Chapter 7

Summary and general discussion
The current thesis advances our understanding of gut microbiome changes in Parkinson’s disease (PD), and explores the possibility of a gastrointestinal origin in a subgroup of PD. Here, the conclusions are summarized per chapter, which will then be integrated with concurrent developments on the topic. Given the clinical subject of this thesis, an overarching aim can be formulated: to move towards disease-modifying therapies for PD. Therefore, the potential of gut microbiota as a treatment for PD will be explored. In addition, the obstacles to assess disease-modifying potential in PD will be assessed and concrete solutions will be suggested.

Summary of the thesis

Chapter 2 provides a systematic review of both the outcomes and methodologies of gut microbiome studies in PD. Since the first PD case-control study by Scheperjans et al., the number of PD microbiome studies has steadily increased. Even in the weeks during which the chapter was written, several new studies were published and subsequently incorporated in the systematic review. All studies concerned sequencing-based methods investigating the fecal microbiome. The majority of studies used 16S rRNA-gene sequencing, one study used shotgun metagenomics and two studies used quantitative polymerase chain reaction (qPCR) of selected taxa. One study also investigated the mucosal gut microbiome from biopsy samples in addition to fecal samples. Even though the results of the included studies showed various inconsistencies, several findings could be replicated in these studies. These included an increased abundance of the family Verrucomicrobiaceae, and a decreased abundance of the families Prevotellaceae and Lachnospiraceae. At the genus level, Akkermansia, Bifidobacterium and Faecalibacterium had an increased abundance in multiple studies, whereas Faecalibacterium, Prevotella and Roseburia were decreased. Besides the large variability inherent to gut microbiome datasets, the inconsistency of several other findings might be attributable to the different methodologies used and the extent to which potential confounders were addressed. Authors of already available and future PD microbiome studies should make the clinical metadata, technical metadata and sequencing data publicly available, to allow for a comprehensive meta-analysis of gut microbiome data in PD. Future studies would benefit from a multi-omics assessment of the gut microbiome for a functional assessment of the gut microbiome rather than a taxonomic assessment. In addition, different control groups, including atypical parkinsonism subjects, are required to assess the utility of gut microbial markers in the differential diagnosis of PD. The a priori identified lack of treatment-naive PD subjects in the first PD microbiome studies, remains of particular concern with only two studies having analyzed gut microbiome data of small subgroups (n=12 and n=39) of treatment-naive subjects separately. One study investigated only levodopa-naive subjects, but these subjects still used other dopaminergic drugs. Therefore, chapter 2 provided additional rational for the studies described in chapter 4 and chapter 5 on the gut microbiome of treatment-naive PD subjects and the effect of PD medication on the gut microbiome.

Chapter 3 provides the rationale and the protocol of the Dutch Parkinson Cohort (DUPARC) study of de novo PD subjects who are treatment-naive at baseline. The DUPARC study is a longitudinal study aimed at deeply phenotyping PD subjects with a focus on three non-motor symptom domains: cognition, vision and gastrointestinal function. For the latter, several concrete objectives were formulated regarding the gut microbiome in PD, including (1) to interrogate the gut microbiome composition of treatment-naive PD subjects compared to healthy control subjects (HC), (2) to determine the effect of dopaminergic medication on gut microbiome composition in PD, (3) to assess possible confounding influences on gut microbiome changes in PD, in particular of dietary habits and constipation, and (4) to investigate gut microbiome composition changes in relevant PD subtypes. Data from the DUPARC cohort is used in the chapters 4-6 to assess the gut microbiome before and after initiation of dopaminergic treatment and to assess clinical and imaging markers in relation to the alpha-synuclein (aSyn) Origin and Connectome (SOC) model.

Chapter 4 describes our gut microbiome study in two large cohorts of treatment-naive de novo PD subjects compared to HC. Fecal samples were analyzed from PD subjects and HC from the DUPARC cohort and from a Finnish cohort consisting of several subsudies. Due to the geographic and methodological differences, both cohorts were analyzed separately. Although none of the findings could be directly replicated, the gut microbiome of PD subjects was characterized by reduced abundances of short chain fatty acids (SCFA) producing taxa in both cohorts, in particular taxa belonging to the family Lachnospiraceae. Also, one amplicon sequence variant (ASV) belonging to Akkermansia muciniphila was increased in one of the PD cohorts. Compared to previous studies, fewer taxonomic differences were found. This suggests a contribution of PD medication and/or disease progression to the gut microbiome changes reported in previous studies. In addition, adequate adjustment for potential confounders, including stool frequency and consistency, and technical batch effects, is required to disentangle disease associated gut microbiome changes from epiphenomena in PD.

Chapter 5 describes the influence of dopaminergic medication on the gut microbiome composition in PD. DUPARC participants who have collected a stool sample at baseline and at the one-year follow-up visit, were included if they have initiated dopaminergic treatment before the follow-up visit. A paired analysis before and after treatment initiation revealed distinct and dose-dependent effects of dopaminergic medication on gut microbiome composition. Interestingly, levodopa and dopamine agonists exerted differential effects on gut microbiome composition. The findings were mostly in line with previous studies contrasting already treated PD subjects with HC, suggesting previous results might be tainted by the effect of dopaminergic medication. At the family level, these included reduced levels of Lachnospiraceae and Ruminococcaceae with increasing levodopa dosage, and increased levels of Bifidobacteriaceae after treatment initiation with pramipexole. At the genus level Lachnospiraceae ND3007 group was inversely related with levodopa dosage. Moreover, reduced levels of a strain belonging to Faecalibacterium prausnitzii and several taxa belonging to the family Lachnospiraceae were found. Although several other PD associated gut microbiome
changes could not be replicated with statistical significance after correction for multiple testing, they did show trends in line with previous studies. These included increased levels of the genera Lactobacillus and Bifidobacterium after initiation of dopamine agonist therapy, and reduced levels of Prevotella after treatment-initiation in the entire study population. Integration of these findings with chapter 4 suggest that PD is already characterized by gut microbiome changes before treatment initiation, including reduced levels of several taxa belonging to the family Lachnospiraceae. Whereas reduced levels of Lachnospiraceae might be further aggravated by the dopaminergic treatment, other associations solely depend on the effect of dopaminergic medication.

