD or Gaucher's disease (GD) were included. In homozygous state, over 400 variants have been reported to cause the lysosomal storage disorder GD.[27,28] Some variants, however, do not cause GD in homozygous state, but do increase the risk to develop PD in heterozygous state, e.g. the relatively common variants E326K and T369M. Generally, these latter variants are considered milder and have a higher residual GCase activity. Within GD, some genotypes are considered worse than others, but the genotype-phenotype correlation is variable.

In this report, we applied nomenclature historically used in GD literature, excluding the 39-amino acid signalling peptide.

MMSE, H&Y and MDS-UPDRS part III

In PD subjects, the mini mental state examination (MMSE) (study 1 and 4), Hoehn & Yahr (H&Y) staging (study 1 and 4) and the Movement Disorders Society – unified Parkinson's disease rating scale (MDS-UPDRS) part 3 (motor score) (study 4 only) were performed. Only screening (MMSE and H&Y study 1, MMSE study 4) or baseline (MMSE, H&Y and MDS-UPDRS study 4) data were used, since these ratings might be influenced by placebo effect.

Statistical testing: variability

Using study 1, to estimate variabilities within a day (measurement 1 to 5 on day 1), the variables were analyzed with a mixed model analysis of variance with fixed factors group (HV, iPD and GBA-PD), measurement and the interaction group by measurement and a random subject factor. The covariance parameter estimates were used to calculate the between- and within-subject variabilities (standard deviation and coefficient of variation). This was repeated for the variability over days (measurement 2 of day 1 and measurements of day 5 and day 8), where measurement was replaced by day.

Collated data of GBA-PD, iPD and HVs

For every subject, the average per parameter was taken of all available measurements (from study 1 all measurements, from study 2, 3 and 4 the

baseline and placebo-treated measurements). Levels are depicted using violin plots. Considering the exploratory nature and extensiveness of measurements, no formal statistical testing was performed to compare means. Means and 95% confidence intervals are given.

Statistical exploration: Principal component analysis

Principal Component Analysis (PCA) is a statistical technique used to emphasize variation and bring out strong patterns within the data. We aimed to explore multivariate association structures in a set of variables including various GSLs in PBMCs, plasma and CSF, and age and BMI. For GBA-PD patients only, we also included H&Y, MDS-UPDRS part III total score, MMSE, age at diagnosis, duration of diagnosis and *GBA1* mutation type. As we did not use any missing data imputation method, we conducted the analysis in four batches (complete cases), to maximize the use of available data. Data were normalized prior to performing each PCA, because variables had different units or scales.

1. PCA 1 (maximized for GCase activity and protein): Explore patterns in GBA-PD vs iPD vs HVs, using GCase activity, GCase protein, GluCer (multiple isoforms) and GluSph in PBMCs and GluCer (multiple isoforms), GluSph and GalSph in plasma, age and BMI, based on data of study 1.
2. PCA 2 (maximized for GluCer, LacCer and GluSph): Explore patterns in GBA-PD vs HVs, using GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, GluSph in plasma and PBMCs, age and BMI, based on data of study 3 and 4.
3. PCA 3. 1 (maximized for GBA-PD): Explore patterns in covariates in GBA-PD, using *GBA1* mutation type, MDS-UPDRS part3 total score, H&Y, age at diagnosis, duration of diagnosis, age, BMI, with GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, GluSph in plasma and PBMCs, based on data of study 4.
4. PCA 3. 2 (maximized for GBA-PD): same as 3. 1, excluding CSF data.
5. PCA 4 (maximized for GluCer): Explore patterns in GBA-PD vs iPD vs HVs, using GluCer (multiple isoforms) in plasma and PBMCs, age and BMI, based on data of study 1, 2, 3 and 4.

PCA is an orthogonal (a.k a. independent) linear transformation that remodels the data to new latent variables, so called principal components. The greatest variance by some scalar projection of the data is presented on the first component, the second greatest variance on the second component, and so on. This represents the optimal subset of highly correlated original variables with fewer independent variables (i. e., the two principal components). Analysis results are presented using biplots, where projection of vectors (a. k. a. loadings) represent their contribution in the given component of the PCA and the angle between a pair of vectors represents their size of correlation (i. e., vectors in the same direction (small angle) have a positive correlation, vectors at a 90-degree angle are not correlated and vectors in opposite direction are inversely correlated). An indirect interpretation is that a short vector, which has a small contribution to the given component, will therefore only represent correlations with a small magnitude with any other vector.

Results

Subjects

In study 1, 8 subjects per group (HV, iPD and GBA-PD) were included. In study 2, 40 HVs were included, of which 2 subjects carried the heterozygous T369M variant and one subject carried the heterozygous E326K variant. In study 3, 39 HVs were included, of which two subjects carried the heterozygous T369M variant; one subject withdrew prior to any measurements for personal reasons and was not replaced. In study 4, 40 people with GBA-PD were included. Demography data of all subjects are summarized in Table 1. Genotypes of GBA-PD subjects in study 1 and 4 can be found in Table 2.

Variability

The within- and between-subject, within- and between-day variability of GluCer (multiple isoforms), GluSph, GalCer, GCase activity and GCase protein in plasma and/or PBMCs, split for HVs, iPD and GBA-PD, are given in Table 3. Generally, plasma measurements were more stable than PBMC

measurements. No diurnal rhythm was observed, based on the samplings from 11:00h to 19:00h in all groups. No acute effect of food intake was observed, based on the first fasted sample in HVs only. GluCer C18:0 (as example), GCase activity and GCase protein measurements can be seen in Figure 1.

Differences between GBA-PD, iPD and HVs

Average levels of various GSLs in HVs, iPD and GBA-PD were compared. Mean values with non-overlapping 95% CI are discussed. GluCer (multiple isoforms), mean (95% CIs) plasma levels were higher in GBA-PD (C16:0 789. 5 [727. 5, 851. 5]; C18:0 85. 9 [78. 3, 93. 4]; C22:0 1724. 9 [1518. 4, 1931. 4]; C24:0 1881. 6 [1649. 2, 2113. 9]; C24:1 1753. 3 [1551. 1, 1955. 5]), compared to both iPD (C16:0 645. 1 [517. 9, 772. 2]; C18:0 59. 4, [47. 1, 71. 6]; C22:0 900. 3 [728. 2, 1072. 4]; C24:0 845 [729. 4, 960. 6]; C24:1 758. 8 [603. 1, 914. 5]) and HVs (C16:0 521. 9 [497. 1, 546. 6]; C18:0 53. 9 [49. 8, 58. 1]; C22:0 839. 5 [798. 2, 880. 9]; C24:0 798. 8 [756. 4, 841. 3]; C24:1 787. 6 [745. 5, 829. 7]). Levels in PBMCs and CSF were similar over all groups (no CSF available from iPD), except GluCer C20:0 in CSF, which was higher in GBA-PD (0. 17 [0. 15, 0. 19]) compared to HVs (0. 12 [0. 10, 0. 14]). See Figure 2 (biomarker: GluCer) and Supplementary table 3.

For LacCer (multiple isoforms, GBA-PD and HVs only), levels in PBMCs did not differ between GBA-PD and HVs. Levels in plasma vary, where in GBA-PD these were elevated for LacCerC18:0 (61. 0 [56. 2, 65. 8] vs 52. 3 [48. 6, 56. 0]), LacCerC22:0 (167. 6 [154. 8, 180. 4] vs 129. 5 [121. 2, 137. 9]) and LacCerC22:1 (51. 5 [46. 8, 56. 1] vs 41. 3 [37. 4, 45. 2]), decreased for LacCerC20:0 (25. 7 [23. 7, 27. 7] vs 33. 6 [31. 2, 36. 0]), and no clear difference for LacCerC16:0, -C24:0 and -C24:1. In CSF, LacCerC18:0 was decreased in GBA-PD (0. 74 [0. 68, 0. 80]) compared to HVs (0. 89 [0. 82, 0. 95]) and LacCerC20:0 was decreased in GBA-PD (0. 113 [0. 099, 0. 127]) compared to HVs (0. 183 [0. 165, 0. 201]), but similar in other isoforms (C16:0, C22:0, C24:0 and C24:1). See Figure 2 (biomarker: LacCer) and Supplementary table 3.

GluSph in plasma was elevated in GBA-PD (1. 36 [1. 21, 1. 52]) compared to HVs (0. 99 [0. 93, 1. 06]) and marginally overlapped with iPD (0. 93 [0. 64, 1. 23]), but was similar over all groups in PBMCs. GalSph (plasma only) was similar in GBA-PD, iPD and HVs. See Figure 2 (biomarker: GluSph and GalSph) and Supplementary table 3.

GCase activity in PBMCs (using 4-MUG) was generally decreased in GBA-PD (2. 29 [1. 21, 3. 37]) compared to HVs (3. 67 [3. 32, 4. 03]), but still slightly overlapped. Activity in iPD (3. 98 [2. 22, 5. 74]) seemed similar to HVs on average, but varied greatly, with both higher and lower levels compared to HVs. GCase protein levels (PBMCs only) were increased in iPD (44180 [31613, 56747]) compared to HVs (25871 [20336, 31407]) and to a lesser extent in GBA-PD (35455 [27510, 43400]) compared to HVs. See Figure 2 (biomarker: GCase activity and GCase protein) and Supplementary table 3.

Principal Component Analysis

Different combinations of data of study 1-4 were used to conduct five PCAs (PCA1, 2, 3. 1, 3. 2 and 4), to maximize sample size for certain parameters. Key observations per PCA are described, while the biplots and a more detailed description can be found in the supplementary material.

PCA1 (Supplementary figure 1) was maximized for GCase activity and protein in PBMCs, but also includes GluCer (multiple isoforms) and GluSph in PBMCs, GluCer, GluSph and GalSph in plasma, age and BMI. Data of study 1 were used, consisting of GBA-PD (n=8), iPD (n=8) and HV (n=7, 1 listwise procedural exclusion of a participant due to missingness of GluCerC22:0 and C24:0 in plasma).

GCase activity and GCase protein level in PBMCs are uncorrelated. GCase activity shows a moderate inverse correlation with GluCer (multiple isoforms), GluSph and GalSph in plasma, but not in PBMCs. GCase protein is positively correlated to GluCer (multiple isoforms) in PBMCs, but not in plasma.

PCA2 (Supplementary figure 2) was maximized for GluCer and LacCer in plasma, PBMCs and CSF and GluSph in plasma and PBMCs, but also includes age and BMI. Data of studies 3 and 4 were used, consisting of GBA-PD (n=40), and HV (n=39).

All CSF measurements (GluCer and LacCer multiple isoforms) are correlated. CSF measurements do not correlate with either PBMC or plasma measurements, and cannot differentiate between GBA-PD and HVs.

PCA3. 1 (Supplementary figure 3) was maximized for GBA-PD, including *GBA1* mutation type, MDS-UPDRS part III total score, H&Y, age at diagnosis, duration of diagnosis, age, BMI, GluCer (multiple isoforms) and LacCer (multiple isoforms) in PBMCs, plasma and CSF and GluSph in plasma and PBMCs. Data of study 4 were used, consisting of GBA-PD (n=38, 2 listwise exclusions due to missing CSF samples).

No differentiation can be made between GBA-PD patients with GD-mutations and non-GD-mutations. Clinical characteristics like MMSE, MDS-UPDRS3, duration of diagnosis and age at diagnosis only show weak correlations at best with GSLs.

PCA3. 2 (Supplementary figure 4) was the same as PCA3. 1, but excluding CSF measurements, to emphasize the relation between plasma and PBMC measurements with the clinical GBA-PD covariates. Data of study 4 were used, consisting of GBA-PD (n=40).

No differentiation can be made between GBA-PD patients with GD-mutations and non-GD-mutations. MDS-UPDRS part III and duration of diagnosis seem weakly inversely correlated to LacCer (multiple isoforms) levels in plasma.

PCA4 (Supplementary figure 5) was maximized for GluCer in plasma and PBMCs, but also includes age and BMI. Data of studies 1, 2, 3 and 4 were used, consisting of GBA-PD (n=48), iPD (n=8) and HV (n=86).

GluCer (multiple isoforms) in plasma and in PBMCs do not correlate. GBA-PD can be differentiated from iPD and HVs based on higher plasma GluCer levels and slightly lower PBMC GluCer levels, however with some overlap. iPD and HVs cannot be differentiated.

Effect of cell subtype

Using data of study 4, effect of granulocyte contamination of isolated PBMCs on LacCer and GluSph levels is depicted in Figure 3. An r-squared of up to 0. 67 was seen, suggesting these GSLs are much more abundant in granulocytes than in monocytes and lymphocytes.

Subsequently, GluCer (multiple isoforms), GluSph and LacCer (multiple isoforms) were determined in separately isolated monocytes (average purity: 84%), lymphocytes (average purity: 94%) and granulocytes (average purity: 94%) from 6 healthy volunteers. Absolute values are presented in Figure 4. Distribution of GluCer over cell types varied per isoform; GluCer C20:0 was twice as abundant in lymphocytes compared to both monocytes and granulocytes, and GluCer C24:0 was more than five times as abundant in monocytes compared to lymphocytes and twice as abundant in monocytes compared to granulocytes. Both GluSph and LacCer (all isoforms) were at least 20 times as abundant in granulocytes compared to both monocytes and lymphocytes, and more abundant in monocytes than in lymphocytes.

Discussion

The GCase mechanism is one of the most promising targets to find a first disease-modifying therapy for Parkinson's disease.[3] Use of biomarkers in early phase clinical trials is crucial for innovative drug development.[29] This paper combined data of five studies to further assess multiple potential biomarkers for GBA-PD. Variability was determined of GluCer (multiple isoforms), GluSph, GalSph, GCase activity (using 4-MUG) and GCase protein in plasma and/or PBMCs. Levels were descriptively compared between GBA-PD, iPD and HVs and effects of various covariates were assessed. GSL levels, primarily of LacCer (multiple isoforms) and GluSph, differ heavily between leukocyte subtypes. This shows that it is of utmost importance to always consider what tissue and cell type is being used when measuring or comparing GSLs.

A low within-subject between-day variability relative to an expected change is favorable for a biomarker when investigating a long-term effect. Measurements in plasma were generally less variable than in PBMCs. Higher variability in PBMCs was likely partly caused by a variable cell type constitution of the PBMC isolation, and/or by contamination of the isolated PBMCs with granulocytes. This variability may be reduced when isolating specific cell types and requires further studies.

A theoretical advantage of using PBMCs as opposed to plasma when measuring GSLs, is that this reflects intracellular levels, which intuitively may be expected to better reflect lysosomal functioning. It should be noted,

however, that the majority of the intracellular GSL content is outside of the lysosome[30] and this intracellular distribution is hard to distinguish. Conversely, measurements in plasma and CSF reflect extracellular GSL, of which the origin is unclear.

To our knowledge, this was the first study to quantitatively evaluate various GSLs in a cell-specific way. After isolating monocytes, lymphocytes and granulocytes separately, it was shown that GluSph and LacCer (multiple isoforms) are much more abundant in granulocytes compared to monocytes and lymphocytes. GluCer (multiple isoforms) varied to a lesser extent over cell types, but these differences may still be clinically relevant. Such differences may affect the sensitivity of these measurements as a biomarker, because the ratios of cell subtypes in a PBMC isolation can vary between blood draws within the same person. Similarly, for GCase activity, others have shown this is decreased in monocytes, but not in lymphocytes, in patients with PD.[22] In neuronal and astrocytic cell cultures, GCase activity was higher in neuronal cells,[31] but this may also be influenced by the culture medium, with higher glucose content, in which cells are dividing.

GCase activity was only measured in PBMCs, since the enzyme is active within the lysosome. Activity was lower in GBA-PD compared to iPD and HV, but this was not observed in all GBA-PD patients. For example, activity was measured in two patients carrying the p. D140H+E326K complex allele, of which one had the highest and the other had the second-to-lowest GCase activity within the GBA-PD group. These measurements were consistent within-subject over seven samples and no technical reason could be identified to explain this between-subject inconsistency in GCase activity in patients with the same mutation. This may be caused by the inconsistency in genotype-phenotype correlation known in Gaucher disease.[32] Similarly, in a patient where no *GBA1* variant could be identified (and was therefore classified as *GBA1* wildtype, i. e. iPD in this paper), the second-to-lowest GCase activity was measured of all participants. It is indisputable that being a carrier of certain *GBA1* variants is associated with an increased risk of developing PD, however, it can be hypothesized that measuring actual GCase activity may be a better predictor. This could also identify patients without a *GBA1* mutation, who could potentially benefit from treatment targeting GCase activity. Measuring GCase activity remains challenging because current techniques use artificial substrates, of which is it unclear how these interact with the >400 described pathological mutations compared to the endogenous substrate. Additionally, and similar to the fact that LacCer and GluSph differ heavily between leukocyte subtypes, measurements of GCase activity may be influenced by cell type composition, but this remains to be confirmed. This would also explain a degree of variability in the commonly used dried blood spot assessment of GCase activity, in which the cell subtype constitution of a drop of blood is unknown and may vary.

Various chain lengths of different GSLs may have different functions, but this is not yet fully elucidated. Absolute quantity of the different isoforms of a specific GSL differs strongly as well, within a cell type (Figure 4). In multiple previous studies,[16,19] all GSL chain lengths are totaled, meaning the signal of sparse isoforms will be overshadowed by those more abundant. For example, LacCerC16:0 is approximately 140 times as abundant as LacCerC20:0 in granulocytes, obscuring any potential change in LacCerC20:0, if these are combined. We therefore advise to consider these different chain lengths, despite their exact function being unknown.

The methodology used for granulocyte isolation can have great influence on the quality and quantity of the cells. Granulocytes, with special attention to neutrophils, are very fragile and easily activated. Blood collection technique, use of anticoagulant and processing time are some factors among others which need to be taken in account. The quantity of collected neutrophils is highly dependent on the time of blood storage and time of processing.[33] All activities, from blood collection to the resuspension of isolated granulocytes in buffer, was done at room temperature. It is favorable to not alter the temperature to preserve quantity and function of granulocytes. Granulocytes were isolated within the first hour after blood draw, processed in 3 hours maximum, and immediately snap frozen at –80°C after resuspension in proper buffer.

When comparing GBA-PD to iPD and HV (Figure 2), all GluCer isoforms in plasma were elevated in GBA-PD. Apart from a decreased GCase activity in PBMCs, this is the clearest expression of an affected GSL metabolism in GBA-PD in a cross-sectional study. This was not seen in PBMCs or CSF. To a lesser

extent, GluSph was also increased in plasma of GBA-PD compared to iPD and HVs, but not in PBMCs. Mean differences in LacCer (multiple isoforms) were less pronounced, showing mostly similar levels over groups, with some increased and some decreased isoform levels in plasma in GBA-PD compared to HVs. This may reflect distinct metabolisms between LacCer isoforms or could be a chance finding. No clear difference was seen in GalSph in plasma between GBA-PD, iPD and HVs, although this may also be explained by a relatively small sample size. Measurements in PBMCs may have been affected by differences in the composition of leukocyte subtypes in the PBMC isolations.

The GluCer C24:1 isoform in plasma best differentiated GBA-PD from iPD and HVs. Out of 48 GBA-PD patients, 32 (66. 7%) had a higher level of plasma GluCer C24:1 than all HVs, suggesting this may be a suitable marker to show a response. However, since the origin of this extracellular GluCer is unknown, it remains unclear how fast a response could be expected. The 32 GBA-PD patients with a high plasma GluCer C24:1 level could not otherwise be distinguished from the 16 patients with 'normal' levels, e.g. based on MDS-UPDRS part III, MMSE, duration of diagnosis, age at diagnosis or genotype (e.g. both p. E326K and p. L444P carriers were present in the high and in the 'normal' group).

Using Principal Component Analysis, it was attempted to uncover underlying correlation structures within the data. A single PCA biplot is a figurative representation of multivariate correlations, which is a powerful tool to identify global patterns in large datasets. Correlations between individual parameters, as opposed to global patterns, should be interpreted with caution. Several patterns based on these biplots are discussed.

Within a matrix (i. e., plasma, PBMCs and CSF), the different isoforms of a GSL (GluCer or LacCer) are interrelated (seen in all PCA biplots). It remains unclear whether these isoforms are affected differently or not when the GSL metabolism is influenced by a pharmacotherapeutic intervention. There is no clear-cut best choice in matrix, since all three investigated matrices have arguments for their usefulness. CSF provides the most direct central nervous system measurement, PBMCs provide the only intracellular measurements, and plasma is the only matrix that showed a clear difference between GBA-PD versus iPD and HVs (for GluCer only).