Chapter 6 explores the clinical and imaging characteristics of the body-first and brain-first subtypes, as proposed by the SOC model, in relation to asymmetry of the dopaminergic deficit. Whereas probable body-first PD (pPD-body) and probable brain-first PD (pPD-brain) is defined by respectively the presence or absence of polysomnography (PSG) proven REM sleep behavior disorder (RBD), they are also characterized by respectively more symmetric and asymmetric involvement of brain hemispheres. Here, we have dichotomized the DUPARC cohort in symmetric PD (PD-sym) and asymmetric PD (PD-asym) by including participants in respectively the lowest and highest tertile of asymmetry of the striatal dopaminergic deficit as measured by FDOPA-PET at the time of diagnosis. Clearly, this limits the interpretation of our findings as no direct associations with pPD-body and pPD-brain can be made. Nonetheless, several hypotheses of the SOC-model can be assessed. For instance, the asymmetry of neurodegeneration should mimic the asymmetry of the dopaminergic deficit in PD-asym, and PD-sym should be associated with more autonomic failure. If contradicted, this would provide a strong argument against the SOC-model. Contrasting PD-sym and PD-asym actually seems to result in a relative clinical overrepresentation of respectively pPD-body and pPD-brain, as PD-sym was associated with more (autonomic) non-motor symptoms with a probable neurological substrate below the substantia nigra. No differences in brain region volumes and asymmetry were found using structural (T1) MRI data after correction for multiple testing. Therefore, the possibility remains that differential neurodegeneration, as predicted by the SOC-model, might be present and detectable with different imaging modalities and/ or longitudinal follow-up of PD-sym and PD-asym.

Gut micro biome in PD

The current thesis mainly advances our knowledge of gut microbiome perturbations in PD by disentangling PD related changes from possible medication effects. Whereas the gut microbiome of treatment-naïve de novo PD subjects was already characterized by a reduction of SCFA producing taxa, in particular belonging to the family Lachnospiraceae, previously reported changes in Bifidobacterium, Lactobacillus and Prevotella seem to be related to PD medication. Clearly, several other advances in PD gut microbiome research are required to explore the significance of gut microbiota in PD, as suggested in our systematic review. Concurrent studies involving meta-analyses of existing data and new metagenomic and metabolic assessments of gut microbiota in PD, allow for a more comprehensive interpretation of our findings. Three meta-analyses of publicly available gut microbiome data in PD have been performed. Although only a limited number of previously reported associations were robustly detected, these still included reduced levels of SCFA-producing taxa belonging to Lachnospiraceae and increased levels of Akkermansia. In addition, intervention trials using probiotics have been performed, aimed at the treatment of constipation in PD. Also, the utility of gut microbiome changes for the diagnosis of PD has been assessed in both taxonomic and shotgun metagenomics studies.

Putative influence of the gut microbiome on PD pathology

SCFAs are the product of bacterial fermentation of dietary fibers and are associated with gut health through their anti-inflammatory properties. As such, reduced levels of SCFAs are not specific for PD and are also found in other disorders including inflammatory bowel disease (IBD). Interestingly, IBD is associated with an increased risk of developing PD later in life. Genetic risk factors for PD have pleiotropic properties with IBD, suggesting a shared genetic background between both disorders. Weaker associations were found between genetic loci associated with celiac disease, whereas opposite associations were found for rheumatoid arthritis and psoriasis. In addition, gut inflammation during a noro-virus infection is associated with increased expression of alpha synuclein (aSyn) in gut wall biopsies of gut transplant recipients. Also, aSyn has chemotaxic properties, resulting in the migration of monocytes, neutrophils and the maturation of dendritic cells. All in all, this suggests a strong association between gut inflammation and PD, with reduced SCFA-levels as possible contributing mechanism. In addition to indirect inferences from gut taxonomy studies, direct measurements of gut microbial metabolites and shotgun metagenomics in PD have also confirmed reduced levels of SCFAs in PD. Also, reduced levels of genes involved in the metabolism of plant-based polysaccharides have been described in PD, indicating a reduced SCFA production capacity. Shotgun metagenomics of the gut microbiome in PD further details a shift to a pro-inflammatory state. Besides reduced SCFA-producing taxa and pathways, pro-inflammatory lipopolysaccharides (LPS) and Gram-negative species were enriched in PD. In addition, gene families responsible for the production
of the Gram-positive pro-inflammatory lipoteichoic acid (LTA), and bacterial lipoprotein (BLP) were increased. However, these associations are either based on already treated PD subjects or subjects with an unknown treatment status. Confirmation of these findings through metabolomic and/or shotgun metagenomic analysis of stool samples of our treatment-naïve de novo PD cohort would further increase our understanding of the role of gut inflammation in PD.

Another robustly replicated finding is an increase in the genus Akkermansia and several lower-level taxa. In our treatment-naïve de novo PD microbiome study, one amplicon sequence variant (ASV) belonging to Akkermansia muciniphila was also increased in PD. However, no trend in the abundances of the genus Akkermansia and the family Akkermansiacaeae could be discerned in our study of treatment-naïve de novo PD subjects or after treatment initiation. Therefore, our studies can only confirm that strains belonging to Akkermansia can already be elevated in de novo PD. The mucin degrading properties of Akkermansia, in particular its species muciniphila, are proposed as pathological mechanisms in PD.17 Mucin is an integral part of the gut wall and degradation of the mucin layer can increase gut wall permeability.18 Besides gut inflammation, increased gut wall permeability has also been associated with PD and is believed to increase the susceptibility of PD subjects to possible pathogens within the gut.19 Two recent PD microbiome studies using shotgun metagenomics showed enrichment of amino acid degradation and proteolytic pathways.20 These pathways included a pathway involved in the degradation of threonine, which includes mucin degradation. Again, these findings are derived from already treated PD subjects or PD subjects whose treatment status is unknown, and require validation in a treatment-naïve de novo PD cohort.