GCase activity in PBMCs showed an inverse correlation with plasma GalSph, and to a lesser extent with plasma GluSph and GluCer. GCase activity was weakly related or unrelated to GluCer in PBMCs (Supplementary figure 1). Plasma GluCer was the only GSL measurement that clearly differed between GBA-PD and HVs (although with overlap) (Figure 2,Supplementary figure 5), where the increased plasma GluCer level in GBA-PD could possibly be explained by a chronically lowered intracellular GCase activity level. It may be hypothesized that GluCer in PBMCs (intracellular) better reflects short-term changes.[25]

It was hypothesized that the GCase protein would be upregulated in patients with a low GCase activity, but these were unrelated (Supplementary figure 1). A p. L444P carrier, with the lowest measured GCase activity, did have the highest GCase protein level, possibly due to compensatory upregulation, but two p. E326K carriers with comparable GCase activity, had a twofold difference in GCase protein, exemplary for the inconsistent relationship with either GCase activity or genotype. GCase protein did positively correlate with GluCer in PBMCs, but not GluSph or any plasma measures. An upregulation of GCase protein may be a response to a relatively higher intracellular GluCer level. GCase protein was measured in PBMCs only, using a commercially available ELISA kit, of which affinity for different mutant proteins is unknown. No differentiation was made between GCase protein in active or in inactive state. Effect of leukocyte subtypes remains to be assessed.

No differentiation between mutation type (Gaucher-associated or PD-only-associated) could be made within the GBA-PD group, based on GluCer (multiple isoforms) and LacCer (multiple isoforms) in plasma, PBMCs and CSF, and GluSph in plasma and PBMCs (Supplementary figure 3, Supplementary figure 4). Conflicting results exist related to clinical differences between certain mutations, meaning these are sometimes apparent, but cannot always be reproduced, likely due to a large variability with small samples sizes.[34–39] This lack of differentiation in the current dataset and the conflicting results in clinical impact may be explained by a variation on a molecular level between patients with the same genotype. Some genotypes may generally be considered more severe than other genotypes based on GD phenotype, but not exclusively so, and vice versa. Due to the high

number of different mutations in the *GBA1* gene, the number of patients with a specific genotype may remain too low to provide sufficient power to adequately detect the, possibly small, molecular or clinical differences between genotypes.

Clinical characteristics assessed in the current GBA-PD study (study 4), MDS-UPDRS III, H&Y, MMSE, age at diagnosis and duration of diagnosis, only showed weak correlations with GSL levels (Supplementary figure 3, Supplementary figure 4). MMSE showed an inverse correlation to CSF levels of GluCer and LacCer. MDS-UPDRS III, H&Y and duration of diagnosis were all aligned and inversely correlated to plasma LacCer. However, the magnitude of these correlations is likely small (as indirectly interpreted based on the short projection vectors on components). A long-term follow-up cohort is required to better quantify a possible relationship between GSL measurements and clinical characteristics.

In addition to what was investigated for the current report, there are of course more potential biomarkers for GBA-PD. The GSL network is extensive and in a previous report, which assessed 520 lipid species in plasma of iPD and controls, monosialodihexosylganglioside (GM3) gangliosides were most significantly increased in iPD [40]. Furthermore, uric acid has been of interest[41] and knowledge about the role of neuroinflammation is quickly expanding [42].

In summary, the *GBA1* mechanism is one of the most promising targets for a first disease-modifying treatment for Parkinson's disease. Use of biomarkers during early-phase trials is crucial for more efficient drug-development. To make best use of biomarkers, any noise in the data should be minimized, which requires an in-depth understanding of the physiology and methodological challenges. Our findings may contribute to this understanding, with key findings given bullet-wise in Box 1. Application of biomarkers in clinical trials may provide novel insights and should be published whenever possible.

Box 1 Bullet-wise summary of the key findings.

KEY FINDINGS

- GluCer, GluSph, GalSph, GCAse activity and GCase protein can be stably measured between-days within-subject, in plasma better than PBMCs.
- Plasma (extracellular) levels of various GluCer isoforms (mainly C24:1 and C24:0) are increased in GBA-PD compared to iPD and HVs.
- GSL levels in plasma (extracellular) are unrelated to levels in PBMCs (intracellular) or CSF (extracellular in central nervous system).
- The relationship between GCase activity, GCase protein level, various GSL levels and *GBA1* genotype, is not straightforward, possibly reflecting a complex and variously adaptive GSL metabolism.
- GBA-PD clinical characteristics do not correlate with GSL levels.
- *GBA1* mutation types do not correlate with GSL levels.
- There can be major differences in GSL levels between cell types, so care is advised when using mixes or tissue homogenates (and may be similarly true for GCase activity and GCase protein).
- GSL isoforms can differ greatly in quantity (within the same cell type) and should not be totaled if possible.

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Table 1 Overview of demographic variables.

Demographics							
	HVs				Patients		
Study	1	2	3	5	1	4	1
Status	HV	HV	HV	HV	GBA-PD	GBA-PD	iPD
n	8	40	39	6	8	40	8
Male/Female	4/4	31/9	26/13	1/5	4/4	20/20	7/1
Age (years), mean (range)	44.4 (22-67)	38.1 (18-64)	67.0 (52-75)	24.3 (23-26)	60.6 (47-72)	61.1 (40-80)	66.0 (54-76)
BMI (kg/2), mean (range)	25.3 (20.4-29.5)	24.1 (18.9-29.8)	25.6 (19.7-31.9)	-	24.0 (18.0-29.1)	24.5 (17.5-35.1)	28.2 (23.2-32.2)
MMSE, mean (range)	-	-	-	-	27.6 (23-30)	27.8 (20.5-30)	28.4 (26-30)
Hoehn & Yahr, mean (range)	N/A	N/A	N/A	N/A	2.3 (1-4)	2.3 (1-4)	1.5 (1-3)
MDS-UPDRS part III, mean (range)	N/A	N/A	N/A	N/A	-	31.4 (14-72)	-
Age at diagnosis (years), mean (range)	N/A	N/A	N/A	N/A	47.0 (38-53)	55.1 (38-76)	60.7 (48-69)
Duration of diagnosis (years), mean (range)	N/A	N/A	N/A	N/A	14.1 (2.8-25)	6.1 (1.5-17.7)	5.7 (1.8-11.8)

BMI=body mass index; max=maximum; MDS-UPDRS=Movement Disorder Society-Unified Parkinson's Disease Rating Scale; min=minimum; MMSE=Mini Mental State Examination; N/A=not applicable; -=not performed in this study.

Table 2 Overview of *GBA1* variants. HVs from study 1 and 5 were not sequenced and genotype is therefore unknown. The *GBA1* allelic names are given, excluding the 39-amino acid signaling peptide. In case of two mutations, variants within the staple signs [ ] are on the same allele, and variants in separate staple signs are on separate alleles. A semicolon in parentheses indicates it is uncertain how these mutations are distributed over alleles.

Study	1	2	3	4	5
GBA1 Genotype	HV, iPD, GBA-PD	HV	HV	GBA-PD	HV
Wildtype	8 (iPD)	37	37	0	0
Unknown	8 (HVs)	0	0	0	6
Mutated	8 (GBA-PD)	3	2	40	0
GBA1 mutations (allelic name)					
p.R120W	0	0	0	2	0
p.[D140H;E326K]	2	0	0	6	0
p.G202R	0	0	0	1	0
p.[L324P];[T369M]	1	0	0	0	0
p.G325R	0	0	0	1	0
p.E326K	2	1	0	9	0
p.[E326K];[E326K]	1	0	0	1	0
p.[E326K];[T369M]	0	0	0	1	0
p.R329C	0	0	0	1	0
p.T369M	1	2	2	4	0
p.T369M(;)L444P	0	0	0	2	0
p.N370S	0	0	0	7	0
p.L444P	1	0	0	5	0

Table 3 Coefficient of variance (= (SD/mean)*100) of GluCer (multiple isoforms), GluSph, GalCer, GCase activity and GCase protein in plasma and/or PBMCs, split for HVs, iPD and GBA-PD, for within/day and between/day assessments. Data is from study 1. Measurements in PBMCs are shaded grey, measurements in plasma are white. PBMCS=peripheral blood mononuclear cells; HV=healthy volunteer; GBA-PD=Parkinson's disease with a *GBA1* mutation; iPD=idiopathic Parkinson's disease (i.e. no *GBA1* mutation); GCase=glucocerebrosidase.

Coefficient of Variation (CV) (study 1)					
		Within day		Over days	
Variable	**Group**	**Within subject**	**Between subject**	**Within subject**	**Between subject**
		PBMCS			
GCase Protein	HV	13.4%	27.4%	17.9%	27.3%
(pg/1.0E+07 cells)	GBA-PD	13.0%	25.9%	19.8%	27.2%
	iPD	11.4%	36.3%	20.0%	32.8%
GCase activity	HV	26.4%	14.0%	14.2%	21.3%
(pmol/h/ug tot prot)	GBA-PD	21.1%	56.2%	17.8%	57.0%
	iPD	21.7%	51.1%	34.2%	53.7%
Glucosylceramide C16:0	HV	20.1%	27.2%	10.8%	15.1%
(pmol/5.0E+05 cells)	GBA-PD	14.9%	15.2%	12.9%	21.1%
	iPD	26.6%	24.3%	18.6%	23.9%
Glucosylceramide C18:0	HV	12.2%	13.3%	10.1%	15.4%
(pmol/5.0E+05 cells)	GBA-PD	12.6%	30.2%	17.2%	21.9%
	iPD	22.4%	22.6%	16.8%	21.1%
Glucosylceramide C22:0	HV	11.1%	7.7%	10.6%	8.1%
(pmol/5.0E+05 cells)	GBA-PD	12.0%	21.0%	13.2%	21.0%
	iPD	21.1%	18.1%	14.3%	13.1%
Glucosylceramide C24:0	HV	13.0%	19.5%	11.9%	19.0%
(pmol/5.0E+05 cells)	GBA-PD	14.4%	23.6%	12.8%	23.8%
	iPD	24.0%	24.1%	15.4%	18.3%
Glucosylceramide C24:1	HV	12.2%	19.8%	12.5%	16.2%
(pmol/5.0E+05 cells)	GBA-PD	12.2%	20.9%	12.9%	25.3%
	iPD	27.1%	27.8%	19.6%	23.8%
Glucosylsphingosine	HV	43.2%	55.3%	35.2%	59.5%
(pmol/5.0E+05 cells)	GBA-PD	32.3%	52.4%	25.9%	51.6%
	iPD	25.9%	38.0%	44.5%	51.7%

Table 3 *(Continuation of previous page)*

Coefficient of Variation (CV) (study 1)					
		Within day		Over days	
Variable	**Group**	**Within subject**	**Between subject**	**Within subject**	**Between subject**
PLASMA					
Galactosylsphingosine	HV	5.2%	30.4%	17.5%	27.6%
(pmol/mL)	GBA-PD	8.4%	17.1%	10.0%	13.3%
	iPD	7.1%	31.7%	7.7%	32.1%
Glucosylceramide C16:0	HV	4.0%	34.6%	6.8%	36.1%
(pmol/mL)	GBA-PD	5.1%	19.7%	6.2%	18.9%
	iPD	5.4%	23.9%	5.8%	23.3%
Glucosylceramide C18:0	HV	6.7%	33.3%	8.5%	34.0%
(pmol/mL)	GBA-PD	7.0%	35.5%	8.3%	36.6%
	iPD	9.2%	25.1%	7.3%	25.1%
Glucosylceramide C22:0	HV	8.6%	31.2%	11.4%	30.3%
(pmol/mL)	GBA-PD	7.7%	25.7%	9.9%	27.5%
	iPD	10.5%	21.2%	8.7%	25.4%
Glucosylceramide C24:0	HV	8.5%	29.4%	11.8%	29.4%
(pmol/mL)	GBA-PD	9.4%	25.0%	12.0%	24.9%
	iPD	10.8%	16.6%	9.1%	17.5%
Glucosylceramide C24:1	HV	6.0%	30.8%	10.1%	31.5%
(pmol/mL)	GBA-PD	6.9%	18.0%	7.0%	18.4%
	iPD	6.8%	25.5%	7.4%	24.6%
Glucosylsphingosine	HV	6.3%	29.9%	19.2%	29.4%
(pmol/mL)	GBA-PD	7.8%	42.5%	13.9%	37.0%
	iPD	7.8%	39.0%	9.3%	36.0%

Figure 1 Measurements over time (first five are within day, last two are on separate days) of GCase activity in PBMCs (top-left), GCase Protein in PBMCs (top-right), GluCer C18:0 in PBMCs (bottom-left) and plasma (bottom-right), for HV (top), iPD (middle) and GBA-PD (bottom). Lines indicate individual participants. PBMCs=peripheral blood mononuclear cells; D=day; M=measurement.

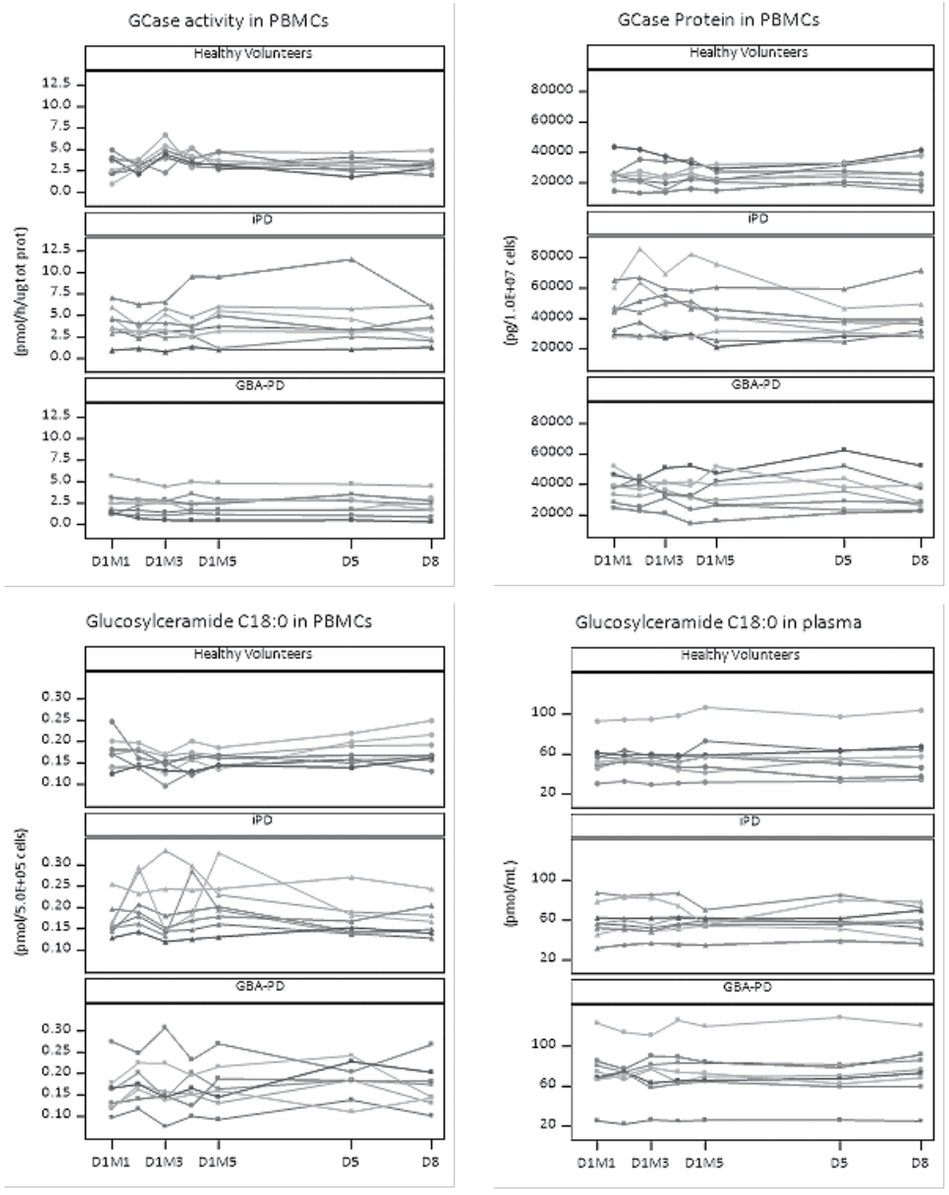


Figure 2 Violin plots given per biomarker (GluCer, LacCer, GluSph, GalSph, GCase activity, GCase protein) and per matrix (PBMCs, plasma and CSF if available), separate for GBA-PD (left), iPD (middle; not available for LacCer) and HVs (right). In the boxplots, the horizontal line indicates the median, the box indicates the 25th and 75th percentiles, and the whiskers indicate the largest/smallest value no further than 1.5 * inter-quartile range from the box. Samples that were <LLOQ were treated as 50% of LLOQ. Some participants have a value between LLOQ and 50% of LLOQ in CSF, if one sample was above LLOQ and one was below (maximum of 2 CSF samples per participant), of which the average was taken.

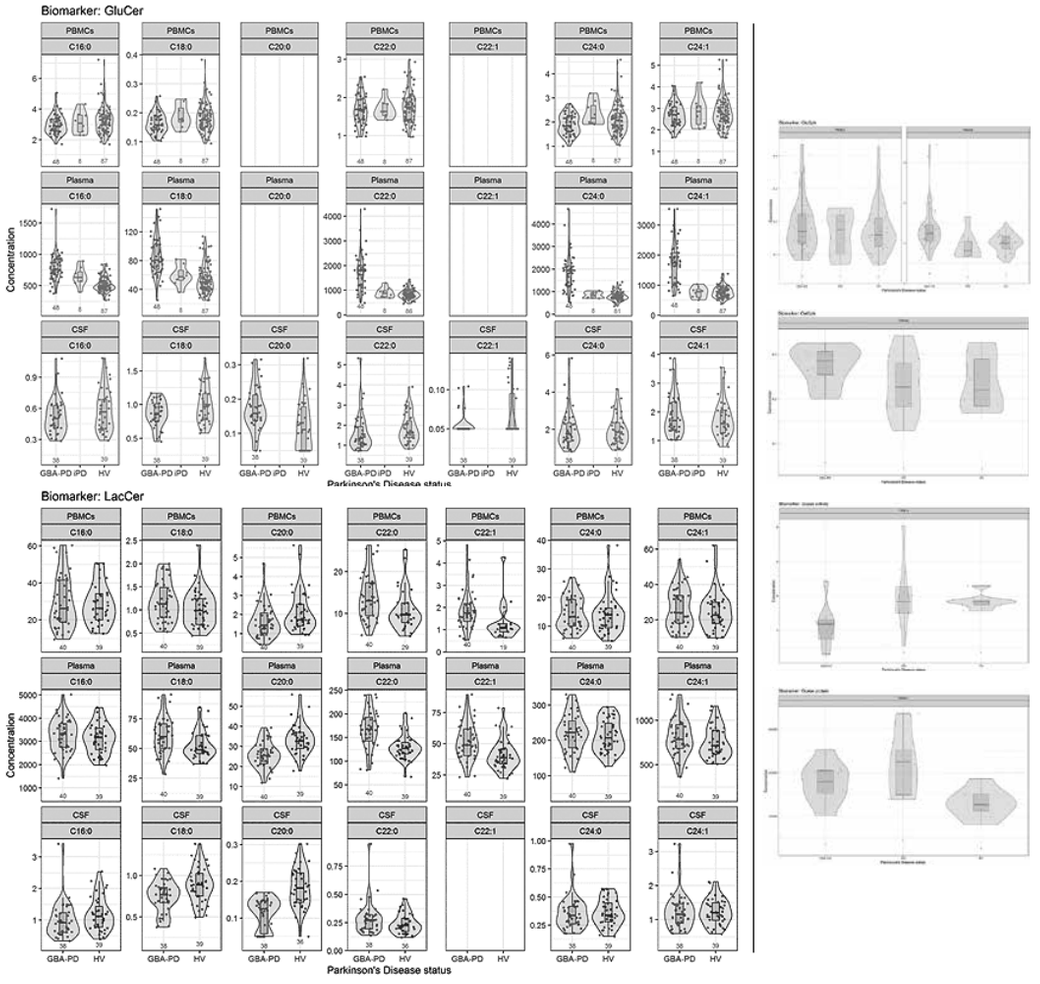


Figure 3 Scatterplots of percentage of granulocytes in a PBMC isolation and the correlation with different LacCer isoforms (pmol per 0.5*10^6 cells) and GluSph (pmol per 1.0*10^6 cells). R-squared is given per plot.

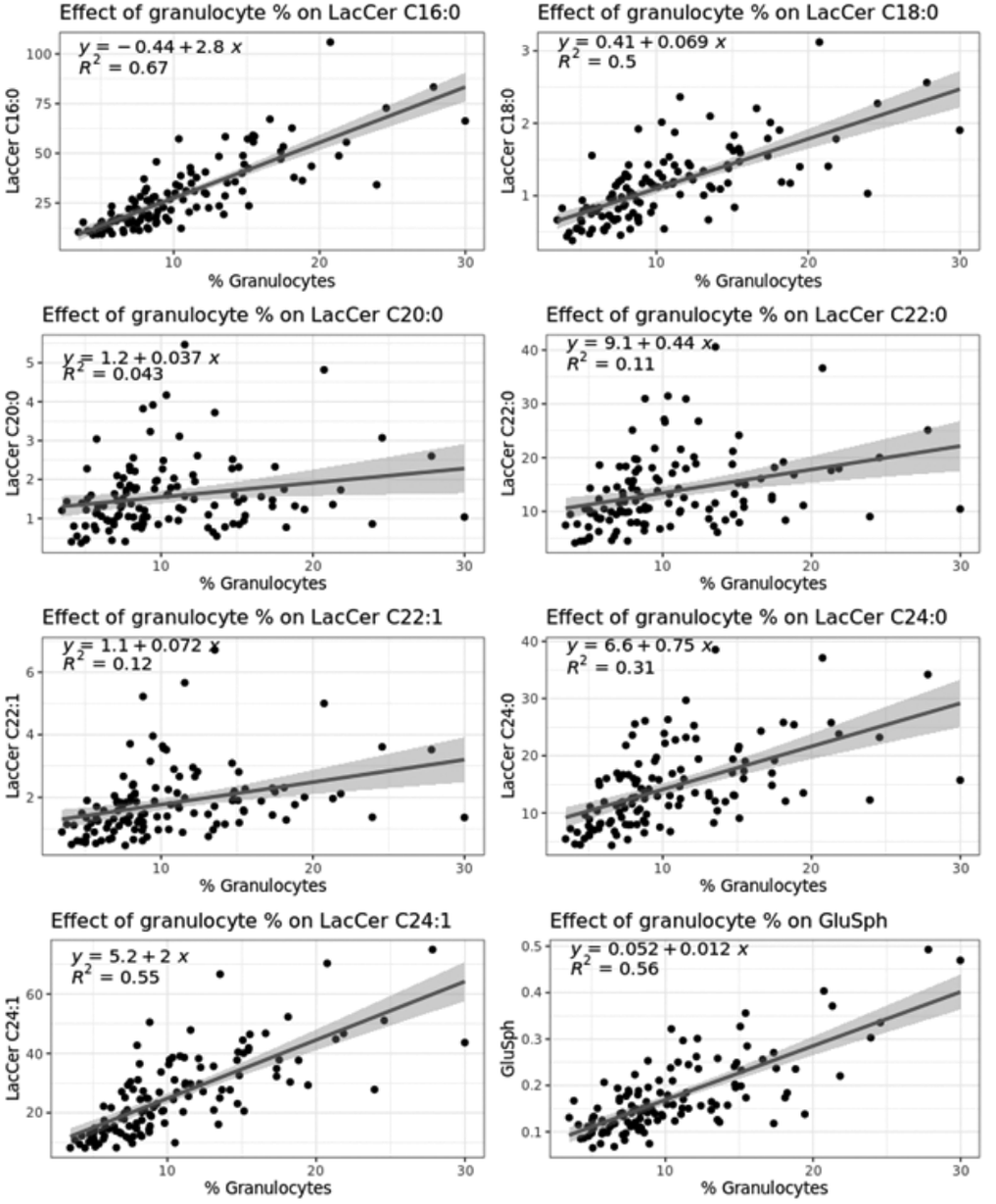
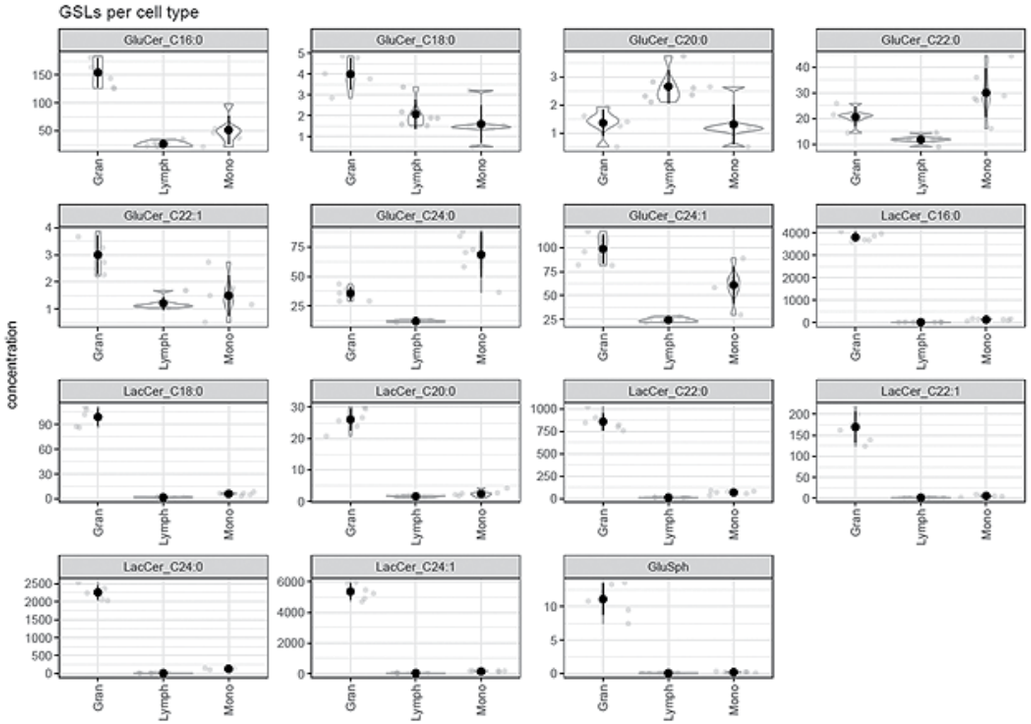


Figure 4 Absolute values per specific GSL in granulocytes, lymphocytes and monocytes, isolated from healthy volunteers (8*10^6 cells mL^{-1}). The same number of cells were isolated per cell type. Due to large differences in Y-axis scaling, each GSL has a separate plot. Within each plot, violin plots are depicted for granulocytes (left), lymphocytes (middle) and monocytes (left). The black point with lines represents the mean±1SD, sometimes not visible due to scaling.



SUPPLEMENTARY MATERIAL

H6ST1 / H6ST2 / H6ST3 / H6SF1 / H6SF2 / H6SF3 / H6SF4 / H6SF5

SCAN ME

CHAPTER 7

A randomized single and multiple ascending dose study in healthy volunteers of LTI-291, a centrally penetrant glucocerebrosidase activator

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The authors confirm that the Principal Investigator for this paper is Geert Jan Groeneveld and that he had direct clinical responsibility for participants.

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Abstract

Aims A mutation in the *GBA1* gene is the most common genetic risk factor for developing Parkinson's disease. *GBA1* encodes the lysosomal enzyme glucosylceramidase beta(glucocerebrosidase, GCase) and mutations decrease enzyme activity. LTI-291 is an allosteric modulator of GCase, enhancing its activity. These first-in-human studies evaluated the safety, tolerability, pharmacokinetics and pharmacodynamics of single and multiple ascending doses of LTI-291 in healthy volunteers.

Methods In the single ascending dose (SAD) study, 40 healthy volunteers were randomly assigned to LTI-291 (n=8 per dose level) or placebo (n=2 per dose level). Single doses of 3, 10, 30, and 90 mg LTI-291 were investigated. In the multiple ascending dose (MAD) study, 40 healthy middle-aged or elderly volunteers were randomly assigned to LTI-291 (n=8 per dose level) or placebo (n=2 per dose level). Fourteen consecutive daily doses of 3, 10, 30, and 60 mg LTI-291 or placebo were administered. In both the SAD and MAD studies, glycosphingolipid levels were measured and a test battery of neurocognitive tasks was performed.

Results LTI-291 was generally well tolerated and no deaths or treatment-related SAEs occurred and no subject withdrew from a study due to AEs. C_{max}, AUC_{0-24} and AUC_{0-inf} increased in a dose proportional manner. The median half-life was 28. 0 hours after multiple dosing. No dose-dependent glycosphingolipid changes occurred. No neurocognitive adverse effects were detected.

Conclusions These first-in-human studies demonstrated that LTI-291 was well tolerated when given orally once daily for 14 consecutive days. This supports the continued clinical development and the exploration of LTI-291 effects in a *GBA1*-mutated Parkinson population.

Statements

What is already known about this subject

- Parkinson's disease is the second most common neurodegenerative disorder.
- Current therapies only alleviate motor symptoms, without addressing non-motor symptoms or influencing disease progression.
- A mutation in the *GBA1* gene, typically as a heterozygous mutation in one allele, affecting 4-20% of patients, is the most common genetic risk factor to develop Parkinson's disease known to date and therefore a potential target for a disease-modifying therapy.

What this study adds

- LTI-291, a glucocerebrosidase enhancer, was shown to be central nervous system penetrant and is intended for use in patients with Parkinson's disease carrying a *GBA1* mutation.
- LTI-291 was generally well tolerated in healthy volunteers.
- LTI-291 has favourable pharmacokinetic characteristics for daily single dosing.

Introduction

Parkinson's disease (PD; MIM: 168600) is the second most common neurodegenerative disorder, with a multifactorial disease etiology, consisting of both environmental and genetic risk factors and their interaction.[1] PD is clinically characterized by both motor and non-motor symptoms, such as mood-, sleep-, autonomic-, cognitive- and olfactory disturbances. Currently available pharmacotherapy is primarily directed at the dopaminergic system and primarily alleviates motor symptomatology, without addressing non-motor symptoms or otherwise influencing disease progression. This leaves a large unmet need for disease-modifying therapy.

A potential target in PD is the lysosomal enzyme glucosylceramidase beta (glucocerebrosidase, GCase; EC 3.2.1.45), encoded by the *GBA1* gene (MIM: 606463). In the mutated state this is the largest genetic risk factor to develop PD known to date, referred to as GBA-PD.[2] In most populations, 4-12% of PD

patients carry a heterozygous *GBA1* mutation and in Ashkenazi Jewish PD patients this is approximately 20%.[3,4] For PD patients, Odds Ratios (OR) of having a *GBA1* mutation compared to people without PD usually vary between 2-7.[3–6] GBA-PD on average presents at a younger age with a higher prevalence of non-motor symptoms, however with a high variability between individual patients.[7,8]

GCase is involved in intralysosomal metabolism of glycosphingolipids (GSLs), a class of lipids, essential for membrane and other cellular functions.[9] It hydrolyses the glycosphingolipid glucosylcerebroside (GluCer) into glucose and ceramide. In the presence of a GCase mutation, this reaction rate is decreased and this may lead to a lower flux of glycolipid metabolism within the lysosome. In case of GCase deficiency, the enzyme acid ceramidase can convert GluCer into glucosylsphingosine (GluSph).[10] GluSph is postulated to have toxic effects and can also be metabolized by GCase,[10] however the turnover rate is much lower than for GluCer.[11]

The immediate precursor to GluCer in the lysosomal degradation pathway is lactosylceramide (LacCer). It is still not fully understood how these molecules are affected in GBA-PD, with conflicting results on whether these accumulate or not.[12–15]

LTI-291 is a central nervous system (CNS) penetrable small-molecule GCase allosteric modulator, enhancing GCase activity, as a potential treatment of GBA-PD. Preclinically, activation of GCase using LTI-291 was shown in recombinant wild type and mutant (the two common variants p. Asn409Ser and p. Glu365Lys, both commonly referred to as N370S and E326K) human GCase, using an *in vitro* micellar environment and the artificial fluorescent 4-MUG substrate. LTI-291 at concentrations of 0. 1 and 1. 0 µM increased wild type GCase activity by 25% and 130%, respectively. GCase resides on the inner membrane surface of the lysosome, and the substrate GluCer resides within the lysosomal membrane. LTI-291 requires a lipidic surface in which to form an active complex with the GCase enzyme. *In vitro*, this surface was supplied by micelles, while *in vivo* activation of GCase probably depends on complex formation within the lysosomal lipid membrane. In transgenic mouse models, in cultured human induced pluripotent stem cell-derived neurons and in *ex vivo* peripheral blood mononuclear cells (PBMCs) from idiopathic PD and GBA-PD patients, GluCer levels decreased by 10-30% after treatment with LTI-291 (observed in GluCer isoforms: C22:0, C22:1, C24:0, C24:1; not observed in GluCer C18:0 and C20:0). Based on the transgenic mouse data, the minimally efficacious plasma concentration lies between 400 and 2500 nM. Higher doses showed comparable reduction of GluCer (~-20%) in transgenic mice, suggesting that once normalization of the pathway flux is achieved, there is a flattening of the dose response. Modelling was used to predict that a dose of 6 mg LTI-291 was needed to achieve a C_{max} of 1000 nM (~360 ng/mL) in humans. The no-observed-adverse-effect level (NOAEL) was determined in rat as the most sensitive species, with first observed adverse effects of frequent salivation and vomiting, at a human equivalent dose of 580 mg per day. Starting dose was determined at 3 mg per day, expected to be pharmacologically inactive and 19-fold lower than the Maximum Recommended Starting Dose (MRSD) of approximately 58 mg. Ascending doses of 10 mg, 30 mg, 60 mg and 90 mg were expected to be pharmacologically active.

GSLs constitute a vast and dynamic network, in which local disturbances can cause multiple changes within the network.[9] GluCer, GluSph and LacCer, as proximal molecules to GCase, were investigated as potential biomarkers in various matrices. Plasma is most conveniently obtained, whereas peripheral blood mononuclear cells (PBMCs), which are more labor intensive to obtain, may better reflect the site of action, namely the lysosome. Cerebrospinal fluid (CSF) was included as matrix most proximal to the brain, despite the limited abundance of lipid substances in this hydrophilic fluid. Healthy volunteers are expected to have normal GCase activity and normal GSL levels. It is hypothesized that LTI-291 may increase the flux through the GSL metabolism by activating GCase and thereby potentially transiently change normal glycosphingolipid levels in healthy volunteers. GSLs were measured to assess potential transient changes and as safety indicator to prevent unexpected unwanted changes. This paper describes the first-in-human doses of LTI-291, assessing safety, tolerability, pharmacokinetics (PK) and pharmacodynamics, in healthy volunteers in a single ascending dose (SAD) study and in healthy middle-aged volunteers in a multiple ascending dose (MAD) study.

Methods

Both the SAD and the MAD study were randomized, double-blind and placebo-controlled. The studies were approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek), Assen, The Netherlands. Both studies are registered in the Dutch Trial Registry (Nederlands Trial Register, NTR) under study number NTR6598 (SAD) and NTR6705 (MAD). The SAD study took place between August and September 2017 and the MAD study took place between September and December 2017. All subjects signed an informed consent form prior to any study-related activity. The authors confirm that the Principal Investigator for this paper is Prof. Dr. G. J. Groeneveld and that he had direct clinical responsibility for participants.

The drug target was referred to in accordance with the IUPHAR/BPS Guide to PHARMACOLOGY nomenclature classification.[16]

Subjects

For the SAD study, healthy men and women of non-childbearing potential were enrolled for a single oral dose of LTI-291. Prior concomitant medication was only allowed at the discretion of the investigator and the sponsor. The following dose levels were investigated in ascending order: 3 mg, 10 mg, 30 mg and 90 mg LTI-291. Treatment was administered as powder in a capsule with 240 mL water. In each cohort 10 subjects were randomized to receive LTI-291 or placebo in an 8:2 ratio. Cohort 1 was dosed using a sentinel approach: the first two subjects (1 placebo and 1 active) were randomized and dosed with a 24 hour observation period prior to dosing the remainder of the cohort. The effect of food on PK was investigated in the 10 mg cohort, using a high-fat breakfast according to FDA standards. In that cohort, subjects returned for a second visit for dosing (in the same 8:2 randomization) in the fed state after a wash-out of at least 1 week following the initial dose.

For the MAD study, healthy middle-aged and elderly (50–75 years of age) men and women of non-childbearing potential were enrolled for 14 consecutive daily oral doses of LTI-291. Prior concomitant medication was only allowed at the discretion of the investigator and the sponsor. The following dose levels were investigated: 3 mg, 10 mg, 30 mg and 60 mg LTI-291. Treatment was administered as powder in a capsule with 240 mL water. In each dose group 10 subjects were randomized to receive LTI-291 or placebo in an 8:2 ratio. Cohort 1 was dosed following a sentinel approach: the first two subjects (1 placebo and 1 active) were randomized and dosed with an observation period equivalent to at least five half-lives prior to dosing the remainder of the cohort.

For the SAD study, dose escalation took place following thorough review of the safety and tolerability data of at least 24 hours post dose, as well as PK assessments of the preceding dose group. In addition, all available pharmacodynamic data were used in this evaluation. For the MAD study, two dose escalation reviews per cohort were performed. The first review after 7 days of dosing, to initiate the next dose level. A second review was performed after 14 days of dosing, to confirm continuation of the subsequent cohort.

Safety

For both studies, a medical screening (medical history, record of prior concomitant medication, subject demographics, height and weight, 12-lead electrocardiography (ECG), vital signs, routine hematology, biochemistry/electrolytes and urinalysis, urine pregnancy test (for females), virology, urine drug screen, ethanol breath test and physical examination) was performed to assess a subject's eligibility. During study periods, safety was assessed using monitoring of adverse events (AEs), concomitant medication, vital signs, ECG, physical examination and safety chemistry and hematology blood sampling.

Pharmacokinetics

LTI-291 levels were measured in K_2EDTA plasma and in cerebrospinal fluid (CSF) (MAD only). LTI-291 levels were measured using a validated high-performance liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection, by PRA Health Sciences (Assen, the Netherlands). The calibration range was 10. 0 – 20000 ng/mL with a coefficient of variation of 5. 2% in

plasma and 0.025 – 50.0 ng/mL with a coefficient of variation of 4. 4% in CSF.

LTI-291 plasma levels were used for non-compartmental PK analysis (NCA) in Phoenix 64 build 7.0.0.2535 using WinNonlin 7.0, by Certara QSP (USA, Inc. Princeton, NJ). If concentrations were below the assay limit of quantification they were treated as having a concentration of 0 ng/mL prior to the first sample with a measurable concentration or as missing at all other time points. PK NCA was performed on the data from each subject as data permitted. The apparent terminal half-life was calculated by log-linear extrapolation of those points determined to be on the apparent terminal phase. The area under the LTI-291 plasma concentration-time curve (AUC) was calculated from 0 to the last measurement point (AUC_{0-last}). In case the terminal phase was sufficiently well characterized and the terminal half-life was estimated, the AUC from 0 to infinity (AUC_{0-inf}) was derived. The apparent clearance (CL/F) and apparent volume of distribution (Vz/F) were calculated following the first dose, where the AUC_{0-inf} was calculated and following the seventh and fourteenth doses, where the AUC_{0-24} was calculated. The C_{max}, AUC_{0-24} and AUC_{0-inf} were plotted against dose level to visually assess dose proportionality. CSF was only collected in the MAD pre-dose and approximately 4 hours after the fourteenth dose, paired with a plasma sample. Due to this limited sampling, only CSF:plasma ratios were calculated.

Pharmacodynamics

GLYCOSPHINGOLIPID LEVELS Biochemical pharmacodynamic markers were measured in K_2EDTA plasma, PBMCs and CSF. PBMCs were isolated from venous blood using cell preparation tubes (CPT) containing sodium heparin (Becton Dickinson, NJ, USA) according to manufacturer's instructions. In short, CPTs were centrifuged at 1800xg for 30 minutes at room temperature. PBMCs were collected and washed twice with Phosphate-buffered saline (PBS) containing 10% heat-inactivated human serum (both Gibco, Thermo Scientific, Waltham, MJ, USA). Cells were snap-frozen at $1*10^7$ cells mL^{-1} in PBS with 0. 1% Bovine Serum Albumin (BSA) buffer, and stored at -80°C until analysis. Cells for GluSph analysis were frozen in glass tubes instead of a cryopreservation tube. For CSF collection, a Pencan® 25G atraumatic needle was used. The first mL CSF was discarded to prevent contamination with blood, after which 4 mL was collected in a 15 mL Falcon® tube. CSF was transferred to a glass tube and 0.2% BSA with ascorbic acid was added. This was centrifuged at 2000xg for 3 minutes at room temperature. Supernatant was transferred to glass tubes, snap frozen and stored at -80°C until shipment for analysis. Time from collection to freezing did not exceed 60 minutes.

GluCer, LacCer and GluSph were measured in K_2EDTA plasma and in PBMCs, using a validated LC-MS/MS method, by Ardena Bioanalytical Laboratory (Assen, the Netherlands). The carbon chain of a ceramide group like in GluCer can be of varying length and saturation. For the SAD, both in plasma and PBMCs, concentrations were measured of GluCer C16:0, C18:0, C22:0, C24:0 and C24:1. In plasma, the assay range was 1. 00 to 2500 pmol. In PBMCs, the assay range was 0.0500 to 12.5 pmol. Biomarker samples were taken pre-dose and 1, 4, 8 and 24 hours post-dose.