Evaluating the disease modifying potential of the gut microbiome in PD

Disease modifying therapies in PD would require the modification of the pathology after its initiation, even in probable prodromal PD subjects. Unfortunately, experimental data from preclinical studies often cannot be translated to clinical PD.21 This is in part due to the study design in which the endpoint is often the creation and severity of a given phenotype. This phenotype is then often rescued by applying the experimental treatment before or during the stimulus that would otherwise culminate in the phenotype of interest. As such, it is not possible to extrapolate the findings of these studies to already established disease, as it is unclear to what extent the phenotype is actually rescued or that the stimulus is attenuated by the treatment. In addition, the provided stimulus in animal PD studies needs to be well-chosen.22 Broadly, animal models in PD rely either on toxins directed at dopaminergic neurons, such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), genetic variants, including SNCA mutations and overexpression, or initiate aSyn aggregation using for instance injections with preformed fibrils or peripheral application of toxins such as rotenone. Of these, the toxin-based models directly targeting dopaminergic neurons greatly mimic the motor symptomatology of PD, but not its pathology and are therefore not suitable to address questions on disease progression. Instead, models initiating aSyn pathology through seeding with preformed fibrils, seem to induce a self-propagating pathology.23 In particular, gastrointestinal injections of aSyn fibrils result in a spreading pattern along the vagal nerve, mimicking the spreading pattern and symptomatology in PD.24 A similar pattern is produced after intragastric administration of the pesticide rotenone, also suggesting a self-propagating pathology. Moreover, the rotenone model would be of additional interest since exposure to pesticides is associated with an increased risk of developing PD.25 Unfortunately, most preclinical studies investigating the therapeutic potential of bacterial strains in PD, are based on stimuli that are not representative of PD pathology, or apply the intervention before the disease-initiating stimulus, or do both. For example, a recent rodent study investigated the neuroprotective effects of deficiency of the Toll-like receptor 4 (TLR-4) signaling pathway, the pathway through which LPS exert their pro-inflammatory effects. TLR-4 deficiency was established through a genetic knockout in mice that received MPTP to establish a PD-like phenotype. Therefore, the TLR-deficiency was already present before the stimulus was provided, whereas the stimulus does not lead to a pathology representative for PD as a whole. As such, conclusions can be drawn about the influence of TLR-4 on the toxic effects of MPTP, but cannot be extrapolated to a disease modifying potential. Similarly, an influential study by Sampson et al. showed critical interactions between gut microbiota, aSyn and associated neuro-inflammation in an aSyn overexpression rodent model.26 Germfree (GF) mice, that did not harbor a gut microbiome, showed far fewer symptoms and pathology after 12 weeks, compared to mice that did harbor a gut microbiome. However, the critical interactions between gut microbiota and aSyn again do not provide evidence for a disease modifying effect after initiation of the pathology. Even though the authors swapped the group status of GF and non-GF mice after five weeks through colonization with mice microbiota and antibiotic treatment in an additional experiment, it remains unclear if the pathology was already present by then. Moreover, genetic overexpression of aSyn provides a continuous stimulus towards aSyn aggregation. Even if an attenuating effect of microbial depletion on the phenotype and pathology after initiation of aSyn aggregation is found, this can still be due to an interaction at the initiation stage of the pathology. Lastly, the human immune system greatly differs from that of rodents,27 which might explain the positive correlation between SCFAs and aSyn pathology in the study by Sampson et al., contrary to the inverse associations found in human studies. To conclude, preclinical models suitable for monitoring the progression of PD should be used to assess the disease modifying potential of gut microbiota after initiation of the pathology.

Nonetheless, the proposed mechanisms through which gut microbiota influence PD pathology can help to assess their disease modifying potential in PD. Besides the aforementioned decrease in anti-inflammatory properties and increase in gut wall permeability, bacterial amyloids are also implicated in the pathogenesis of PD.28,29 Bacterial amyloids are proteins with a propensity to aggregate. Most prominently, the bacterial amyloid curli is capable of cross-seeding aSyn and thereby initiating Lewy body pathology.30 Although a continuous...
contribution of newly initiated αSyn aggregation in the gut might contribute to disease progression, this seems negligible given the self-propagating nature and central nervous system involvement of the αSyn pathology in established disease. Therefore, therapies directed at reducing bacterial amyloids do not seem prime targets for disease modification in PD. In contrast, inflammatory factors within the gut exert systemic effects, including neuroinflammation, which increases neuronal vulnerability to αSyn pathology. In addition, increased gut wall permeability increases exposure of the host to inflammatory factors within the gut. Shifting the gut microbiome to a more anti-inflammatory state might therefore still influence disease progression in established PD. Interventions in line with the philosophy of selective digestive decontamination (SDD) can be envisioned. SDD is used to prevent infectious disease in critically ill patients through an antibiotic regimen aimed at eliminating potentially pathogenic Gram-negative bacteria, whilst not affecting potentially favorable microbes. Clearly, an antibiotic regimen would be less suitable for disease modification in PD. The antibiotic regimen in SDD is short, given once, and potential antibiotic resistance is offset by the prevention of additional infections requiring more extensive antibiotic treatment. These characteristics seem not applicable to a disease modifying course of antibiotics in PD. Instead, positive selection mechanisms might be employed through increased intake of plant-based polysaccharides, in particular dietary fiber, to increase anti-inflammatory SCFA production. An additional benefit could be a shift away from proteolytic bacteria, using protein as their main source of energy, towards species using polysaccharides as main source of energy. Since proteolytic species might use protein content from the host, including mucin, a reduction of proteolytic species might increase gut wall integrity. However, increased dietary fiber intake does not always lead to increased SCFA production, dependent on the type and quantity of dietary fiber used. Moreover, a relative increase in species using polysaccharides might not necessarily lead to a concurrent reduction of proteolytic species, in particular in the mucosal microenvironment where mucin degradation takes place. Gut microbiome interventions for PD symptomatology