For the MAD, GluCer and LacCer were measured in plasma, PBMCs and CSF. GluSph was measured in plasma and PBMCs. GluSph is present at too low levels in CSF to be accurately measured. Both in plasma and PBMCs, the same GluCer isomers as in the SAD were measured in the MAD. Additionally, concentrations were measured of LacCer C16:0, C18:0, C20:0, C22:0, C22:1, C24:0 and C24:1. In plasma, the assay range was 1. 00 to 4000 pmol. In PBMCs, the assay range was 0.0500 – 50.0 pmol. GluSph was measured in plasma with an assay range of 0.0500 – 10.0 pmol and in PBMCs with an assay range of 0.00500 – 1.00 pmol. In CSF, GluCer C16:0, C18:0, C22:0, C24:0 and C24:1 were measured and LacCer C16:0, C18:0, C20:0, C22:0, C22:1, C24:0 and C24:1 were measured. The lower limit of quantification (LLOQ) was 0. 100 pmol for GluCer C20:0, C22:1, C24:0 and C24:1 and LacCer C20:0, C22:1 and C24:1, 0. 300 pmol for GluCer C16:0, C18:0 and C22:0 and LacCer C18:0, C22:0, C24:0 and C24:1 and 4. 00 pmol for LacCer C16:0. The upper limit of quantification (ULOQ) was 100 pmol for all analytes. Biomarker samples were taken twice pre-dose, 2 hours after the seventh dose and 2 hours after the fourteenth (last) dose.

NEUROCOGNITIVE BIOMARKERS (FOR SAFETY) Both in SAD and MAD, the NeuroCart®, a CNS test battery, was used to exclude any adverse effects of LTI-291 on CNS functioning. The test battery consists of neurophysiological, psychomotor and cognitive tests and has been extensively used

previously in clinical drug development.[17–21] In short, measurements consist of saccadic and smooth pursuit eye movements, the adaptive tracking test (a visuo-motor task sensitive to disturbances in vigilance and attention), the body sway (a test of postural stability), the Bond and Lader test (visual analogue scale (VAS) of alertness, calmness and mood), the Visual Verbal Learning Test (VVLT) (a test of immediate and delayed memory), and pharmaco-EEG. Tests were performed in a quiet room with ambient illumination with only one subject in the same room (and a research assistant) per session. For the SAD, all measurements were performed at baseline and 1 hour, 3 hours and 6 hours post-dose. For the MAD, all measurements were performed at baseline, 1 hour, 2 hours and 4 hours after the first dose, 2 hours prior to and 1, 2 and 4 hours after the fourteenth (last) dose. The VVLT, part of the test battery, was only performed at 1 hour post-dose after first and last dose, to minimize the learning-effect for this memory-based task. All subjects underwent a training session of all CNS tests within 21 days preceding study start to minimize learning effects and to make sure subjects were familiar with the test procedure.

GBA1 Genotyping

The full *GBA1* gene was sequenced in all subjects, with a validated method accounting for the *GBA1* pseudogene, described in detail elsewhere.[22] Considering the low predictive clinical value of having a *GBA1* mutation, results of the genotyping were not shared with subjects, unless the subject specifically requested this.

Statistical analysis

Repetitively measured pharmacodynamic data were analysed with a mixed model analysis of variance with fixed factors treatment, time and treatment by time, random factor subject and the average pre-value as covariate. Single measured VVLT data were compared with a one-way ANOVA with factor treatment (for SAD). Repetitively measured VVLT data without pre-value were analysed with a mixed model analysis of variance with fixed factors treatment, time and treatment by time and random factor subject (for MAD). Single measured CSF data with pre-value were compared with a one-way ANOVA with factor treatment and pre-value as covariate (MAD only).

Biomarkers are measured in an exploratory hypothesis-generating setting and are therefore not corrected for multiple testing.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

Demographics and baseline characteristics

In the SAD study, a total of 40 healthy subjects received a single dose of LTI-291 or placebo. The cohorts were comparable regarding mean body mass index (BMI). Age varied both within and between cohorts. In the 90 mg group, 3 subjects with a heterozygous *GBA1* mutation were identified, while in all other subjects no *GBA1* mutation could be detected (referred to as wild type). Of the three subjects with a *GBA1* mutation, two carried the p. Thr408Met variant and one carried the p. Glu365Lys variant. For many *GBA1* mutations it is known that in homozygous state they can potentially cause the lysosomal storage disorder Gaucher's disease (GD). The two currently found variants are both relatively mild variants that do not cause GD in homozygous state, but are a risk factor for developing Parkinson's disease (in GD literature these mutations are historically referred to as T369M and E326K, respectively).[23,24] Demographic data of the SAD study are summarized in Table 1.

In the MAD study, a total of 39 healthy middle-aged subjects received 14 consecutive daily doses of LTI-291 or placebo. One subject randomized to 3 mg LTI-291 withdrew pre-dose due to personal reasons and was not replaced. The cohorts were comparable regarding mean age and BMI. Two subjects (one randomized to 10 mg LTI-291 and one to 60 mg LTI-291) had a heterozygous *GBA1* mutation, both carrying the relatively mild p. Thr408Met variant. Demographic data of the MAD study are summarized in Table 2.

Safety and tolerability

Single administrations of LTI-291 up to the highest dose of 90 mg and 14 consecutive daily administrations of LTI-291 up to the highest dose of 60 mg were generally well tolerated in healthy volunteers. No serious AEs (SAEs) were reported and no AEs led to discontinuation. No clinically relevant changes in blood chemistry, hematology, urinalysis, vital signs, ECG or CNS tests were identified. In the SAD study, all AEs were mild, except for a single moderate AE, which was considered unlikely related to treatment with LTI-291 (vasovagal collapse after venipuncture). In the MAD study, dose dependent increases in AE frequency could be seen in the nervous, gastrointestinal and musculoskeletal system organ classes. Headache, somnolence and myalgia were relatively highly reported in LTI-291 dose groups in general and in the 60 mg LTI-291 dose group specifically compared to placebo. Headache was reported by 39% of all LTI-291 treated subjects versus 0% of placebo subjects, however no dose-dependent increase was observed. None of the reported headaches ameliorated in supine position or otherwise seemed related to a lumbar puncture. Somnolence was reported by 50% of 60 mg LTI-291 subjects versus 12. 5% of placebo subjects, however, for most subjects this was of short duration and no abnormalities were found in CNS measurements that are known to be associated with a decline in arousal. Myalgia was reported by 37. 5% of 60 mg LTI-291 subjects versus 12. 5% of placebo subjects. No clinically significant creatine kinase abnormalities were found in the 60 mg group, thereby excluding rhabdomyolysis. These AEs were assessed as possibly related to administration of LTI-291. Three moderate AEs were reported, all three assessed as unlikely related to LTI-291 treatment. One subject (3 mg LTI-291) had a transient increase in liver enzymes after dose 13, preceded by abdominal pain in the upper right quadrant, sweating, increased blood pressure and an urge to move around, assessed as a passing gallstone. In this subject transaminases were highest pre-dose on day 14 of dosing (AST 702 U/L (reference range: 0-35 U/L), ALT 994 U/L (reference range: 0-45 U/L), GammaGT 286 U/L (reference range: 0-55 U/L) and Alkaline Phosphatase 143 U/L (reference range: 0-115 U/L). Total bilirubin was not significantly elevated: 20 umol/L (reference range: 0-17 umol/L), decreased on day 15 (AST 85 U/L, ALT 376 U/L, GammaGT 212 U/L and Alkaline Phosphatase 126 U/L) and normalized at follow-up. A second subject (10 mg LTI-291) had hypertension on day 1 of dosing, most likely due to suboptimal use of anti-hypertensives, which normalized after increasing the prior concomitant medication. A third subject (60 mg LTI-291) had an asymptomatic urinary tract infection on day 1, treated with antibiotics. An overview of all AEs by treatment is given in Table 3.

Pharmacokinetics

Following oral dosing of LTI-291 to fasted subjects plasma concentrations increased immediately with no apparent lag time. The average T_{max} was 2.2 hours (range 1.0 - 8. hours) following single oral administration and was similar following multiple administrations at 2. 3 hours (range 1.0 - 8.8 hours) (Figure 1 for SAD and Figure 2 for MAD). In the SAD study group geometric mean half-life varied between 21. 2 and 23. 3 hours and plasma concentrations declined in a mono or biphasic manner following C_{max}. Other PK parameters estimated for both SAD and MAD studies are summarized in Table 4 and Table 5. In the fed state there was a lag in absorption of LTI-291 resulting in an average T_{max} of 8.0 hours (range 4.0-12.0 hours) vs 1 hour (range 1. 0-4.0 hour) in the fasted state, but there appeared to be no difference in AUC_{0-inf}, AUC_{0-last}, elimination rate constant (λz) or half-life between fed and fasted dosing, as assessed in the 10 mg SAD cohort (Figure 1, Table 4). Plasma exposure was dose-proportional both in single and in multiple doses (Table 4 and Table 5). For example, in the SAD study over a 30-fold dose range (3 to 90 mg) the C_{max}, AUC_{0-last} and AUC_{0-inf} all increased in a dose proportional manner. Steady state plasma concentrations were reached after seven days of daily dosing, resulting in an approximate 1. 8-fold increase for C_{max} and 2. 5-fold increase in AUC_{0-24}. The mean CSF:plasma concentration ratios ranged from 0. 0124 to 0. 0134 at 4-5 h after the 14^{th} dose and were similar at all dose levels (Table 6). Over the 20-fold dose range (3 to 60 mg) the CSF concentrations increased in a dose proportional manner relative to the plasma concentrations.

Pharmacodynamics

GLYCOSPHINGOLIPID LEVELS In the SAD study, different GluCer isomers were measured in plasma and PBMCs. No significant dose-dependent effects of LTI-291 were detected. See Supplementary table 1 for details. The three subjects with a *GBA1* mutation showed a comparable pattern of GluCer variables in plasma and PBMCs to the subjects in the same cohort without a *GBA1* mutation before and after a single dose of LTI-291 (data not shown).

In the MAD study, different GluCer and LacCer isomers were measured in plasma, PBMCs and CSF. GluSph was measured in plasma and PBMCs. No significant dose-dependent effects of LTI-291 were detected. Some isolated decreases and increases in GSLs were detected at different dose levels in different matrices, but no consistent pattern was observed. See Supplementary table 2 and 3 for details. Similar as in the SAD study, the two subjects with a *GBA1* mutation (randomized to 10 mg and 60 mg) did not show a distinct pattern of glycosphingolipid variables compared to controls (data not shown).

For both studies, no correction for multiple testing was performed, considering the hypothesis-generating nature of these measurements. Isolated glycosphingolipid changes are therefore at risk of being chance findings.

NEUROCOGNITIVE BIOMARKERS There were no significant, dose-dependent effects of LTI-291 on any of the CNS tests, neither after a single dose, nor after prolonged daily dosing. Some isolated differences from placebo were seen in single, mostly submaximal, dose levels, but due to the lack of dose dependency these were considered chance findings due to multiple testing. Details can be found in Supplementary table 4 for the SAD study and Supplementary table 5 for the MAD study.

Discussion

Here we report the results of the first in human administration of a first in class drug aimed at enhancing glucocerebrosidase activity with the goal to slow down disease progression of Parkinson's disease. In these studies, safety, tolerability, PK and pharmacodynamics of LTI-291 were evaluated in healthy volunteers. LTI-291 was administered as a single dose at 3, 10, 30 and 90 mg and as 14 consecutive daily doses at 3, 10, 30 and 60 mg. LTI-291 was generally well tolerated and no treatment-related SAEs or deaths occurred and no subject withdrew from a study due to AEs.

Drug levels as measured by C_{max}, AUC_{0-24} and AUC_{0-inf} increased in a dose proportional manner (Figures 1 and 2). In the SAD study at the 3 mg dose the mean C_{max} and AUC_{0-last} was 110 ± 13.2 ng/mL and 1600 ± 383 ng*h/mL, respectively, whereas at the 90 mg dose the mean C_{max} and AUC_{0-last} was 2750 ± 786 ng/mL and 48400 ± 8790 ng*h/mL, respectively, demonstrating a 25-fold increase in Cmax and 30-fold increase in AUC_{0-last} (Table 4). Similarly in the MAD study on day 14 at the 3 mg dose the mean C_{max} and AUC_{0-24} was 159 ± 46.6 ng/mL and 3200 ± 1070 ng*h/mL, respectively, whereas at the 60 mg dose the mean C_{max} and AUC_{0-24} was 2900 ± 896 ng/mL and 52300 ± 13800 ng*h/mL, respectively, demonstrating a 18-fold increase in Cmax and 16-fold increase in AUC_{0-last} (Table 5). With a median half-life of 28. 0 hours after multiple dosing, LTI-291 is a suitable candidate for daily single dosing. In a single subject in the 30 mg MAD cohort an estimated half-life of 250 hours at day 14 sampling was observed. This subject had a relatively low C_{max} this day with an apparently slowed absorption, possibly contributing to the long half-life based on the 48 hours post-dose sampling, combined with a variable elimination. This was seen to a lesser extent in other subjects. Within the 30 mg dose group on day 14, the half-lives recorded were 15.5, 52.9, 56.1, 70. 8,85. 2,99. 4,145 and 250 hours. Nothing unusual in the higher-range subjects or in the cohort happened that could explain this wide range, so it is likely an expression of a variable elimination, or possibly of flip-flop kinetics, despite the powder in a capsule formulation. The 24-hour concentrations following all the multiple doses were consistent, as were the AUC_{0-24} values following the 7^{th} and 14^{th} doses. Pharmacokinetic steady state is reached by the 7^{th} daily dose (Table 5). The median ratios of the AUC_{0-24} following multiple doses were 2. 52 and 2. 59 after the 7^{th} and 14^{th} dose, respectively whereas the median ratios of the C_{max} were 1.62 and 1.55 after the 7^{th} and 14^{th} dose. The median accumulation index was 2.28 and 2. 70 after the 7^{th} and 14^{th} dose 9 (Table 5).

Since LTI-291 is a highly permeable molecule and based on the preclinical rat neuro PK data where the unbound partition coefficient (K_{puu}) between brain and plasma was determined to be 1. 02 the compound has excellent brain penetration and is at distribution equilibrium between the plasma, brain and CSF compartments. On this basis one can anticipate that in human at steady state the CSF concentration will be similar to unbound drug in brain ($C_{u,b}$) and plasma ($C_{u,p}$). In the MAD study the mean CSF:plasma concentration ratios ranged from 0. 0124 to 0.0134 at 4-5 h after the 14^{th} dose and were similar at all dose levels (Table 6). Over the 20-fold dose range the CSF concentrations increased in a dose proportional manner relative to the plasma concentrations. For example, at the 3 mg dose the LTI-291 concentration was 1.82 ± 0.687 ng/mL whereas at the 60 mg dose the LTI-291 concentration was 30.7 ± 7.56 ng/mL demonstrating a 17-fold increase. These results show that LTI-291 has good CNS/brain distribution.

The LTI-291 drug levels achieved in the MAD study suggest that all doses could potentially result in GCase activation. Based on preclinical in vitro studies (unpublished), 18% activation of GCase occurs at drug levels of 36 ng/mL (0. 1 µM) and 100% activation of GCase occurs at drug levels of 360 ng/mL (1 µM). The 10 mg daily dose would achieve 100% GCase activation, sinceat day 14 the average plasma C_{max} was 509 ± 141 ng/mL and based on the long half-life the C_{min} was 239 ± 56 ng/mL (Table 5).

LTI-291 is expected to increase GCase activity, which might lead to increased cleavage of GCase's substrates GluCer and GluSph, thus leading to lower levels of these substrates in the lysosome. This could indirectly affect the upstream glycosphingolipid LacCer upon stimulation of this sphingolipid pathway. It is still unclear how this would translate to levels measured in whole cells, since e.g. GluCer is primarily located outside of the lysosome.[25] Lack of a clear treatment effect in this study can be explained by LTI-291 having been given to healthy subjects, who can be expected to have a normally functioning GCase enzyme. Additionally, it is unclear how a change in intralysosomal levels is reflected in total cellular levels. No clear transient effects on GSLs were detected. It is expected that the drug would have a stronger effect in patients with a compromised GCase activity. In the MAD study, 2 healthy subjects with a mild *GBA1* mutation (T369M) were identified, of which one randomized to the highest dose. Even though these subjects might have had an altered GCase activity, the variability in biomarker effects between subjects was too large to be able to conclude that these two *GBA1* mutated subjects respond differently on GluCer, LacCer and GluSph variables compared to the non-mutated subjects. Several GluCer and LacCer isoforms in CSF decreased in 10 mg or 60 mg LTI-291 treated subjects compared to placebo. However, this could be a chance finding, because the placebo group had an average increase compared to baseline for these isoforms, whereas LTI-291 treated subjects remained unchanged. Glycosphingolipid levels are not expected to significantly change in this timeframe in untreated subjects, so this was likely a result of natural variability.

The desired mode of action for treating GBA-PD is an ongoing debate.[26–30] More than 400 different mutations in the *GBA1* gene are known from GD.[31,32] These different mutations can have different effects, e.g. enzymes that reach the lysosome albeit with decreased activity, or retention in the endoplasmic reticulum (ER), possibly resulting in an ER stress response. Correction of protein misfolding, assisted transport to the lysosome and substrate reduction therapies have also been suggested.[30] The multitude of different mutant enzymes makes enzyme activation challenging. Since most GBA-PD patients have a heterozygous mutation, a common factor is the remainder of the wild type GCase enzyme. Enhancement of the wild type enzyme, as is shown preclinically for LTI-291, seems crucial when targeting enzyme activity. GCase activation by LTI-291 could not directly be measured *ex vivo* in cell lysate, because LTI-291 requires a lipidic surface to function (in vivo the lysosomal membrane). The live cell assay for lysosomal GCase activity (using 5-(Pentafluorobenzoylamino) Fluorescein Di-beta-D-Glucopyranoside (PFB-FDGlu) substrate) is not compatible with LTI-291 since it blocks the allosteric site. It remains unclear what the best biomarkers would be for future studies in GBA-PD.

In conclusion, LTI-291 was observed generally to be well tolerated when given orally once daily for 14 consecutive days at all dose levels tested in this healthy subject population. Results from this study support the continued clinical development of LTI-291 and the exploration of LTI-291 effects in a *GBA1*-mutated Parkinson population.

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Table 1 SAD study summary of baseline subject characteristics.

LTI-291 or placebo	3 mg LTI-291	10 mg LTI-291	30 mg LTI-291	90 mg LTI-291	Placebo
n	8	8	8	8	8
Male/Female	6/2	4/4	7/1	8/0	6/2
Age (years), mean (range)	29.5 (18-56)	49.5 (22-63)	27.5 (18-56)	38.1 (23-64)	46.0 (30-63)
BMI (kg/m²), mean (range)	23.8 (19.7-27.3)	23.9 (18.9-28.3)	23.7 (19-28.6)	24.9 (19.7-29.8)	24.2 (22.3-27.6)
Race					
Caucasian	8	6	7	8	7
Black	0	1	1	0	1
Asian	0	1	0	0	0
GBA1 wild type/mutation	8/0	8/0	8/0	5/3	8/0

Table 2 MAD study summary of baseline subject characteristics.

LTI-291 or placebo	3 mg LTI-291	10 mg LTI-291	30 mg LTI-291	60 mg LTI-291	Placebo
n	7	8	8	8	8
Male/Female	7/0	4/4	4/4	5/3	6/2
Age (years), mean (range)	65.0 (57-74)	69.4 (60-75)	67.3 (57-74)	65.9 (56-75)	67.4 (52-73)
BMI (kg/m²), mean (range)	24.9 (22.2-28.1)	24.9 (21.7-29.5)	25.4 (19.7-28.7)	26.7 (23.1-31.9)	26.1 (22.8-30.8)
Race					
Caucasian	6	7	7	8	8
Asian	0	1	0	0	0
Latino	1	0	0	0	0
Mixed	0	0	1a	0	0
GBA1 wild type/mutation	7/0	7/1	8/0	7/1	8/0

The subject of mixed race was of Dutch-Indonesian and German-Indonesian descent.

Table 3 MAD study adverse events by treatment. All AEs were coded using the Medical Dictionary for Regulatory Activities (MedDRA) version 20.0. Greyed rows depict system organ classes and numbers are a summation of all preferred terms in that class. Multiple AEs could be reported by the same subject.

MAD adverse events	3mg LTI-291		10mg LTI-291		30mg LTI-291		60mg LTI-291		Placebo	
	N=7		N=8		N=8		N=8		N=8	
System Organ Class/ Preferred Term	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))
ANY EVENTS	17	5 (71.4)	15	6 (75.0)	19	7 (87.5)	27	8 (100.0)	11	5 (62.5)
CARDIAC DISORDERS	1	1 (14.3)	-	-	-	-	-	-	-	-
Bundle branch block right	1	1 (14.3)	-	-	-	-	-	-	-	-
EYE DISORDERS	-	-	1	1 (12.5)	-	-	1	1(12.5)	1	1 (12.5)
Ocular hyperaemia	-	-	1	1 (12.5)	-	-	-	-	-	-
Vision blurred	-	-	-	-	-	-	1	1 (12.5)	1	1 (12.5)
GASTROINTESTINAL DISORDERS	3	3 (42.9)	1	1 (12.5)	3	2 (25.0)	4	2 (25.0)	-	-
Abdominal pain upper	1	1 (14.3)	1	1 (12.5)	-	-	1	1 (12.5)	-	-
Constipation	1	1 (14.3)	-	-	-	-	-	-	-	-
Diarrhoea	1	1 (14.3)	-	-	-	-	-	-	-	-
Faeces soft	-	-	-	-	-	-	1	1 (12.5)	-	-
Flatulence	-	-	-	-	-	-	1	1 (12.5)	-	-
Frequent bowel movements	-	-	-	-	1	1 (12.5)	-	-	-	-
Nausea	-	-	-	-	2	1 (12.5)	1	1 (12.5)	-	-
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	4	3 (42.9)	2	2 (25.0)	2	2 (25.0)	3	3 (37.5)	2	2 (25.0)
Fatigue	1	1 (14.3)	-	-	-	-	-	-	1	1 (12.5)
Feeling cold	-	-	-	-	-	-	1	1 (12.5)	-	-
Feeling hot	-	-	1	1 (12.5)	-	-	-	-	-	-
Medical device site eczema	-	-	-	-	1	1 (12.5)	-	-	1	1 (12.5)
Medical device site erythema	-	-	-	-	-	-	1	1 (12.5)	-	-
Medical device site reaction	2	2 (28.6)	-	-	1	1 (12.5)	-	-	-	-
Pain	-	-	-	-	-	-	1	1 (12.5)	-	-
Puncture site pain	1	1 (14.3)	-	-	-	-	-	-	-	-
Vessel puncture site vesicles	-	-	1	1 (12.5)	-	-	-	-	-	-
INFECTIONS AND INFESTATIONS	1	1 (14.3)	-	-	-	-	1	1 (12.5)	-	-
NASOPHARYNGITIS	1	1 (14.3)	-	-	-	-	-	-	-	-
Urinary tract infection	-	-	-	-	-	-	1	1 (12.5)	-	-

Table 3 *(Continuation of previous page)*

MAD adverse events	3mg LTI-291		10mg LTI-291		30mg LTI-291		60mg LTI-291		Placebo	
	N=7		N=8		N=8		N=8		N=8	
System Organ Class/ Preferred Term	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))
INVESTIGATIONS	1	1 (14.3)	1	1 (12.5)	-	-	1	1 (12.5)	-	-
Gamma-glutamyltransferase increased	-	-	-	-	-	-	1	1 (12.5)	-	-
Hepatic enzyme increased	1	1 (14.3)	1	1 (12.5)	-	-	-	-	-	-
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	1	1 (14.3)	4	4 (50.0)	4	2 (25.0)	9	6 (75.0)	5	4 (50.0)
Arthralgia	-	-	-	-	2	2 (25.0)	-	-	-	-
Back pain	-	-	2	2 (25.0)	-	-	2	2 (25.0)	2	2 (25.0)
Muscle fatigue	-	-	1	1 (12.5)	-	-	1	1 (12.5)	-	-
Muscle spasms	-	-	-	-	1	1 (12.5)	2	2 (25.0)	-	-
Musculoskeletal stiffness	-	-	-	-	-	-	1	1 (12.5)	1	1 (12.5)
Myalgia	1	1 (14.3)	-	-	1	1 (12.5)	3	3 (37.5)	1	1 (12.5)
Neck pain	-	-	1	1 (12.5)	-	-	-	-	-	-
Pain in extremity	-	-	-	-	-	-	-	-	1	1 (12.5)
NERVOUS SYSTEM DISORDERS	4	3 (42.9)	3	2 (25.0)	6	5 (62.5)	8	5 (62.5)	3	3 (37.5)
Dizziness	1	1 (14.3)	-	-	1	1 (12.5)	-	-	2	2 (25.0)
Head discomfort	-	-	1	1 (12.5)	-	-	1	1 (12.5)	-	-
Headache	2	2 (28.6)	2	2 (25.0)	5	5 (62.5)	3	3 (37.5)	-	-
Somnolence	1	1 (14.3)	-	-	-	-	4	4 (50.0)	1	1 (12.5)
RENAL AND URINARY DISORDERS	1	1 (14.3)	-	-	-	-	-	-	-	-
Polyuria	1	1 (14.3)	-	-	-	-	-	-	-	-
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	1	1 (14.3)	1	1 (12.5)	2	1 (12.5)	-	-	-	-
NAsal congestion	1	1 (14.3)	1	1 (12.5)	1	1 (12.5)	-	-	-	-
Oropharyngeal pain	-	-	-	-	1	1 (12.5)	-	-	-	-
SKIN AND SUBCUTANEOUS TISSUE DISORDERS	-	-	1	1 (12.5)	2	1 (12.5)	-	-	-	-
Hyperhidrosis	-	-	1	1 (12.5)	2	1 (12.5)	-	-	-	-
VASCULAR DISORDERS	-	-	1	1 (12.5)	-	-	-	-	-	-
Hypertension	-	-	1	1 (12.5)	-	-	-	-	-	-

Table 4 Arithmetic means ± standard deviation of different pharmacokinetic parameters of the SAD study, measured in plasma. SAD=single ascending dose.

LTI-291 dose	3 mg (n=8)	10 mg (n=8)	10 mg (n=8)	30 mg (n=8)	90 mg (n=8)
Nutritional state	fasted	fasted	fed	fasted	fasted
C_{max} (ng/mL)	110 ± 13	350 ± 131	243 ± 75	997 ± 283	2750 ± 786
$t_{½}$ (h)	23.6 ± 11.1	23.6 ± 8.3	25.3 ± 10.1	24.3 ± 7.7	23.9 ± 6.1
λ_z (h-1)	0.0366 ± 0.0185	0.0334 ± 0.0137	0.0324 ± 0.0156	0.0314 ± 0.0109	0.0304 ± 0.0064
AUC_{0-last} (ng*h/mL)	1600 ± 383	5760 ± 965	5950 ± 1130	18700 ± 3920	48400 ± 8790
AUC_{0-inf} (ng*h/mL)	2350 ± 703	7820 ± 2300	8860 ± 2670	24800 ± 3050	64000 ± 10200
CL/F (L)	1.38 ± 0.40	1.37 ± 0.35	1.22 ± 0.36	1.23 ± 0.15	1.44 ± 0.21
Vz/F (L)	42.7 ± 15.8	43.5 ± 9.9	40.7 ± 9.9	43.4 ± 15.8	49.1 ± 12.4
C_{max}/dose (ng/mL/mg)	36.7 ± 4.4	35 ± 13.1	24.3 ± 7.5	33.2 ± 9.4	30.5 ± 8.7
AUC_{0-inf}/dose (ng*h/mL/mg)	783 ± 234	782 ± 230	886 ± 267	827 ± 102	711 ± 113
Median T_{max} (h) (min, max)	1.48 (1.00, 2.00)	1.01 (1.00, 4.02)	8.00 (4.00, 12.00)	3.02 (1.00, 4.00)	2.00 w(1.00, 8.17)

Table 5 Arithmetic means ± standard deviation of different pharmacokinetic parameters of day 1, day 7 and day 14 of the MAD study, measured in plasma. All PK doses were in fasted state. MAD=multiple ascending dose.

	Day 1				Day 7				Day 14			
LTI-291 dose	3 mg (n=7)	10 mg (n=8)	30 mg (n=8)	60 mg (n=8)	3 mg (n=7)	10 mg (n=8)	30 mg (n=8)	60 mg (n=8)	3 mg (n=7)	10 mg (n=8)	30 mg (n=8)	60 mg (n=8)
C_{max} (ng/mL)	91 ± 18	316 ± 80	927 ± 340	2070 ± 702	145 ± 33	516 ± 109	1610 ± 186	3290 ± 891	159 ± 47	509 ± 141	1510 ± 204	3200 ± 1070
C_{min} (ng/mL)	32 ± 9	92 ± 18	312 ± 22	547 ± 109	77 ± 30	237 ± 70	797 ± 183	1580 ± 368	95 ± 38	239 ± 56	847 ± 220	1650 ± 493
$t_{½}$ (h)	35.2 ± 30.2	20.9 ± 3.0	27.4 ± 9.9	26.0 ± 7.5	32.6 ± 13.1	29.9 ± 9.9	26.2 ±5.0	38.6 ± 17.7	40.1 ± 19.7	33.0 ± 8.3	96.9 ± 72.5	33.5 ± 6.9
λ_z (h-1)	0.0296 ± 0.017	0.0338 ± 0.0051	0.0280 ± 0.0090	0.0287 ± 0.0088	0.0231 ± 0.0093	0.0251 ± 0.0072	0.0276 ± 0.0071	0.0206 ± 0.0069	0.0207 ± 0.0089	0.0222 ± 0.0053	=0.0128 ± 0.013	0.0214 ± 0.0042
AUC_{024} (ng*h/mL)	1060 ± 182	3450 ± 625	10700 ± 1470	19900 ± 4080	2010 ± 805	7900 ± 1750	26900 ± 4930	53000 ± 10200	2900 ± 896	7650 ± 1580	26500 ± 4410	52300 ± 13800
CL/F (L)	1.41 ± 0.77	1.66 ± 0.29	1.35 ± 0.29	1.59 ± 0.50	1.63 ± 0.65	1.33 ± 0.32	1.15 ± 0.22	1.17 ± 0.25	1.14 ± 0.41	1.36 ± 0.28	1.17 ± 0.24	1.21 ± 0.27
Vz/F (L)	49.9 ± 9.1	49.4 ± 8.0	50.4 ± 9.4	56.2 ± 11.9	70.5 ± 0.0322	54.7 ± 12.5	42.2 ± 5.2	61.3 ± 17.3	60.3 ± 16.2	63.6 ± 15.8	149 ± 95.6	57.2 ± 12.7
C_{max}/dose (ng/mL/mg)	30.2 ± 5.9	31.6 ± 8.0	30.9 ± 11.3	34.5 ± 11.7	48.2 ± 11.1	51.6 ± 10.9	53.6 ± 6.19	54.9 ± 14.9	53.0 ± 15.5	50.9 ± 14.1	50.4 ± 6.8	53.3 ± 17.9
AUC_{0-24}/dose (ng*h/mL/mg)	353.3 ± 60.7	345.0 ± 62.5	356.7 ± 49.0	331.7 ± 68.0	670.0 ± 268.3	790.0 ± 175.0	896.7 ± 164.3	883.3 ± 170.0	966.7 ± 298.7	765.0 ± 158.0	883.3 ± 147.0	871.7 ± 230.0
Median T_{max} (h) (min, max)	2.0 (1.0, 4.0)	1.0 (1.0, 2.0)	1.0 (1.0, 8.0)	1.0 (1.0, 8.1)	4.0 (1.0, 4.0)	1.0 (1.0, 8.0)	1.5 (1.0, 4.0)	1.0 (1.0, 4.0)	1.0 (1.0, 8.8)	1.1 (1.0, 8.0)	1.5 (1.0, 8.0)	1.0 (1.0, 2.0)
Accumulation Index	-	-	-	-	2.51 ± 0.77	2.35 ± 0.58	2.13 ± 0.28	2.86 ± 1.05	2.95 ± 1.17	2.53 ± 0.49	6.35 ± 4.34	2.56 ± 0.41

Table 6 Arithmetic means ± standard deviation of CSF concentrations of LTI-291 and CSF:plasma ratios of the MAD study. Three CSF samples could not be obtained (due to a repeated dry tap, a refusal of a subject and an error in lab handling). MAD=multiple ascending dose; CSF=cerebrospinal fluid; SD=standard deviation.

MAD (day 14) CSF pharmacokinetics				
LTI291 dose	3 mg (n=6)	10 mg (n=8)	30 mg (n=7)	60 mg (n=7)
	LTI-291 CSF concentration (ng/mL)			
Mean ± SD	1.82 ± 0.69	4.77 ± 1.40	18.2 ± 6.73	30.7 ± 7.56
Minimum, maximum	1.01, 3.08	2.51, 6.40	7.95, 26.5	19.8, 43.3
	CSF:plasma concentration ratio ((ng/mL)/(ng/mL))			
Mean ± SD	0.0130 ± 0.00262	0.0127 ± 0.00317	0.0134 ± 0.00414	0.0124 ± 0.00267
Minimum, maximum	0.0105, 0.0171	0.00904, 0.0175	0.00642, 0.0194	0.00947, 0.0176

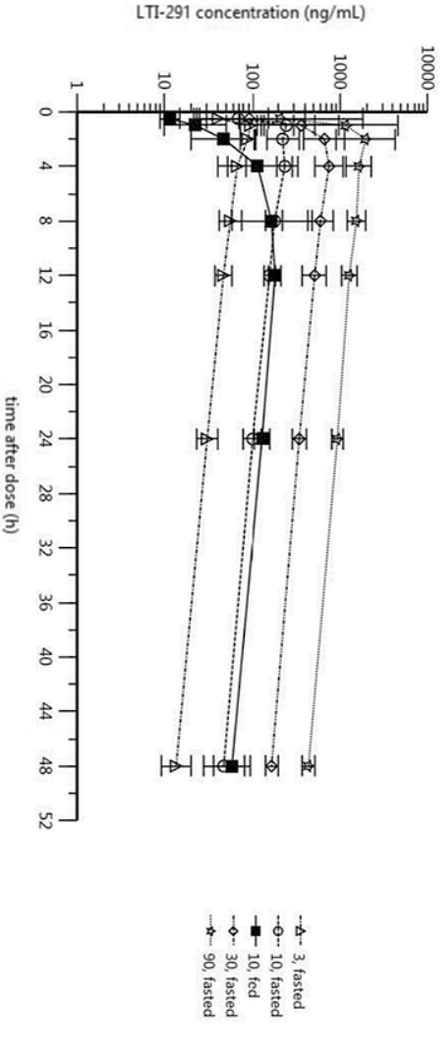
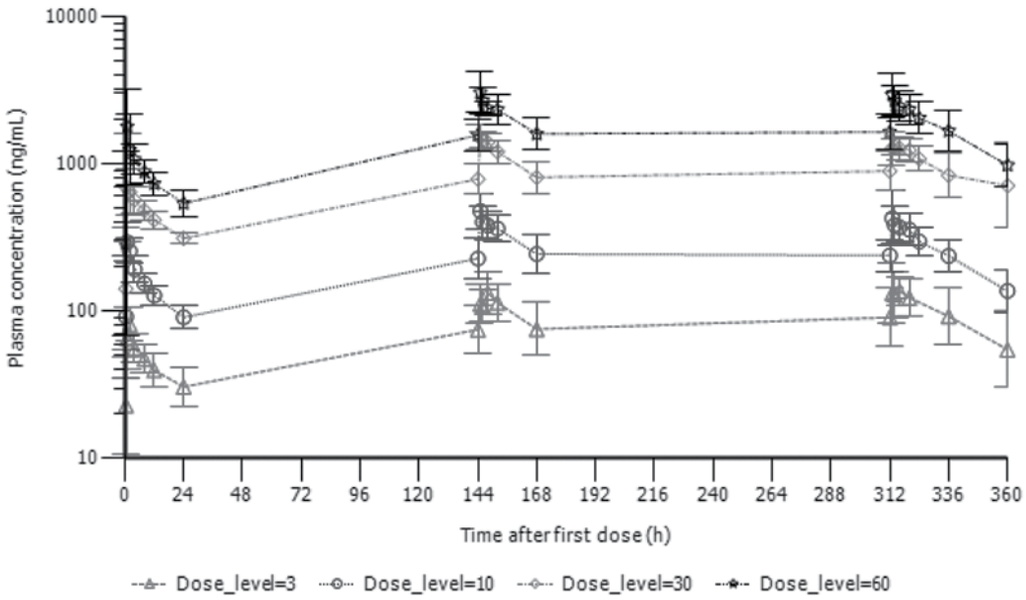


Figure 1 Figure 1 SAD study: Geometric mean (± geometric SD) plasma concentration of LTI-291 against time (h) after dose following a single oral dose of 3, 10, 30 and 90 mg LTI-291 in the fasted state and 10 mg LTI-291 in the fed state.

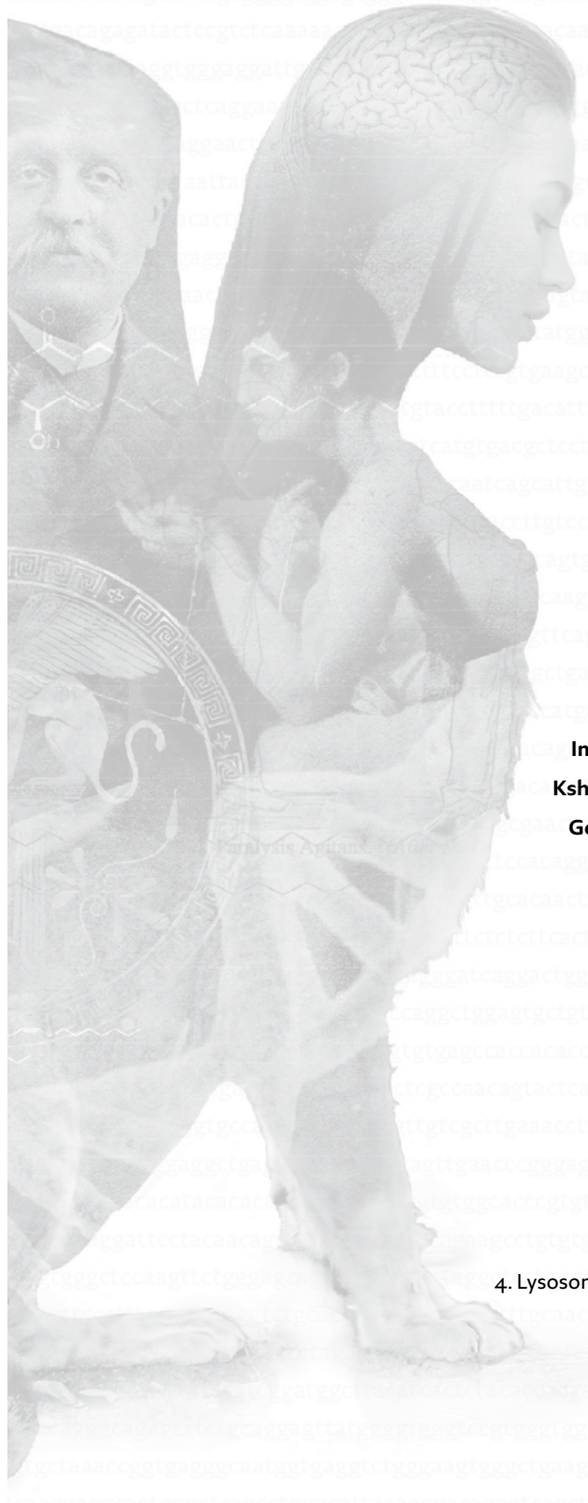
Figure 2 MAD study: Geometric mean (± geometric SD) plasma concentration of LTI-291 against time (h) after dose following the first, seventh and fourteenth oral dose of 3, 10, 30 or 60 mg LTI-291.



SUPPLEMENTARY MATERIAL

H7ST1 / H7ST2 / H7ST3 / H7ST4 / H7ST5

SCAN ME



CHAPTER 8

A phase 1B trial in *GBA1*-associated Parkinson's disease of LTI-291, a glucocerebrosidase activator

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Abstract

Background Loss-of-function mutations in the *GBA1* gene are one of the most common genetic risk factors for onset of Parkinson's disease and subsequent progression (GBA-PD). *GBA1* encodes the lysosomal enzyme glucocerebrosidase (GCase); a promising target for a possible first disease-modifying therapy. LTI-291 is an allosteric activator of GCase, which increases the activity of normal and mutant forms of GCase.

Objectives This first-in-patient study evaluated the safety, tolerability, pharmacokinetics and pharmacodynamics of 28 daily doses of LTI-291 in GBA-PD.

Methods This was a randomized, double-blind, placebo-controlled trial in 40 GBA-PD participants. 28 consecutive daily doses of 10, 30 or 60mg LTI-291 or placebo were administered (n=10 per treatment allocation). Glycosphingolipid (GluCer and LacCer) levels were measured in peripheral blood mononuclear cells, plasma, and CSF, and a test battery of neurocognitive tasks, the MDS-UPDRS and the MMSE were performed.

Results LTI-291 was generally well tolerated and no deaths or treatment-related SAEs occurred, and no participants withdrew due to AEs. $C_{max,}$ and AUC_{0-6} of LTI-291 increased in a dose proportional manner, with free CSF concentrations equal to the free fraction in plasma. A treatment-related transient increase of intracellular glucosylceramide (GluCer) in peripheral blood mononuclear cells was measured.