Gut microbiota might also play a role in the symptomatic treatment of PD by reducing constipation and increasing the bioavailability of orally administered levodopa. Several studies have investigated the effect of probiotic strains on gastrointestinal complaints in PD and all found a reduction of gastrointestinal complaints, including constipation, bloating and incomplete defecation. Most probiotic strains that were investigated belonged to the genera Lactobacillus and Bifidobacterium, which are decreased in participants with idiopathic constipation. However, gut microbiome changes in patients with idiopathic constipation seem to be different from the gut microbiome changes found in PD, although no direct comparison between PD and idiopathic constipation is available. The levels of Lactobacillus and Bifidobacterium even seem opposite, as increased levels of both genera in PD are among the most replicated findings of gut microbiome studies in PD. Additional supplementation with Lactobacillus and Bifidobacterium therefore seems contra-intuitive, unless higher levels of these two genera are a compensatory mechanism, do not reflect higher levels of the specific strains used in probiotic studies, or are related to PD medication. Both genera have indeed been associated with disease progression and levodopa dosage. In addition, a mendelian randomization study concluded the relationship between Bifidobacterium and PD to be a result of reverse causation. Our follow-up study both genera showed an increasing trend after initiating treatment with a dopamine agonist, further strengthening the idea of reverse causation, in particular due to treatment effects. It is therefore less likely for supplementation with Lactobacillus and Bifidobacterium to have a detrimental effect on PD pathophysiology, making these probiotics safe options for the treatment of gastro-intestinal complaints in PD.

Another study found reduced motor symptoms after treatment with a probiotic mix containing four strains belonging to Lactobacillus and Bifidobacterium. Whereas improved motor scores can possibly be explained by improved gastro-intestinal motility and thereby an increased uptake of PD medication, there might also be a direct interaction between gut microbiota and levodopa. Levodopa is a precursor of dopamine which is capable of passing the blood-brain-barrier, after which it gets decarboxylated to dopamine. To prevent the conversion of levodopa outside of the brain, levodopa therapy is supplemented with decarboxylase inhibitors. Nonetheless, bacterial decarboxylases can convert levodopa in the gut, despite the presence of a decarboxylase inhibitor, thereby decreasing the bioavailability of levodopa in the brain. Tyrosine decarboxylase genes (tdc), and genes relevant for further metabolism of dopamine, are present in the genome of strains belonging to Lactobacillus, Enterococcus and Eggerthella. Except for a trend with higher levels of Lactobacillus none of these taxa were in our follow-up study after treatment initiation. A cross-sectional study found a positive correlation between tdc abundance in feces and levodopa dosage, whereas a longitudinal study found correlations with other PD medications, but not levodopa. Possibly, a direct measurement of tdc in fecal samples of our treatment-naïve de novo PD cohort can reveal trends of positive selection over time.

The gut microbiome as diagnostic marker of PD

Besides an increasing body of knowledge concerning the possible influence of the gut microbiome on the pathophysiology and symptomatology of PD, the gut microbiome might also serve as a diagnostic marker. Several studies have tried to differentiate PD from HC based on taxonomic gut microbiota differences, yielding an area under the curve around 0.82. However, the utility of these markers is questionable, as an independent validation cohort was lacking, there is no clear added value to differentiating PD from HC, and the included PD subjects already had established PD. Ideally, the performance of a diagnostic marker should be tested around the time of diagnosis, against relevant differential diagnoses and validated in a separate cohort. Our gut microbiome study in treatment-naïve de novo PD subjects would fit two of these criteria. However, we performed no formal analysis to differentiate between PD and HC, as none of the findings could be directly replicated between the two cohorts and fewer
differences were found compared to studies using already treated subjects. Therefore, we concluded that taxonomic differences down to genus level are unlikely to provide an adequate diagnostic marker. Especially considering the fact that relevant differential diagnoses of PD, like multiple system atrophy (MSA) and progressive supranuclear palsy (PSP), share similar gut dysbiosis compared to PD.45 One shotgun metagenomic study identified marker genes, rather than taxonomies, and outperformed previous studies in differentiating between PD and HC (AUC 0.896). The marker genes were validated in separate cohorts, including MSA and Alzheimer’s disease (AD) subjects, resulting in an AUC of 0.83 and 0.90, respectively. Nonetheless, the study concerned almost exclusively already treated PD subjects with an average disease duration around 7 years for both the discovery and validation cohorts, making it impossible to adequately account for the effects of disease progression and treatment. Therefore, shotgun metagenomics sequencing in treatment-naïve de novo PD subjects would still be of added value to discover and validate gut microbiome markers for the diagnosis of PD.

The gut as site-of-origin in body-first Parkinson’s disease

The SOC-model integrates pathological and clinical evidence to predict clinical progression in PD based on a probably site of origin, resulting in the proposed body-first and brain-first subtypes. Given the different origins of the pathology in both subtypes, RBD present before motor onset has been used by Borghammer et al. to define probable body-first PD, as one of the neurological substrates of RBD – the locus coeruleus, would be affected before the substantia nigra.46,47 However, isolated RBD (iRBD) might also not provide a perfect classification of body-first and brain-first PD. First, RBD might not be present in all probable body-first PD subjects, as REM sleep without atonia might not be seen on polysomnography (PSG) or the loss of atonia is not associated with dream enactment behavior.48 Second, dream enactment is also present in other disorders, including narcolepsy and drug-induced (eg. selective serotonin reuptake inhibitors) dream enactment.49 Last, aSyn pathology might arise in the substantia nigra or the locus coeruleus, making it impossible to establish the chronology of RBD and motor symptoms.50 The main driver behind the different clinical pictures of body-first and brain-first PD is the asymmetry of the pathology.51 In body-first, the pathology is hypothesized to originate in the gut. Based on preclinical data, the neuronal innervation of the gut is cross-linked and pathology would thus spread along both the left and right vagal nerve and result in symmetric involvement of both hemispheres.52 In contrast, brain-first PD is hypothesized to originate in one hemisphere and subsequently spread mainly within the same hemisphere, as intra-hemispheric neuronal connections greatly outnumber inter-hemispheric connections.53 Indeed, the dopaminergic deficit as measured by FDOPA-PET is more symmetrically affected in RBD-positive than RBD-negative PD subjects at the time of diagnosis.54 Given the continuing depletion of striatal dopaminergic terminals, the asymmetry of the dopaminergic deficit on FDOPA-PET is expected to disappear within a few years after diagnosis, when a floor effect is reached.55 The DUPARC cohort therefore provides a unique opportunity to assess several hypotheses posed by the SOC-model by comparing participants with the most and least asymmetric dopaminergic deficit at the time of diagnosis. According to the SOC-model, this would lead to enrichment with probable brain-first and body-first PD subjects, respectively. Clearly, dopaminergic asymmetry remains a cruder classifier than PSG-proven RBD, especially in the absence of prevalence estimates of body-first and brain-first PD. Associations might therefore be obscured as the two groups are not optimally enriched. Also conceptually, the interpretation of our findings is limited, as only a relation with dopaminergic asymmetry can be established, rather than the probable body-first and brain-first subtypes. At the most, several trends in our dataset seem to be in line with the SOC-model, whereas none of our findings contradicted the SOC-model. These include more non-motor symptoms with a probable neurological substrate caudal from the substantia nigra in symmetric PD, and several brain regions with lower volumes in the most affected hemisphere in asymmetric PD. However, the differences in brain region volumes were not statistically significant after correction for multiple testing. Further assessment of the possible origin of the brain-first subtype, objective markers of symptomatology, different imaging modalities, and integration with gut microbiome research are required to direct future research concerning the SOC-model.

Brain-first PD

The body-first subtype is relatively well-defined by means of PSG-proven RBD and has a probable site-of-origin in the gut, based on the clinical and pathological progression of prodromal PD. In contrast, the brain-first subtype is a category by exclusion in the absence of PSG-proven RBD. It is therefore unclear to what extent brain-first PD represents a homogenous subtype with a well-defined site-of-origin. Two of the earliest classification systems of Lewy-related pathology (LRP) were proposed by Braak et al. and McKeith et al.56,57 Braak described a caudo-rostral staging system with pathology starting in the dorsal motor nucleus of the vagal nerve, from where it sequentially spreads towards the locus coeruleus, the substantia nigra, the basal forebrain and the neocortex. In addition, the olfactory bulb pathology precedes either a brainstem predominant or limbic predominant classification.58 More recently, two patterns of LRP spreading were proposed in a post-mortem study in the elderly population with an amygdala-based and caudo-rostral progression.59 The above classification systems are not based exclusively on PD subjects and include subjects with dementia with Lewy bodies (DLB), subjects with Alzheimer’s pathology in addition to LRP and non-affected individuals. Nonetheless, according to the SOC-model, the different distribution patterns of LRP is informative of possible sites of origin, in particular since LBD and PD seem to be part of a spectrum and are only differentiated based on the chronology of cognitive and motor symptoms.60 Intuitively, an amygdala-centered pattern seems to be related to early cognitive decline. However, autonomic dysfunction and RBD are also associated with a higher risk of dementia, suggesting a relation with the body-first subtype.61 As such, PD and
Recently, the open-implemented. In our study, objective assessment of olfactory function was performed as a crude measure of non-motor symptoms. Ideally, more objective assessments should be applicable, self-administered questionnaire, that allows for clustering of symptoms with the Movement Disorders Society Non Motor Symptom Questionnaire (NMSQ).

Non-motor symptom assessment in our study was performed by means of the Movement Disorders Society Non Motor Symptom Questionnaire (NMSQ) and brainstem-proven RBD to classify body-first PD. One of the limitations of our analysis contrasting symmetric and asymmetric PD, could be the use of T1 structural MRI in de novo PD subjects. Structural brain atrophy might occur relatively late in the neurodegenerative process, as neurons are hypothesized to degenerate in a “dying-back-fashion” in PD, starting at the axonic terminals. In the DUPARC cohort, cholinergic brain imaging with FEBV-PET scanning is used to characterize PD participants. FEBV is a radio tracer of the vesicular acetylcholine transporter that allows for the assessment of the axonal integrity of cholinergic projections in PD. Differential cholinergic denervation between asymmetric and symmetric PD suggestive of the brain-first and body-first subtype might be visualized using FEBV, as cholinergic denervation is already present in de novo PD. However, interpretation of functional PET imaging is complicated by the possibility of compensatory mechanisms. Another imaging modality we suggested to be more suitable for early detection of differential denervation is diffusion-weighted or diffusion tensor imaging (DWI/DTI), which allows for the assessment of microstructural abnormalities and tractography of white matter. A recent study contrasted RBD subjects with PD and HC. Interestingly, RBD was characterized by more pronounced damage to the cerebellar peduncles and brainstem compared to PD. Since the PD group was not assessed for the presence of RBD, it is likely that the PD group contained both RBD-positive and RBD-negative subjects.