Conclusion These first-in-patient studies demonstrated that LTI-291 was well-tolerated when given orally for 28 consecutive days to patients with GBA-PD. Plasma and CSF concentrations were reached that are considered pharmacologically active (*i. e.,* sufficient to at least double GCase activity). Intracellular GluCer changes were detected that suggest target engagement. Clinical benefit will be assessed in a larger long-term trial in GBA-PD.

Introduction

Parkinson's disease (PD; MIM: 168600) is the second most common neurodegenerative disorder and has a likely multifactorial disease etiology, consisting of both environmental as well as genetic risk factors (Kalia and Lang 2015). A disease-modifying treatment is lacking. Mutations in the *GBA1* gene are one of the most common genetic risk factors for Parkinson's disease (GBA-PD)(Gasser 2015; Schapira 2015; Gan-Or et al. 2015; Ruskey et al. 2019; den Heijer, Cullen, et al. 2020). GBA-PD presents at a slightly younger age than idiopathic PD, with a greater prevalence of non-motor symptoms (Petrucci et al. 2020; Mata et al. 2016; den Heijer, van Hilten, et al. 2020). *GBA1* encodes the lysosomal enzyme glucocerebrosidase (GCase; EC 3.2.1.45) and risk-associated *GBA1* mutations cause a loss of enzymatic activity. Greater relative risk is associated with a decreased activity of the mutant enzyme; the greatest relative risk is associated with a mutant allele that is not translated (hence residual activity is *ca* 50% of normal). In addition to their role in PD risk, *GBA1* mutations have also been linked to more rapid progression of motor (Davis et al. 2016; Ortega et al. 2021) and cognitive (Cilia et al. 2016; Liu et al. 2021) symptoms of PD. Activation of GCase is therefore a promising strategy for a possible first disease-modifying therapy in PD.

GCase functions at the luminal face of the lysosomal membrane and catalyzes one step in the multi-step hydrolytic degradation of glycosphingolipids (GSLs), leading ultimately to sphingosine, the building block for the synthesis of new GSLs (Kitatani, Idkowiak-Baldys, and Hannun 2008; Boer et al. 2020). GSLs are essential for maintenance of membrane properties that play a role in many diverse cellular functions (Merrill 2011). GCase hydrolyzes glucosylceramide (GluCer), producing glucose and ceramide. Ceramide is subsequently deacylated to produce sphingosine, which is transported to the cytosol for elaboration. Ceramide and all of its conjugates, including GluCer, comprise a group of acyl chain isomers that are produced from sphingosine, by acylation with activated fatty acids of diverse chain lengths by the ceramide synthases (Tidhar et al. 2018). The acyl chain isomers of ceramide do *not* interconvert (*e.g.*, by addition or removal of carbons from

the acyl chain). All ceramide isomers can be glucosylated by glucosylceramide synthase (GCS) to produce the GluCer isomers, the starting points for ganglioside synthesis in the Golgi.

It is not possible to measure lysosomal activation of GCase by LTI-291 with a fluorogenic probe, since the leaving group of the available probe occupies the allosteric binding site of LTI-291. As an alternative, time-dependent changes in the levels of substrate GluCer can be used to infer enzyme activity/activation. It is important to note that, as predicted by Michaelis-Menten kinetic theory (Conzelmann and Sandhoff 1983), GluCer levels are not sensitive to GCase activity when GCase activity exceeds *ca.* 30% of the normal, or average, level (Gegg et al. 2015), as is the case in GBA-PD (*in contrast*, Gaucher disease, which is characterized by very low GCase activity, is characterized by accumulation of GluCer isomers in peripheral cells and tissue).

LTI-291 (now designated BIA-28) is a small-molecule GCase allosteric activator that increases V_{max} and decreases K_m of wild-type and at least some mutant enzymes, such that *in vitro* activity is increased by up to 3-fold (LTI, unpublished). When administered to healthy volunteers for fourteen days, with a maximal single dose of 90 mg and multiple daily doses of 60 mg, LTI-291 was generally well-tolerated, without any treatment emergent serious adverse events (SAEs) or any AEs that led to discontinuation (den Heijer, Kruithof, et al. 2021). No AEs were attributed as being related to the administration of LTI-291/BIA-28. CSF unbound drug concentrations were estimated to be in an approximate 1:1 ratio with the unbound plasma drug concentration, across all doses, indicating excellent central penetrance. Based on *in vitro* studies, the central exposures reached by multiple LTI-291 doses of 10 mg to 60 mg were sufficient to *at least* double *in vitro* GCase activity (LTI, unpublished). Doubling of GCase activity is expected to restore 100% of average non-GBA-PD activity in most, if not all, GBA-PD patients. In an earlier study of LTI-291/BIA-28 in healthy elderly (den Heijer, Kruithof, et al. 2021), intracellular GluCer isomers (in PBMCs) did not change significantly over 14 days of dosing. This may be attributable to the possibility that these healthy volunteers had 'normal' GSL flux, which cannot be increased by further GCase activation. The same GluCer isomers were again measured as exploratory biomarkers for the current 28-day study in GBA-PD patients, with significantly different results. This paper describes these studies, assessing safety, tolerability, pharmacokinetics (PK) and pharmacodynamics, in GBA-PD in a 28-day-treatment trial of LTI-291.

Methods

This was a randomized, double-blind and placebo-controlled trial. The study was approved by the Independent Ethics Committee of the Foundation 'Evaluation of Ethics in Biomedical Research' (Stichting Beoordeling Ethiek Biomedisch Onderzoek), Assen, The Netherlands. The trial is registered in the Dutch Trial Registry (Nederlands Trial Register, NTR) under study number NTR6960. The trial took place between January and June 2018 at the Centre for Human Drug Research, Leiden, the Netherlands. All participants signed an informed consent form prior to any study-related activity, in accordance with the Declaration of Helsinki.

Participants

GBA-PD patients (minimum age of 18 years), with Hoehn and Yahr (H&Y) stage 1-4 and a mini mental state exam (MMSE) score ≥18, male and female of non-childbearing potential were enrolled for 28 consecutive daily oral doses of LTI-291 or placebo. Stable treatment with antiparkinsonian treatments from 1 month prior to the screening (2 months for monoamine oxidase B inhibitors) was allowed. Other prior concomitant medication was only allowed at the discretion of the investigator. The following dose levels were investigated: 10 mg, 30 mg and 60 mg LTI-291. Treatment was administered as powder in a capsule. Each treatment arm consisted of 10 patients. Patients were randomized in 10 blocks of 4 to receive one of the three dose levels of LTI-291 or placebo in a 1:1:1:1 ratio. The randomization code was generated using SAS version 9. 4 by a study-independent statistician. Patients visited the clinical research unit at start of dosing, after one week, two weeks and four weeks. A safety call was performed after three weeks. Between visits, patients self-administered LTI-291 daily. A safety follow-up visit was performed 7-14 days after last dose.

Safety

A medical screening (medical history, record of prior concomitant medication, participant demographics, height and weight, 12-lead electrocardiography (ECG), vital signs, routine hematology, biochemistry/electrolytes and urinalysis, urine pregnancy test (for females), virology, urine drug screen, ethanol breath test, physical examination, MMSE and H&Y staging) was performed to assess a participant's eligibility. During study periods, safety was assessed using monitoring of adverse events (AEs), concomitant medication, vital signs, ECG, physical examination and safety chemistry and hematology blood sampling.

Pharmacokinetics (PK)

LTI-291/BIA-28 levels were measured in K_2EDTA plasma and in cerebrospinal fluid (CSF). Plasma PK samples were taken predose, 2-, 4- and 6-hours after first and last dose and a single sample after seventh (±2) and fourteenth (±2) dose. CSF was taken predose and 4-hours after the last (28^{th}) dose. Non-compartmental analysis was performed on the plasma data from each participant as data permitted.

Pharmacodynamics

GLYCOSPHINGOLIPID LEVELS Biochemical pharmacodynamic markers were measured in K_2EDTA plasma, PBMCs and CSF, as described previously (den Heijer, Kruithof, et al. 2021). GluCer and LacCer were measured in plasma, PBMCs and CSF. GluSph was measured in plasma and PBMCs. The acyl chain of the ceramide group in GluCer and LacCer can be of varying length and saturation. Both in plasma and PBMCs, concentrations were measured of GluCer C16:0, C18:0, C22:0, C24:0 and C24:1 and of LacCer C16:0, C18:0, C20:0, C22:0, C22:1, C24:0 and C24:1. In CSF, GluCer C16:0, C18:0, C22:0, C24:0 and C24:1 were measured and LacCer C16:0, C18:0, C20:0, C22:0, C22:1, C24:0 and C24:1 were measured but are not reported here. In the first-in-human multiple dose studies, GluCer (five isomers) and GluSph were investigated as potential biomarkers in PBMCs, plasma, and CSF in healthy elderly (55+) volunteers. No significant changes were detected. Biological (intra-individual) variability of all GluCer isomers was determined to be < 13. 3%, except for GluCer C18:0 (17. 2%) (in draft: den Heijer, Pereira, et al. 2022). Inter-individual variability is much greater (see below).

MDS-UPDRS PART III, MMSE AND NEUROCOGNITIVE BIOMARKERS No clinical effect was expected after 28 days of LTI-291/BIA-28 dosing, but the Movement Disorder Society – Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (Goetz et al. 2008) part III (motor assessment) in ON state and the MMSE were performed at baseline and at end of dosing as pharmacodynamic parameters for safety.

The NeuroCart®(Groeneveld, Hay, and Van Gerven 2016), a CNS test battery, was used to exclude any adverse effects of LTI-291/BIA-28 on CNS function. This was performed at baseline and after two weeks of dosing, to spread the burden of different measurements over different visits. Steady state exposures of LTI-291/BIA-28 were expected to be achieved after seven days of dosing. The test battery consists of neurophysiological, psychomotor and cognitive tests and has been extensively used previously in clinical drug development. (Muehlan et al. 2019; Baakman et al. 2019; Groeneveld, Hay, and Van Gerven 2016; Van Steveninck et al. 1991; Chen et al. 2012)

In short, measurements consist of saccadic and smooth pursuit eye movements, the adaptive tracking test (a visuo-motor task sensitive to disturbances in vigilance and attention), the body sway (a test of postural stability), the Bond and Lader test (visual analogue scale (VAS) of alertness, calmness and mood), the Visual Verbal Learning Test (VVLT) (a test of immediate and delayed memory), and pharmaco-EEG (measured separately after last dose instead of after two weeks). Tests were performed in a quiet room with ambient illumination with only one participant in the same room (and a research assistant) per session.

GBA1 genotyping

GBA1 genotype was determined in a previous large-scale *GBA1* screening in the Netherlands. (den Heijer, Cullen, et al. 2020) In short, full gene sequencing was performed on saliva-derived DNA, using next generation sequencing

and a primer set unique for the functional gene, thereby preventing amplification of the nearby pseudogene. For this trial, *GBA1* genotypes were confirmed by repeating sequencing in a whole blood sample (Table 1).
GBA1 genotypes were categorized into two categories:

1. carriers of one allele that has been reported in at least a single Gaucher's disease (GD), or
2. carriers of a non-GD *GBA1* allele linked to PD-risk, for alleles associated with PD or reported in PD patient(s), but never GD. It is important to emphasize that, although *GBA1* genotype is related to *average* residual GCase activity, there is considerable inter-individual variation and overlap between genotypes.

Statistical analysis

Neurocognitive pharmacodynamic data were analyzed with an analysis of covariance with fixed factor treatment and average predose value as covariate.

All safety and neurocognitive pharmacodynamic statistical programming were conducted with SAS 9. 4 for Windows (SAS Institute Inc., Cary, NC, USA). All PK analyses were performed in Phoenix 64 build 8. 0. 0. 3176 using WinNonlin 8. 0 (Certara L.P.). Statistical analysis of PK was performed using R version 3. 3. 1 ((2016-06-21).

Biochemical pharmacodynamic data were analyzed with a linear mixed model, with fixed factors treatment, time and treatment by time, random factor participant, and covariates average baseline value, sex, age and *GBA1* type (GD or non-GD carriers). An overall treatment effect was assessed and an effect over time, both for all active dose levels combined and per dose level compared to placebo. Statistical programming was conducted with R version 3.6.2 (2019-12-12).

This was an exploratory study; therefore, the sample size was not based on statistical considerations. 10 patients per dose level and 10 placebo patients were considered adequate to define initial safety and tolerability and to explore pharmacodynamics in the target patient population over 28 days of dosing.

Biomarkers were measured in an exploratory hypothesis-generating setting and were therefore not corrected for multiple testing.

Data availability

Data are available upon reasonable request.

Results

Forty-nine participants signed the informed consent form and underwent a medical screening. Seven participants were not enrolled because they were excluded based on the inclusion and exclusion criteria or withdrew consent to participate. A total number of 42 participants were enrolled. Two participants were withdrawn prior to the first dose based on physician decision (significant ECG abnormalities, not visible at screening). In total 40 participants were treated in the study, and all completed the study including the follow-up visit (Supplementary Figure 1).

Demographics and baseline characteristics

In total 20 males and 20 females were included in the study. The mean weight ranged from 69.0 kg (10 mg LTI-291) to 81. 0 kg (30 mg LTI-291/BIA-28). Participants in the different dose levels were comparable regarding mean age, mean height, mean MMSE and mean MDS-UPDRS part III score. Demography data are summarized in Table 1.

Safety and tolerability

28 consecutive daily administrations of LTI-291/BIA-28 up to the highest dose of 60 mg were generally well tolerated in people with GBA-PD. No serious AEs (SAEs) occurred after dosing and no AEs led to discontinuation. No clinically relevant changes in blood chemistry, hematology (Supplementary table 1), urinalysis, vital signs, ECG or CNS tests were identified. See Table 2 for a full listing of all AEs after dosing. Back pain was only reported in LTI-291/BIA-28

dose groups (N=4) and not in placebo, however there is no clear rationale for this and there was no dose-dependent increase in frequency, therefore this was considered unlikely related to administration of LTI-291/BIA-28. Other frequently reported AEs like fatigue and headache occurred in a similar or higher frequency in the placebo group and no dose-dependent increase was observed, therefore these are also considered unlikely related to administration of LTI-291/BIA-28. Five participants (LTI-291 n=4; placebo n=1) reported a mild subjective worsening of Parkinson's disease symptoms. Three participants related this to a stressful period. In four out of five participants this subjective worsening of symptoms resolved prior to the last dose of LTI-291/BIA-28 or placebo. Most AEs were mild in severity. Only 3 moderate AEs were reported, namely urinary tract infection (10 mg LTI-291), tendonitis (placebo) and paronychia (30 mg LTI-291). Both infections were successfully treated with antibiotics and the participant with tendonitis was referred for physiotherapy. These three AEs were all considered to be unlikely related to LTI-291/BIA-28 treatment.

Pharmacokinetics

Pharmacokinetic analysis of LTI-291/BIA-28 showed a maximum plasma concentration (T_{max}) ranging from 2 to 6 hours. $C_{max,}$ and AUC_{0-6} increased in a dose proportional manner. Half-life could not be determined due to limited sampling, but the pharmacokinetic profile otherwise was similar to results from previous studies in healthy volunteers (den Heijer, Kruithof, et al. 2021). The CSF:plasma concentration ratios range from 0.00634 to 0.0187 and were similar at all the dose levels. See Supplementary Table 2 and Supplementary Table 3 for details.

Pharmacodynamics

GROUP AVERAGE LEVELS OF INTRACELLULAR GLUCER ISOMERS IN PBMCS SIGNIFICANTLY INCREASE 14 DAYS AFTER DOSING WITH LTI-291/BIA-28, THEN PARTIALLY RETURN TO THE PRE-DOSE LEVEL In PBMCs, GluCer C16:0, C22:0, C24:0 and C24:1 showed a statistically significant overall treatment-associated *increase* in all active treatment groups (doses were combined since all doses were expected to at least double activity based on estimated brain exposure) at all times combined, compared to placebo (Table 3). The effect was significant in the 10 mg LTI-291/BIA-28 and the 60 mg LTI-291/BIA-28 dose groups, but not in the 30 mg treated group (Table 3). The effect was largest on Day 14 (Table 4, Figure 1) (Supplementary Table 4). Age, sex and *GBA1* genotype were not significant covariates. LacCer and GluSph in PBMCs were omitted from analysis, because of influence of leukocyte subtype ratios (including granulocyte contamination), which vary between blood draws. No significant changes were detected in extracellular GluCer levels at any time (plasma or CSF; data not shown).

MDS-UPDRS PART III, MMSE AND NEUROCOGNITIVE BIOMARKERS WERE UNCHANGED BY 28 DAYS OF DOSING No clinically significant changes were seen in MDS-UPDRS-Part III (ON state) or MMSE total score in any dosing group compared to placebo (Table 5). See Supplementary Table 5 for details.

There were no dose-dependent effects of LTI-291 on any of the neurocognitive biomarkers, indicating that 28 consecutive oral doses in participants with GBA-PD were not observed to cause any effects on CNS functioning. Some isolated differences from placebo were seen in single, mostly submaximal, dose levels, but due to the lack of dose dependency these were considered chance findings due to multiple testing (Supplementary Table 6).

Discussion

Here we report the first administration of LTI-291 (now designated BIA-28), a centrally penetrant small molecule, aimed at increasing glucocerebrosidase activity in patients with GBA-PD. Safety, tolerability, PK and pharmacodynamics of LTI-291/BIA-28 were evaluated. LTI-291/BIA-28 was administered in 28 consecutive daily doses at 10, 30 or 60 mg. This was generally well tolerated, no treatment-related SAEs or deaths occurred, and no participants withdrew due to AEs.

A significant and transient increase in 4/5 *intracellular* GluCer isomers was detected in PBMCs in dosed participants as compared to placebo (the fifth, which is also the lowest in abundance, was also increased, but not

statistically significantly so). No change of *extracellular* GluCer was observed in plasma at any time or in CSF at 28 days (not shown). Intracellular GluCer levels and plasma GluCer levels do not correlate (in draft: den Heijer, Pereira, et al. 2022). Drug-associated elevation of intracellular PBMC GluCer isomers also occurred at the lowest dose of 10mg, which may be expected, since measured exposures at the lowest dose were sufficient to double GCase activity *in vitro* (den Heijer, Kruithof, et al. 2021). The observed increase in intracellular GluCer was slow, with no change at 6h, a mild increase after seven days of dosing and a significant increase after 14 days of dosing. A second phase of the response was suggested by the fact that GluCer levels seemed to return towards pre-dose levels by day 28 (Table 4). Two unpublished observations from previous trials are pertinent to the analysis of the response. First, the initial increase in intracellular GluCer was *not* observed in a previous 14 day phase 1 trial in healthy elderly (den Heijer, Kruithof, et al. 2021). Second, all analyzed clinical data demonstrated that *intracellular* GluCer levels in GBA-PD, non-GBA-PD patients and healthy controls are comparable, with possibly a trend for slightly *lower intracellular* GluCer levels in GBA-PD compared to healthy controls (in draft: den Heijer, Pereira, et al. 2022). This temporary elevation may therefore be a response selective to individuals with a chronic suboptimally functioning GluCer recycling, like in GBA-PD.

The observed response to BIA-28 constitutes two phases; a slow (7-14 days) increase in intracellular GluCer, followed by an even slower decrease/return to pre-dose levels. It should be noted that intracellular GluCer measures include lysosomal GluCer (the *GBA1* substrate), as well as non-lysosomal or cytoplasmic GluCer (Fuller et al. 2008). Since the majority of GluCer is non-lysosomal, we propose that the activation of GCase activity by dosing with LTI-291/BIA-28 may cause a transient increase of salvaged ceramide available for GluCer synthase in the cytosol (Kitatani, Idkowiak-Baldys, and Hannun 2008; Boer et al. 2020). As the systemic ceramide (and GluCer) levels increase, de novo synthesis, which is known to be sensitive to ceramide (Wattenberg 2021), is down-regulated. This effect subsides as the system returns to a new homeostasis.

GluCer transient elevation was seen for the 10mg and 60mg treated groups, but small increases in the 30mg group did not reach statistical significance. Based on preclinical experiments, an effect was expected with a C_{max} of ~360 ng/mL, with similar responses for higher dose levels, indicating a flattening of the dose response. In the 10mg group, the mean C_{max} was 554 ng/mL, showing all dose levels reached expected active concentrations. Lack of a clear signal in the 30mg group could be explained by the inherent variability of the biomarker, combined with a small sample size of subgroups.

Variability of GluCer in PBMCs as a biomarker can also be seen in placebo data. No change over 28 days is expected in placebo treated participants, so fluctuations in the placebo group likely reflect natural variability. The strongest signal at day 14 seems driven by both a GluCer increase in LTI-291 treated participants and a random trough in the placebo-treated group (Figure 1). Nevertheless, the overall treatment effect is still statistically significant different in LTI-291 treated participants compared to placebo, accounting for this variability over time (Table 2). Considering the exploratory setting of these pharmacodynamic measurements, without correction for multiple testing, these effects require validation in a larger cohort.