Suggestions for future research

Future research can further assess the SOC-model by implementing different classification methods, more objective non-motor symptom assessments, and imaging modalities more appropriate for early phase PD. As described above, PSG-proven RBD is the gold standard to identify probable body-first subjects. Given that PSG is a cumbersome method for both researchers and participants, different methodologies should be validated against PSG for the detection of RBD. Questionnaire assessments of RBD do not provide the diagnostic power of PSG, are considered invalid for de novo PD, and have a greatly reduced hazard ratio for the prediction of phenoconversion to PD in probable prodromal PD.

Recently, the open-source software RBDtector was developed to identify REM sleep without atonia based on PSG. Reliable software solutions should ideally be integrated with wearable or self-applicable sensors for the assessment of RBD at home. Non-motor symptom assessment in our study was performed by means of the Movement Disorders Society Non Motor Symptom Questionnaire (NMSQ). Although this is an easily applicable, self-administered questionnaire, that allows for clustering of symptoms with a probable neurological substrate caudal to the substantia nigra, the NMSQ is a very crude measure of non-motor symptoms. Ideally, more objective assessments should be implemented. In our study, objective assessment of olfactory function was performed using Sniffin’ Sticks. However, more objective assessments of non-motor symptoms are available, including a standard clinical assessment of orthostatic hypotension and colonic transit time assessments by counting sequentially ingested radiopaque markers on abdominal imaging. In the DUPARC dataset a stool diary, measuring stool consistency and frequency, provided a more objective measurement of constipation compared to the NMSQ-related items. Nonetheless, we deemed the absence of a statistically significant difference (data not shown) as not informative. Even though stool frequency and consistency were relevant variables that were adjusted for in the de novo PD gut microbiome study, they did not differentiate between PD and HC, despite a statistically significant difference (data not shown). In contrast, Knudsen et al. showed that colon transit time can differentiate between PD and HC. Therefore, objective markers of non-motor symptoms, in particular stool transit time as measure of constipation, might reveal otherwise obscured differences in non-motor symptomatology between symmetric and asymmetric PD. However, it might still be possible that the constellation of non-motor symptoms, rather than individual non-motor symptoms, is a better proxy of body-first associated symptomatology.

In line with the suggestion to objectively measure non-motor symptoms, an assessment of the underlying neurological substrate might show ascending denervation along the enteric nervous system, the vagal nerve and sympathetic nerves in body-first PD. Multimodal imaging could entail the use of 13C-donepezil for intestinal innervation, 18F-MIBG scintigraphy for cardiac innervation, and neuromelanin-sensitive MRI for the locus coeruleus. These imaging modalities were all deployed in the study used to define body-first and brain-first PD by Horsager et al. Besides the relation between peripheral denervation and asymmetry of the dopaminergic deficit, these imaging markers might also provide alternatives to PSG-proven RBD to classify body-first PD.
Gut microbiome changes in body-first versus brain-first PD

Gut microbiome changes can be present in both the brain-first and the body-first subtype, as microbiota-gut-brain signaling is bidirectional. Nonetheless, according to the SOC-model, gut microbiome changes might be more apparent in the body-first subtype, as the early gastrointestinal involvement provides additional triggers for compositional changes and gut microbiota might exclusively play a role in the etiology of body-first PD. To date, Heintz-Buchart et al. performed the only comparison of gut microbiome composition between probable body-first and probable brain-first PD. The authors compared PSG-proven RBD-positive and RBD-negative PD subjects and found higher levels of Akkermansia and Prevotella in RBD-positive PD. The same study also investigated the gut microbiome of iRBD subjects, which showed similar deviations as the PD group. Interestingly, no differences in the abundances of Akkermansia and Prevotella were found between iRBD and PD subjects, suggesting these differences are independent of PD medication in RBD-positive PD. One other study investigated the gut microbiome of iRBD, including a meta-analysis with data from Heintz-Buchart et al., and also found increased abundances of Akkermansia. However, no evidence was found for a reduction of SCFA producing taxa. This challenges the idea that reduced SCFA production contributes to the etiology of PD, but instead SCFA production potential might decline as prodromal PD progresses to PD. Nonetheless, shotgun metagenomic sequencing or metabolomics analysis in iRBD is still lacking and could provide a direct assessment of the SCFA production potential in iRBD subjects. Moreover, sample sizes of iRBD microbiome studies thusfar have been rather low (n=21 and n=26) and require replication in larger cohorts.

The DUPARC dataset could provide additional data on gut microbiome composition contrasting probable body-first and probable brain-first PD. The already available gut microbiome data can be compared between symmetric and asymmetric PD as defined in chapter 6 to assess whether our findings in chapter 4 are related to a subgroup of treatment-naive de novo PD subjects. However, this will reduce the sample sizes to a third of the original DUPARC microbiome dataset and intra-PD differences might be lower. Possibly, higher resolution data or direct functional readouts of the gut microbiome might be required to assess differences between symmetric and asymmetric PD.

Towards disease modifying treatment in PD

Gut microbiome alterations might contribute to PD pathology and are therefore a potential therapeutic target for disease modifying trials in PD. Based on our findings and recent literature, most evidence points towards a concurrent reduction in anti-inflammatory and increase in pro-inflammatory components, as well as degradation of the gut wall barrier function. However, as described above, the assessment of proposed therapeutic targets through mechanistic research in preclinical models is often not adequate, as these models are often not suitable to translate to already established pathology in (prodromal) PD. This is in part due to the use of non-compatible pathological stimuli, and initiation of the rescue therapy before or during application of the pathological stimulus. Development of preclinical studies designed to assess disease progression in PD will allow for a better selection of viable therapeutic targets. Simultaneously, translational efforts in clinical trials aimed at disease-modification have all failed so far, which means there is no disease-modifying treatment for PD to date. Failed translation might in part be attributable to the lack of adequate preclinical endpoints to measure disease progression. However, the clinical heterogeneous progression of PD poses another reason for failed translation, as large clinical trials are required to provide evidence of clinical efficacy. In case of underpowered clinical trials, the absence of an effect renders the trial inconclusive, as an effect cannot be proven nor rejected. This section explores the sample size requirements of clinical trials aimed at disease modification. Currently active clinical trials identified at ClinicalTrials.gov are assessed for their primary outcome and sample size. Suggestions to decrease sample size requirements are provided, including adequate subtyping in line with the SOC-model.

Sample size requirements for disease-modifying trials in PD

Ideally, a disease-modifying effect is established by showing a slower clinical progression of PD. The most used clinical scale for measuring the symptomatic burden in PD is the Movement Disorders Society Unified Parkinson Disease Rating Scale (MDS-UPDRS), consisting of four parts: I) non-motor aspects of experiences of daily living, II) motor aspects of experiences of daily living, III) motor examination, IV) motor complications. In particular, part III seems to be a valid assessment of the progression of motor symptomatology, as a standardized motor examination is expected to worsen over time in PD, whereas short-term test-retest reliability of the MDS-UPDRS is adequate. Moreover, part III can be performed in the practically defined OFF-state: a predetermined amount of time before the motor examination during which no dopaminergic medication can be used, to limit the confounding effect of dopaminergic treatment. Data on the natural progression of PD is required to assess the utility of the MDS-UPDRS part III as progression marker. For this purpose, the Parkinson’s Progression Markers Initiative (PPMI) was founded, a multicenter study aimed at finding markers of disease progression. The MDS-UPDRS part III in the practically defined OFF-state (>6 hours after the last dose of dopaminergic medication in the PPMI protocol) was part of the clinical assessment. Holden et al. performed sample size...
estimates based on the 12-month follow-up visit during which 318 participants had completed an MDS-UPDRS part III examination of which 195 participants had started dopaminergic treatment and 123 were still treatment-naïve. The treatment-naïve group had an increase (mean ± standard deviation) in MDS-UPDRS part III scores of 6.35 ± 6.66 points, whereas those who had initiated dopaminergic treatment had an increase of 1.54 ± 9.0 points. Sample size estimates were based on the untreated group and suggested that a complete halting of progression would be detectable after one year with 90% power, when including 34 participants per treatment arm. A more modest reduction of 37% of progression would require 240 participants per treatment arm. However, the use treatment-naïve PD subjects would not be sustainable, as abstinence of symptomatic treatment will become undesirable or even unethical in most participants. No sample size calculations were provided based on the data of the treated group, but when performing our own sample size calculations using the data of Holden et al., a complete halting of progression would only be detectable with 90% power when including 718 participants per treatment arm. A 37% reduction of progression would even require over 5000 participants per treatment arm. Also, the predicted annual increase of MDS-UPDRS part III scores, based on a follow-up period of five years, differed greatly between medicated and unmedicated subjects, with an increase of 1.77 and 4.02 points, respectively. Therefore, dopaminergic medication clearly increases clinical heterogeneity and thereby sample size requirements for clinical trials aimed at disease modification using the MDS-UPDRS part III in the practically defined OFF-state. Power calculations for phase III clinical trials aimed at establishing efficacy in PD are often based on the results of smaller phase II clinical trials that provided a proof of concept. Considering the progression of MDS-UPDRS part III scores, sample size calculations based on smaller phase II trials should be critically reviewed. Especially if the trial shows a reversal of progression, whereas the mechanism under investigation would only slow down disease progression, the trial design should be reviewed for differences in baseline characteristics, (unintended) unblinding due to adverse effects or a concurrent symptomatic effect of the treatment.  

Current disease modifying trials in PD

A search at the trial registry ClinicalTrials.gov for phase III interventional trials in PD that are not terminated (either not yet recruiting, recruiting, enrolling by invitation or are active, but not recruiting) using the term “Parkinson Disease” resulted in 32 trials (December 2022). Manual curation of these trials, based on a formulated aim regarding disease modification or a primary outcome tracking disease progression, resulted in 11 remaining studies aimed at disease modification, of which 8 were including PD subjects. The medical interventions included traditional Chinese herbal medicine, inhibition of αSyn transmission and reducing mitochondrial damage, among others. Of these 8 PD trials, 6 used the MDS-UPDRS part III as (part of the) primary outcome. Other primary outcomes were the Rey auditory verbal learning test (RAVLT) and the MDS-UPDRS part II. To our knowledge, the RAVLT is a cognitive assessment that has not been validated as a marker of progression in PD, whereas the slope of the MDS-UPDRS part II over time is less steep than that of part III, making it a less suitable progression marker. Not all studies mentioned whether motor examination would take place in a practically defined OFF-state. Duration of treatments versus placebo ranged from three months up to 180 weeks, including trials with a delayed start design. Sample sizes per study arm ranged from 25 to 200. All studies aimed at including already treated PD subjects. Therefore, when assuming 37% reduction of disease progression over one year, the aforementioned sample size of 240 participants per treatment arm provides a very optimistic estimate, since the inclusion of already treated subjects likely increases sample size requirements, as described above. Three trials might still be viable given this effect size, as their duration of differential treatment exceeds one year. However, with sample sizes ranging from 90 to 200, it remains questionable whether the longer exposure time will offset the additional variability in clinical progression due to dopaminergic treatment.