Measurements in PBMCs of GluSph and various LacCer isoforms were heavily influenced by the cell subtype composition of the PBMC isolate (in draft: den Heijer, Pereira, et al. 2022). This composition also varied within-individual between samples. Because this variation could not be distinguished from a potential treatment effect, these were omitted from analysis.

GBA1 genotype category (GD-risk (n=25) vs PD-risk (n=15)) was investigated as covariate. GD-risk showed a trend for a stronger effect in all GluCer isoforms in PBMCs, but did not reach statistical significance (data not shown). It can be speculated that patients with a larger GCase deficiency, may have more benefit of treatment. Subgroups were small however, and GCase activity is known to vary between individuals with the same mutation. Whether this translates to a clinical effect will be determined in an upcoming trial.

Pharmacokinetic sampling was limited with three plasma samples up to six hours post-dose on day 1 and day 28, showing a dose-proportional increase in C_{max} and AUC_{0-6}. The mean CSF:plasma concentration ratios ranged from 0.0113 to 0.0122 at 4 hours after the 28^{th} dose and were similar

at all dose levels (Supplementary Table 3). This ratio corresponds with a free distribution of unbound LTI-291 between plasma and CSF, which again is in distribution equilibrium with brain tissue, as was shown in preclinical rat neuro PK experiments. This PK profile is similar to what was determined in healthy volunteers (den Heijer, Kruithof, et al. 2021), which also showed a median half-life of 28. 0 hours, favoring daily single dosing.

A neurocognitive test-battery showed no adverse effect on CNS functioning, performed after the 14th dose, during which steady state LTI-291 plasma concentration was already achieved. No clinical improvement was expected after 28 days of dosing and no deterioration was observed, as confirmed by MDS-UPDRS part III (motor assessment) and MMSE testing. The MDS-UPDRS was performed in ON state, since the burden of testing in OFF state was not considered justified, as no clinical change was expected. In a long-term study to assess clinical improvement, OFF state measures will be appropriate.

In five participants, a mild subjective worsening of PD symptoms was reported, which resolved before end of dosing in four participants (three active treatment, one placebo). Considering the natural variation in Parkinson's disease symptom severity and the progressive disease course, these complaints were considered unlikely to be caused by administration of LTI-291.

In conclusion, LTI-291 was observed to be well tolerated when given orally once daily for 28 consecutive days at all dose levels tested in this GBA-PD population. Plasma concentrations were reached that are expected to be active in at least doubling glucocerebrosidase activity. Exploratory pharmacodynamic markers suggest peripheral target engagement. A long-term (one year) dosing study is being planned to assess clinical benefit.

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Table 1 Overview of demographic variables. The *GBA1* allelic names are given, excluding the 39-amino acid signaling peptide. In case of two mutations, variants within the staple signs [ ] are on the same allele, and variants in separate staple signs are on separate alleles. A semicolon in parentheses indicates it is uncertain how these mutations are distributed over alleles. GD mutations are designated (1) and non-GD mutations (2).

Demographics and baseline characteristics					
Demographic variables	**All participant (N=40)**	**10 mg LTI-291 (N=10)**	**30 mg LTI-291 (N=10)**	**60 mg LTI-291 (N=10)**	**Placebo (N=10)**
AGE (YEARS)					
Mean (SD)	61.1 (9.3)	59.9 (11.5)	62.6 (8.2)	59.1 (9.1)	62.8 (8.9)
Min, Max	40, 80	40, 79	51, 80	46, 80	47, 73
HEIGHT (CM)					
Mean (SD)	172.2 (8.9)	170.1 (9.7)	172.0 (7.1)	174.8 (8.8)	172.1 (10.5)
Min, Max	156.1, 189.2	156.1, 189.0	161.6, 184.1	162.4, 189.2	157.3, 183.5
WEIGHT (KG)					
Mean (SD)	73.9 (13.4)	69.0 (15.2)	81.0 (14.0)	74.0 (13.1)	71.6 (9.5)
Min, Max	46.25, 110.05	46.25, 88.2	63.9, 110.05	55.5, 92.85	60.2, 87.3
BMI (KG/M^2)					
Mean (SD)	24.8 (3.6)	23.6 (3.1)	27.3 (3.9)	24.1 (3.5)	24.3 (3.4)
Min, Max	17.9, 35.1	19, 27.9	22.6, 35.1	19.4, 29	17.9, 28.9
MMSE Total					
Mean (SD)	27.9 (2.6)	27.7 (4.1)	27.1 (1.9)	29.0 (1.3)	27.7 (2.3)
Median	29.0	30.0	27.0	29.5	28.5
Min, Max	19, 30	19, 30	25, 30	26, 30	24, 30
MDS-UPDRS part III Total (ON STATE)					
Mean (SD)	31.4 (14.0)	31.1 (17.4)	28.4 (12.6)	32.8 (14.3)	33.4 (12.7)
Median	27.5	27.5	22.5	26.5	31.0
Min, Max	14, 72	14, 72	17, 54	20, 65	17, 52
SEX					
Female	20 (50.0%)	6 (60.0%)	4 (40.0%)	5 (50.0%)	5 (50.0%)
Male	20 (50.0%)	4 (40.0%)	6 (60.0%)	5 (50.0%)	5 (50.0%)
RACE					
White	40 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)

Table 1 *(Continuation of previous page)*

Demographics and baseline characteristics					
Demographic variables	**All participant (N=40)**	**10 mg LTI-291 (N=10)**	**30 mg LTI-291 (N=10)**	**60 mg LTI-291 (N=10)**	**Placebo (N=10)**
GBA1 mutation (allelic name)[disease association]					
P.[D140H;E326K][1]	6 (15.0%)	1 (10.0%)	3 (30.0%)	2 (20.0%)	0 (0%)
P.E326K[2]	9 (22.5%)	4 (40.0%)	2 (20.0%)	2 (20.0%)	1 (10.0%)
P.[E326K];[E326K][2]	1 (2.5%)	0 (0%)	0 (0%)	0 (0%)	1 (10.0%)
P.G202R[1]	1 (2.5%)	0 (0%)	1 (10.0%)	0 (0%)	0 (0%)
P.G325R[1]	1 (2.5%)	0 (0%)	1 (10.0%)	0 (0%)	0 (0%)
P.L444P[1]	5 (12.5%)	2 (20.0%)	1 (10.0%)	1 (10.0%)	1 (10.0%)
P.T369M(;)L444P[1]	2 (5.0%)	0 (0%)	0 (0%)	1 (10.0%)	1 (10.0%)
P.N370S[1]	7 (17.5%)	2 (20.0%)	1 (10.0%)	1 (10.0%)	3 (30.0%)
P.R120W[1]	2 (5.0%)	0 (0%)	0 (0%)	1 (10.0%)	1 (10.0%)
P.R329C[1]	1 (2.5%)	0 (0%)	0 (0%)	1 (10.0%)	0 (0%)
P.T369M[2]	4 (10.0%)	1 (10.0%)	1 (10.0%)	1 (10.0%)	1 (10.0%)
P.[E326K];[T369M][2]	1 (2.5%)	0 (0%)	0 (0%)	0 (0%)	1 (10.0%)

BMI=body mass index; min=minimum; max=maximum; SD=Standard deviation, MMSE=Mini Mental State Examination, MDS-UPDRS=Movement Disorder Society-Unified Parkinson's Disease Rating Scale.

Table 2 All treatment emergent adverse events by treatment. All AEs were coded using the Medical Dictionary for Regulatory Activities (MedDRA) version 20.0. Greyed rows depict system organ classes and numbers are a summation of all preferred terms in that class. Multiple AEs could be reported by the same participant. *Preferred term Parkinson's disease was used for a participant-reported worsening of Parkinson's disease related symptoms.

	10mg LTI-291		**30mg LTI-291**		**60mg LTI-291**		**Placebo**	
	N=10		**N=10**		**N=10**		**N=10**	
System Organ Class/ Preferred Term	**Events (N)**	**Subjects (N (%))**	**Events (N)**	**Subjects (N (%))**	**Events (N)**	**Subjects (N (%))**	**Events (N)**	**Subjects (N (%))**
ANY EVENTS	17	9 (90.0)	20	7 (70.0)	9	8 (80.0)	12	7 (70.0)
EAR AND LABYRINTH DISORDERS	3	2 (20.0)	-	-	-	-	-	-
Tinnitus	2	2 (20.0)	-	-	-	-	-	-
Vertigo	1	1 (10.0)	-	-	-	-	-	-
GASTROINTESTINAL DISORDERS	2	2 (20.0)	2	1 (10.0)	-	-	-	-
Abdominal pain	-	-	1	1 (10.0)	-	-	-	-
Diarrhoea	-	-	1	1 (10.0)	-	-	-	-
Gastroenteritis viral	2	2 (20.0)	-	-	-	-	-	-
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	2	2 (20.0)	5	3 (30.0)	1	1 (10.0)	2	2 (20.0)
Fatigue	1	1 (10.0)	4	3 (30.0)	1	1 (10.0)	2	2 (20.0)
Feeling hot	-	-	1	1 (10.0)	-	-	-	-
Oedema	1	1 (10.0)	-	-	-	-	-	-
INFECTIONS AND INFESTATIONS	1	1 (10.0)	1	1 (10.0)	1	1 (10.0)	1	1 (10.0)
Influenza	-	-	-	-	1	1 (10.0)	-	-
Paronychia	-	-	1	1 (10.0)	-	-	-	-
Urinary tract infection	1	1 (10.0)	-	-	-	-	1	1 (10.0)
INJURY, POISONING AND PROCEDURAL COMPLICATIONS	1	1 (10.0)	-	-	-	-	-	-
Vessel puncture site phlebitis	1	1 (10.0)	-	-	-	-	-	-
Soft tissue injury	-	-	1	1 (10.0)	2	2 (20.0)	-	-

Table 2 *(Continuation of previous page)*

	10mg LTI-291		30mg LTI-291		60mg LTI-291		Placebo	
	N=10		N=10		N=10		N=10	
System Organ Class/ Preferred Term	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))	Events (N)	Subjects (N (%))
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	-	-	6	5 (50.0)	4	4 (40.0)	3	3 (30.0)
Back pain	-	-	3	3 (30.0)	1	1 (10.0)	-	-
Muscle spasms	-	-	1	1 (10.0)	-	-	-	-
Muscle strain	-	-	-	-	1	1 (10.0)	-	-
Musculoskeletal pain	-	-	-	-	-	-	1	1 (10.0)
Myalgia	-	-	1	1 (10.0)	-	-	1	1 (10.0)
Tendonitis	-	-	-	-	-	-	1	1 (10.0)
NERVOUS SYSTEM DISORDERS	5	3 (30.0)	4	3 (30.0)	2	2 (20.0)	5	3 (30.0)
Headache	5	3 (30.0)	1	1 (10.0)	-	-	4	3 (30.0)
Parkinson's disease*	-	-	2	2 (20.0)	2	2 (20.0)	1	1 (10.0)
Restless legs syndrome	-	-	1	1 (10.0)	-	-	-	-
PSYCHIATRIC DISORDERS	-	-	-	-	-	-	1	1 (10.0)
Stress	-	-	-	-	-	-	1	1 (10.0)
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	2	1 (10.0)	2	2 (20.0)	1	1 (10.0)	-	-
Cough	-	-	2	2 (20.0)	-	-	-	-
Sinusitis	-	-	-	-	1	1 (10.0)	-	-
Viral upper respiratory tract Infection	2	1 (10.0)	-	-	-	-	-	-
VASCULAR DISORDERS	1	1 (10.0)	-	-	-	-	-	-
Epistaxis	1	1 (10.0)	-	-	-	-	-	-

Table 3 Summary of linear mixed model analysis results per GluCer isoform. The table shows the overall treatment effect (all dose groups (n=30) compared to placebo (n=10)) and treatment effect per dose level (n=10 per level). Estimates of the difference with 95% confidence intervals and p-values are shown per comparison. Log-transformed parameters were back-transformed after analysis and therefore these parameters are shown as percentage change.

Overall treatment effect on GluCer isoforms of LTI-291 vs placebo								
	LTI-291 overall vs placebo		LTI-291 10mg vs placebo		LTI-291 30mg vs placebo		LTI-291 60mg vs placebo	
Isoform	Contrast (95%CI)	p	Contrast (95%CI)	p	Contrast (95%CI)	p	Contrast (95%CI)	p
GluCer C16:0	6.7% (1.2%-12.6%)	0.018	8.3% (1.4%-15.7%)	0.019	4.6% (-2.0%-11.7%)	0.170	7.3% (0.1%-15.0%)	0.047
GluCer C18:0	3.6% (-3.0%-10.7%)	0.284	4.8% (-3.2%-13.4%)	0.237	0.4% (-7.2%-8.6%)	0.925	7.2% (-1.9%-17.2%)	0.121
GluCer C22:0	6.4% (0.1%-13.0%)	0.046	9.8% (2.6%-17.5%)	0.008	0.3% (-6.3%-7.4%)	0.923	9.7% (2.0%-17.9%)	0.014
GluCer C24:0	8.8% (1.5%-16.7%)	0.018	12.5% (3.8%-21.8%)	0.005	2.4% (-5.5%-11.0%)	0.549	12.5% (3.4%-22.3%)	0.007
GluCer C24:1	0.197 (0.065-0.329)	0.004	0.238 (0.084-0.391)	0.003	0.102 (-0.052-0.257)	0.188	0.266 (0.103-0.428)	0.002

Table 4 Summary of analysis over time results per GluCer isoform. The table shows the overall treatment effect over time (all dose groups compared to placebo). Estimates of the difference with 95% confidence intervals and p-values are shown per comparison. Log-transformed parameters were back-transformed after analysis and therefore these parameters are shown as percentage change.

Treatment effect over time on GluCer isoforms of all LTI-291 dosed vs placebo								
	Day 1		Day 7		Day 14		Day 28	
Isoform	Contrast (95%CI)	p-value	Contrast (95%CI)	p-value	Contrast (95%CI)	p-value	Contrast (95%CI)	p-value
GluCer	6.3%		7.1%		15.6%		7.2%	
C16:0	(-3.7%–17.2%)	0.224	(-2.9%–18.1%)	0.170	(4.8%–27.5%)	0.004	(-2.8%–18.2%)	0.166
GluCer	6.4%		0.2%		10.5%		3.3%	
C18:0	(-4.8%–18.9%)	0.273	(-10.3%–11.9%)	0.974	(-1.1%–23.5%)	0.078	(-7.6%–15.4%)	0.567
GluCer	5.2%		6.4%		17.5%		5.2%	
C22:0	(-5.5%–17.1%)	0.354	(-4.4%–18.5%)	0.252	(5.5%–30.8%)	0.003	(-5.5%–17.1%)	0.353
GluCer	5.5%		12.4%		22.6%		5.4%	
C24:0	(-6.4%–18.9%)	0.376	(-0.3%–26.6%)	0.056	(8.8%–38.1%)	0.001	(-6.5%–18.8%)	0.388
GluCer	0.099		0.570		0.143		0.195	
C24:1	(-0.168–0.367)	0.293	(0.303–0.838)	0.153	(-0.124–0.411)	<0.001	(-0.073–0.462)	0.465

Table 5 Summary of MDS-UPDRS part III (motor assessment, ON state) and MMSE total scores.

MDS-UPDRS part III and MMSE scores at baseline and after 28th dose				
	10 mg LTI-291 (N=10)	30 mg LTI-291 (N=10)	60 mg LTI-291 (N=10)	Placebo (N=10)
MDS-UPDRS PART III (ON STATE) (MEAN (SD))				
Predose	31.1 (17.4)	28.4 (12.6)	32.8 (14.3)	33.4 (12.7)
EOT	31 (13)	30 (9)	33 (12)	35 (15)
Change from baseline	-0.1 (7.6)	1.2 (7.1)	0.3 (8.9)	1.6 (6)
MMSE (MEAN (SD))				
Predose	27.3 (3.2)	27.4 (2.5)	28.7 (2.4)	27.7 (1.8)
EOT	27.3 (4.0)	28.0 (1.8)	29.0 (1.3)	27.5 (1.9)
Change from baseline	0.0 (2.0)	0.6 (2.3)	0.3 (2.0)	-0.2 (1.3)

MDS-UPDRS=Movement Disorder Society-Unified Parkinson's disease rating scale, MMSE=mini mental state examination, SD=standard deviation, EOT=end of treatment.

Figure 1 (A) The GCase reaction targeted by LTI-291, part of the sphingolipid (SL) recycling pathway. The SL pathway is a closed system, with de novo synthesis as input and sphingosine lyase as output. The former pathway is endogenously inhibited, but may play a role in maintaining GSL flux when GCase activity is low. Two pools of GluCer (lysosomal and non-lysosomal) exist, which are not distinguishable in our measures. (B) GBA-PD patients recycle ceramide slowly, so de novo synthesis of ceramide is increased and GluCer levels are maintained (left panel). Treatment with LTI-291 increases availability of cytosolic ceramide, resulting in a transient increase in GluCer synthesis (middle panel). However, increased ceramide levels are known to result in decreased de novo synthesis, bringing steady-state levels of GluCer and Cer back to the pre-dose levels at 28 days. However, the pre-dose and day 28 pathways differ in that the day 28 pathway has greater GSL flux (comparable amount, but faster rate) and reduced de novo synthesis. GCase = glucocerebrosidase, Cer = ceramide.

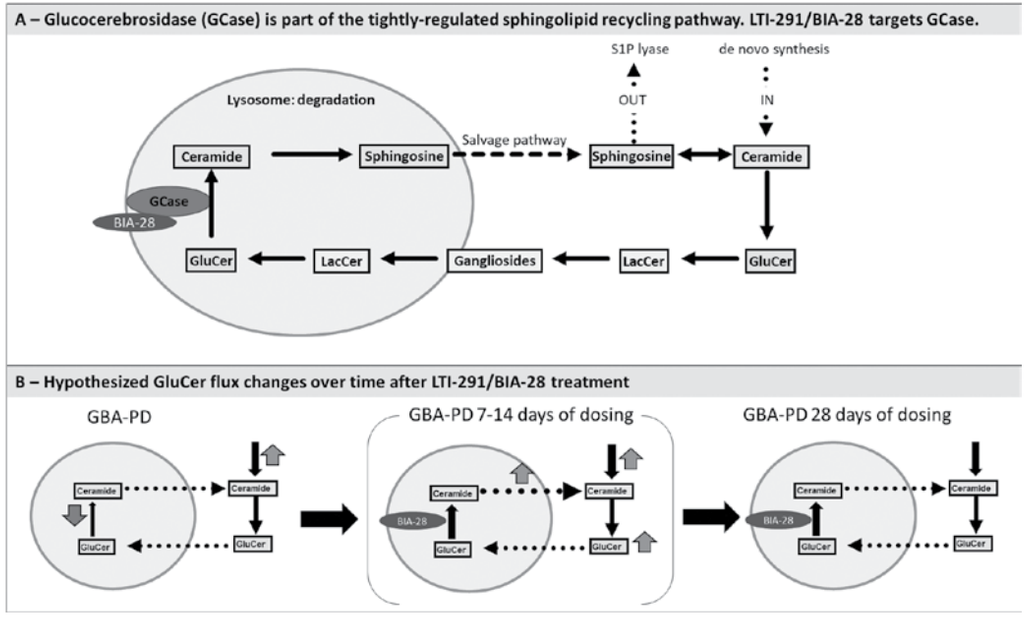
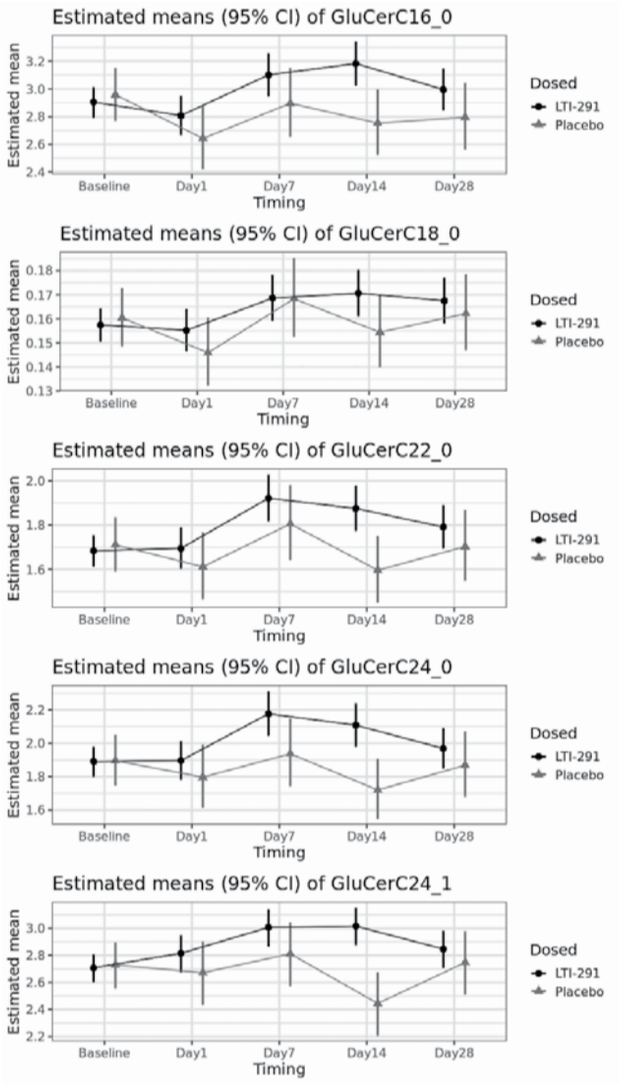


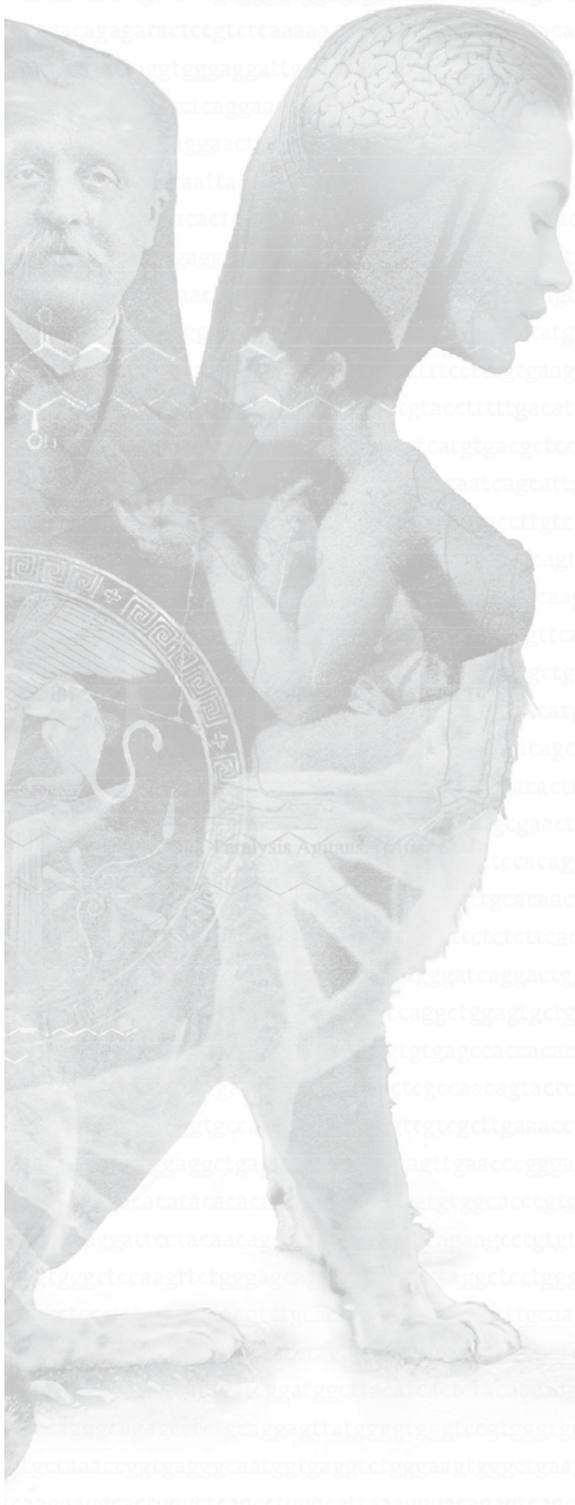
Figure 2 Graphs depicting the estimated means (95% confidence interval) of different GluCer isoforms over time, separate for participants treated with LTI-291/BIA-28 (all dose levels combined, n=30, dark bars) or placebo (n=10, light bars). The Day1 sample was taken 6 hours after dosing. Sample timing was the same for all participants, offset of the means and bars is for readability.



SUPPLEMENTARY MATERIAL

H8SF1 /H8ST1 / H8ST2 / H8ST3 / H8ST4 / H8ST5 / H8ST6

SCAN ME



CHAPTER 9

Discussion

This thesis describes a series of studies related to GBA-PD and development of a novel pharmacotherapeutic intervention. As one of the most common neurodegenerative diseases worldwide, Parkinson's disease is vigorously being researched, to better understand and consequently better treat this debilitating progressive affliction. Despite the fact that this disease was first described already more than 200 years ago by James Parkinson, no disease-modifying treatment is yet available. This also explains why all new clues regarding the pathogenesis will be turned upside down and inside out, to explore any possibility to design a disease-modifying treatment targeting this factor of the pathogenesis. One such example is the *GBA1* gene, which is considered one of the most promising targets currently known for a new disease modifying therapy for PD. The chapters in this thesis describe studies that have contributed to exploration of this therapeutic target and will hopefully support future research to be more effective.

Apart from the novel results yielded by the various studies, described in the respective chapters, there are several overarching lessons that need to be taken into account in future studies. These include methodological and physiological challenges, which one needs to be aware of, because these can be relevant for the design, execution, and interpretation of a drug trial involving GCase as the target. Methodological challenges were present in most papers of this thesis.

A serendipitous methodological finding hugely impacted the results of the *GBA1* screening (**Chapter 3**). The *GBA1* gene is susceptible to an imbalanced amplification of alleles, which initially resulted in a large number of false-negative results which was corrected by changing the type of polymerase used. Sequencing of the *GBA1* gene was already known for its risk of false-positive results due to the nearby pseudogene, but we have shown that false-negative results should be considered as well. This could also explain conflicting results in existing literature. Additionally, it is another confirmation that sequencing of the *GBA1* gene can only reliably be performed using dedicated conditions, and not with coarser techniques like GWAS and imputation without validating this first.

In accordance with this first lesson, care was taken in selecting international cohorts that allow a valid comparison of our *GBA1* screening results (**Chapter 2**). Many papers only screened for 'the most common' variants in the *GBA1* gene which is primarily based on studies performed in the Ashkenazi Jewish population who have a remarkably high incidence of certain *GBA1* mutations. Several papers, including our own, have shown the existence of population-specific variants, underscoring the relevance of performing full-gene screenings in populations of sufficient size. Preferably, costs for full-gene sequencing are reduced so this can be standard practice in the future.

Because *GBA1* variants behave as risk-factors with limited penetration, they require a less straight-forward type of counseling than in case of an autosomal dominant or recessive disorder. Since *GBA1* variants lack therapeutic consequences, there is little experience in informing patients on the potential relevance of *GBA1* variants. Our large-scale *GBA1* screening therefore underscored the need to develop documentation to guide clinicians in this process (**Chapter 4**).

Implementation of novel biomarkers is challenging since there is no reference to guide expectations. The LTI-291 trials were the first to determine various glycosphingolipids in plasma, PBMCs and CSF (**Chapter 7 and 8**). The findings of these trials yielded an unexpected increase in GluCer levels, requiring an adaptation of the hypothesis of how these biomarkers should be interpreted. We hypothesize that lowering of the intralysosomal sources may have led to an increase in total intracellular (including extralysosomal) presence of GCase substrates. Alternatively, the findings may reflect a chance finding.

Lastly, another serendipitous finding was the significant impact of cell types for certain biomarkers related to GCase activity (**Chapter 6**). Peripheral blood mononuclear cells (PBMCs) consist of monocytes and lymphocytes and are a valuable and easily accessible source to determine biomarkers in a cell-based environment. Inherent to the isolation methodology, there will be a varying amount of granulocyte contamination, rendering PBMC isolation susceptible to collecting at least three different cell types. This issue may be solved by more costly procedures that subtype PBMCs. Knowledge of the relevance of these different cell types for a biomarker determination is therefore essential to adequately interpret PBMC based data.

Several reasons underlie the challenge in proving efficacy of a novel disease-modifying treatment for neurodegenerative diseases like Parkinson's disease. First, the inherent typically slow progression of neurodegenerative

diseases, and the relatively large day-to-day variability of measurements assessing disease progression (like the MDS-UPDRS in PD). Both issues hamper the detection of a disease modifying effect and require adequate powering of studies and a long follow-up. Development and use of biomarkers in early stages of drug development may contribute to the decision-making on whether to proceed with such large trials. Use of wearables or measurements at home (either using video, or tasks on computers or tablets etc.) will hopefully reduce variability associated with snapshot measurements of less frequent in-hospital clinical assessments. Efforts to allow a more reliable estimate of the progression rate should be pursued but are complicated given the non-linear progression of PD. Additionally, only using fixed baseline characteristics (like a gene variant) may introduce noise if dynamic characteristics (like age and duration of disease) are not considered.

The development of a successful trial requires an effective recruitment strategy highlighting the need of adequate communication and collaboration. An excellent example is the *GBA1* screening (**Chapter 2**), which was a collaborative effort of ten medical centres, local patient groups and the national patient association, which was essential for successfully finding 40 GBA-PD patients for the first-in-patient trial of LTI-291 (**Chapter 8**). In just three months, the genetic screening evolved from a first idea into a fully approved protocol with a first reach-out to patients. In the course of months, ten movement disorders neurologists sent letters to the PD patients from their center, informing them about the study. Every patient that applied, received an information letter. Every patient that signed, received a saliva tube. All in all, tens of thousands of letters were sent (our corporate outgoing mail had to be upgraded...) to achieve a successful screening of the *GBA1* gene in 3402 patients over a period of less than one year - a large operation associated with a relatively minor effort for collaborators and patients. Unanticipated, this project gave rise to the earlier discussed serendipitous finding (**Chapter 3**), but also to new international collaborations, both published (**Chapter 5**, New Zealand/Australia) and still unpublished (*GBA1* and *LRP10* interaction, Italy; *GBA1* intron variant, United Kingdom).

In the future, treatment of Parkinson's disease may be based on a profile of genetic and/or biological markers that guide the development of a personalized approach. Certain glycosphingolipid levels and GCase activity may contribute to such a profile which may also include other potential drug targets, involving endosomal, mitochondrial and cellular trafficking functioning, inflammation or alpha-synuclein subtypes. Involvement of different biological pathways may vary between patients and therefore determine the personalized content of a cocktail of drugs targeting different pathways. This may lead to new dilemmas, e.g. biological profiles frequently do not provide a clear (black and white) cut-off as to when to treat or not. Considering the unmet need of a disease-modifying treatment in PD, every patient would likely want to try all of these drugs, however small the benefit might be, assuming it has a favorable safety profile. Which will make it a matter of costs.

This highlights another topic of public debate, which is drug development in general, its costs, pricing of new drugs and maximizing profit (e.g. by making creative use of patents). It is justified to make profit for drug discovery and development. But it is difficult to determine what is reasonable and what is excessive. Because it pertains to medical needs of humans, there is an additional moral pressure. Most people engage in research because they are interested in the science and want to help others, but unfortunately there are also examples of abuse of this medical need for (excessive) profits. Increasing transparency of the full drug development process, and reducing defensive bureaucratic obligations, could stimulate a fair healthcare and reimbursement process. Additionally, drug studies should be performed by transparent and professional organizations, where employees receive a reasonable wage, with a clearly defined maximum, for like a medical specialty, running clinical trials is a specialization on its own. Only by such in-depth knowledge of the drug development process, can e.g. trial design pitfalls be prevented and can redundant bureaucratic requests be identified and omitted.

In conclusion, this thesis is a collection of studies that together form another tiny, yet necessary, step toward a better care for a growing patient population. This goal can only be achieved through the continuous dedicated efforts of researchers, patients and other caregivers.

CHAPTER 10

Nederlandse samenvatting

GENETISCHE EN KLINISCH FARMACOLOGISCHE ONDERZOEKEN NAAR *GBA1*-GEASSOCIEERDE ZIEKTE VAN PARKINSON Dit proefschrift omvat een serie onderzoeken gerelateerd aan de ziekte van Parkinson en mutaties in het *GBA1* gen.

De ziekte van Parkinson (ZVP) is een van de meest voorkomende neurodegeneratieve aandoeningen. Dit zijn aandoeningen waarbij bepaalde delen van het brein sneller dan normaal achteruitgaan. De ZVP kenmerkt zich door problemen in het bewegen, zoals traagheid, stijfheid en trillen, maar juist ook door veel andere problemen in bijvoorbeeld de stemming, het geheugen en het reguleren van automatische processen in het lichaam (zoals de bloeddruk en de spijsvertering). Het is nog onbekend hoe het kan dat iemand de ZVP ontwikkelt. Het komt waarschijnlijk door een samenspel van omgevingsfactoren en factoren in de erfelijke code (de genen). Eén van de vele genen in ons lijf is het *GBA1* gen. Dit stukje code zorgt voor de aanmaak van het stofje GCase (glucocerebrosidase), een stofje dat in bijna alle cellen van het lijf zit en helpt bij een bepaalde afvalverwerking. Van alle genen hebben we twee versies, één van vader en één van moeder. Als er een verandering (mutatie) in beide versies van het *GBA1* gen zit, waardoor het gen niet meer goed werkt, kan iemand de zeldzame stapelingsziekte van Gaucher ontwikkelen.

In de afgelopen twee decennia is geconstateerd dat mensen met de ZVP relatief vaak een mutatie hebben in één van de twee versies van het *GBA1* gen. Omdat slechts één van de twee versies zo een mutatie bevat, krijgen mensen niet deze stapelingsziekte van Gaucher, maar is er wel een *iets verhoogd risico* om de ZVP te ontwikkelen (GBA-ZVP). Sinds dit aan het licht is gekomen, wordt er veel onderzoek gedaan naar het *GBA1* gen, in de hoop een middel te kunnen ontwikkelen dat aangrijpt op dit proces en ook de hersenen bereikt, om hopelijk de achteruitgang van de ZVP af te remmen. Dit proefschrift omvat een kleine bijdrage aan de verheldering van dit proces.

In **hoofdstuk 1** wordt een samenvatting gegeven van verschillende bekende erfelijke factoren die betrokken kunnen zijn bij de ZVP. Als we weten dat een mutatie in een bepaald gen een grotere kans geeft op de ZVP, kan er gericht gekeken worden naar de functie van dat gen, om aanwijzingen te krijgen over welke cellulaire processen er betrokken zijn bij het ontwikkelen van de ZVP. Zodra bekend is welke processen er fout gaan, kan hier ook gericht een medicamenteuze therapie voor worden ontwikkeld (klinkt wel een stuk makkelijk dan het is, helaas). Het kleine Amerikaans bedrijf Lysosomal Therapeutics Inc., heeft een eerste middel ontwikkeld (LTI-291) dat in preklinisch onderzoek (dus aanvankelijk nog niet in mensen) het stofje GCase weer actiever kan maken en ook in de hersenen kan komen.

Als voorbereiding om het middel LTI-291 te onderzoeken bij mensen met de ZVP, specifiek met ook een mutatie in het *GBA1* gen, hebben we in een bijzonder omvangrijke patiëntenpopulatie genetische screening gedaan in Nederland (**hoofdstuk 2**). Hiervoor konden mensen met de ZVP via de post een buisje met speeksel inleveren, wat geanalyseerd kon worden. Meer dan 3500 patiënten hebben dit gedaan. Uit deze analyse bleek dat in Nederland ongeveer 15% van de patiënten een mutatie heeft in het *GBA1* gen, terwijl in een controlegroep (van 655 mensen) slechts ongeveer 6.4% van de mensen een mutatie heeft.

Dit was een groot samenwerkingsproject, waaraan 10 verschillende ziekenhuizen hebben meegewerkt, evenals de Parkinson Vereniging. Wereldwijd was dit dan ook het grootste onderzoek naar het *GBA1* gen binnen één land.

De analyse kwam wel met de nodige methodologische uitdagingen in het lab, die we gelukkig hebben kunnen oplossen (**hoofdstuk 3**). Omdat het *GBA1* gen (nog) niet standaard wordt gescreend, hebben we een protocol voor genetische begeleiding van patiënten m.b.t. het *GBA1* gen opgesteld (**hoofdstuk 4**). Naar aanleiding van onze resultaten is in samenwerking met een onderzoeksgroep in Australië en Nieuw-Zeeland nog gekeken of bepaalde specifieke varianten in het *GBA1* gen invloed hebben de debuutleeftijd van de ZVP, maar deze vraag behoeft verder onderzoek (**hoofdstuk 5**).

Een grote uitdaging in de geneesmiddelenontwikkeling is om aan te tonen dat een middel daadwerkelijk werkt en ook helpt. Bij neurodegeneratieve aandoeningen zoals de ZVP is dat extra uitdagend, omdat mensen inherent aan de aandoening relatief langzaam achteruitgaan, dus moet er ook lang gemeten worden om aan te kunnen tonen dat deze achteruitgang wordt afgeremd. In combinatie met een grote natuurlijke variatie is dit erg langdurig en kostbaar onderzoek. Om al eerder een indruk te kunnen krijgen of een middel aangrijpt op het beoogde proces, kunnen biomarkers worden gebruikt.

In **hoofdstuk 6** zijn verschillende potentiële biomarkers geanalyseerd, in verschillende lichaamsstoffen (bloedplasma, witte bloedcellen en hersenvocht). Deze biomarkers zijn allemaal stoffen die nauw gerelateerd zijn aan het proces van GCase, zoals glucosylceramide (GluCer), lactocylceramide (LacCer) en glucosylsphingosine (GluSph). Bij mensen met GBA-ZVP lijkt met name GluCer in plasma gemiddeld iets hoger te zijn, maar de spreiding van de waardes overlapt wel nog met die van gezonde controles. Het blijft echter moeilijk te zeggen wat de beste biomarker is, omdat de verschillende lichaamsstoffen verschillende analysevoordelen hebben: in bloedplasma is het duidelijkste verschil tussen groepen te zien, in witte bloedcellen kunnen intracellulaire niveaus bepaald worden, en hersenvocht komt vanuit de hersenen zelf (waar het ziekteproces zich ook afspeelt). Verder werd per toeval ontdekt dat de samenstelling van een isolatie van witte bloedcellen erg kan variëren en dat dit grote invloed kan hebben op metingen van met name LacCer en GluSph.

Hoofdstuk 7 en 8 beschrijven de eerste toedieningen van LTI-291 aan gezonde vrijwilligers en vervolgens aan mensen met GBA-ZVP. Deze eerste toedieningen zijn goed verlopen, zonder duidelijke of ernstige bijwerkingen die te wijten zijn aan het gebruik van LTI-291. Het ziet er dus naar uit dat het middel goed verdragen wordt. Verschillende eigenschappen van het middel zijn onderzocht, zoals hoe snel het middel wordt opgenomen door het lichaam en ook weer uitgescheiden. Deze onderzoeken zijn nodig om een dosering te kunnen vaststellen. De vraag of het middel ook werkt en helpt is helaas nog onbeantwoord. De biomarkers die gemeten zijn bij gezonde vrijwilligers lieten geen veranderingen zien, wat verklaard zou kunnen worden door het feit dat dit gezonde mensen zijn. In het onderzoek bij GBA-ZVP werden wel veranderingen gezien in bepaalde biomarkers (een tijdelijke stijging van GluCer in witte bloedcellen, maar niet in plasma of hersenvocht, en ook niet van LacCer of GluSph), maar interpretatie van deze constatering vormt een nieuwe uitdaging. Op basis van deze uitslagen kunnen we wel een hypothese vormen als mogelijke verklaring, maar het bewijst een effect nog niet (het kan alsnog een toevalsbevinding zijn). Het lijkt dus wel een aanwijzing dat het middel aangrijpt op het beoogde proces (dat het werkt), maar dit moet bevestigd worden. Of het middel ook daadwerkelijk helpt, dus dat patiënten er ook profijt van hebben in het dagelijks leven (en dat niet alleen bloedwaardes veranderen), zal pas in een lange termijn vervolgonderzoek aangetoond kunnen worden.

Samenvattend is dit proefschrift een verzameling van onderzoeken, die samen een kleine, basale stap vormen naar beter ziekte-inzicht en hopelijk kunnen leiden tot betere zorg voor een groeiende patiëntenpopulatie. Dit kan alleen worden bereikt door aanhoudende toewijding van onderzoekers, patiënten en zorgverleners.

CURRICULUM VITAE

Jonas Matthias den Heijer was born on March 1st, 1991, in Roermond, the Netherlands. After completing secondary school at B.C. Broekhin in Roermond in 2009, he studied Medicine at Utrecht University. Since his second year he was involved in extracurricular scientific research. During his master he focused his clinical rotations on neurology and scientific research on epilepsy and Parkinson's disease. He obtained his doctoral degree in 2016 and started working as a research physician at the Centre for Human Drug Research (CHDR) in 2017. In this position under supervision of prof. dr. G.J. Groeneveld his focus was on clinical studies related to disorders of the central nervous system, with a special interest in Parkinson's disease, including the research described in this thesis. In 2021 he worked as a neurology resident at the Rijnstate hospital in Arnhem and in 2022 he started as neurology resident at the Radboud University Medical Centre in Nijmegen. He is finalizing his clinical pharmacologist certification.

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