Reducing sample size requirements

Sample size requirements for disease modifying trials in PD might be reduced by decreasing variability in the assessment of PD subjects or by reducing the clinical heterogeneity of the study population by means of subtyping. First, although the MDS-UPDRS part III in the practically defined OFF-state is the most used primary outcome of disease-modifying trials in PD, further validation is required. Improvements compared to the PPMI dataset can be made, including using fewer raters and defining a stricter OFF-state with a longer duration since the last dosage of dopaminergic medication. A study aimed to disentangle noise (i.e. error variance), from long-lasting differences in progression rates (i.e. true variance) in the PPMI dataset, indeed found a higher true variance and lower error variance in OFF-state assessments >14 hours postdose. However, attaining a complete washout of the dopaminergic response to for instance levodopa seems unrealistic, as levodopa has a long-duration response, lasting several days, in addition to the short-duration response. Also, subscores of relevant items of the MDS-UPDRS might be used instead of the total scores, as factors related to gait, mobility and resting tremor also showed a favorable profile with relatively low error variance. However, the temporal progression of individual motor symptoms should be taken into account. Progression of dopamine-responsive symptoms might be more apparent in the OFF-state during the later stages of PD, after the so-called honeymoon period, during which early phase PD subjects often have a broader therapeutic window. In contrast, dopamine-resistant tremor might reach a floor-effect after a few years and could therefore be of particular relevance for measuring progression in early PD.  

Second, in addition to the MDS-UPDRS part III, other clinical endpoints might also show a measurable progression over time. One of the trials found on ClinicalTrials.gov used a marker of cognition, the RAVLT. Even though no validation of the RAVLT as progression marker could be found, measuring cognitive performance and the associated heterogeneity over time, might lead to a valid progression marker by means of a neuropsychological test battery. Moreover, cognitive decline is one of main worries of PD patients expressed in the clinic,
making cognitive decline an impactful endpoint. Third, in addition to the assessment of progressive non-motor symptoms, efforts are made to provide more objective measurements of motor symptomatology. In particular, resting tremor, gait and mobility seem suitable candidates due to their relatively low error variance. Wearables tracking tremor or insoles tracking gait speed and stride might provide more accurate and reliable assessments of motor symptomatology by eliminating the variability inherent to a clinical assessment.

Last, imaging markers might provide insights in the actual pathophysiological progression without the noise associated with clinical assessments. Dopaminergic imaging has been investigated the most in this regard. Even though the decline in dopaminergic signal on for instance FDOPA-PET and DAT-SPECT scans is less variable than a clinical motor examination, there are limitations to the interpretation of functional dopaminergic imaging. Dopaminergic imaging can be confounded by compensatory mechanisms and dopaminergic medication, and shows limited correlation with clinical progression. Other imaging markers, including PD related patterns of hypo- and hypermetabolism on FDG-PET also show a correlation with disease progression, but require further validation as a progression marker. Ideally, a direct assessment of the spreading of aSyn pathology would be possible, but currently no imaging marker of actual aSyn pathology exists. Moreover, it remains unclear if aSyn aggregation on its own is enough to cause neurodegeneration in PD, and which aSyn conformation would be neurotoxic. Therefore, regardless of the proposed imaging modality, the need remains for a clinical effect to actually impact patients, caregivers and society. Clinical trials are the only ascertainment that the change in pathology, as measured by for instance aSyn imaging, also leads to a change in clinical outcome. Subsequently, imaging markers might become a stepping stone to invest in larger observational, longitudinal, monocenter studies aimed at discovering and validating clinical and imaging markers might provide insights in the actual pathophysiological progression. This would reduce the sample size by 24% for a disease-modifying trial that would slow disease progression by 50%. Regardless of disease stage, the identification of PD subtypes can reduce sample size requirements through selection of persons with PD with a more homogeneous progression of disease. PD subtypes might also represent different etiologies and study populations can be enriched based on the action of mechanism of the investigational product. Of the trials selected from ClinicalTrials.gov, only one trial defined an enriched population of PD subjects, being PD subjects with an LRRK2 mutation. Genetic subtyping enriches study populations for investigational products modulating the genetic risk factor. Moreover, genetic subtypes of PD can also be characterized by a more homogeneous disease progression. Whereas LRRK2-positive PD is characterized by a less steep increase in UPDRS III scores, the clinical progression of PD subjects with a GBA1-mutation seems to be characterized by a more rapid clinical progression. GBA1 encodes for the lysosomal enzyme glucocerebrosidase and heterozygous mutations are hypothesized to increase the risk of PD by a reduced lysosomal activity targeting misfolded and aggregated aSyn. Trials targeting glucocerebrosidase would therefore greatly benefit from the inclusion of PD participants with a GBA1-mutation. Within the scope of the current thesis, enriching trials with body-first PD subjects might increase viability of trials targeting the microbiota-gut-brain axis in PD. Gastrointestinal modulators of PD pathology will more likely contribute to body-first PD compared to brain-first PD. In addition, body-first PD is the more malignant subtype, probably due to a more symmetric spreading of aSyn pathology. Given the homogeneous spreading of pathology in body-first PD, as suggested by post-mortem studies identifying a brainstem-dominant pattern, the faster symptom progression might be accompanied by less variability. Longitudinal follow-up of body-first and brain-first PD will aid to assess the utility of both subtypes to reduce sample size requirements for disease-modifying trials in PD. Within the DUPARC cohort, the three-year follow-up could already provide valuable information in this regard, through the relative enrichment of both subtypes based on dopaminergic asymmetry at the time of diagnosis. Inclusion of IRBD subjects might provide even more favorable characteristics for disease-modifying trials in synucleinopathies. First, the clinical endpoint for measuring rate of progression would be phenoconversion. Although diagnostic uncertainty might introduce variability, it is a relatively strict endpoint compared to MDS-UPDRS III motor assessments. Especially when the diagnosis can be confirmed using biomarkers, such as dopaminergic imaging in PD. Second, IRBD would be prodromal to the body-first subtype according to the SOC-model and might therefore be characterized by a relative homogenous progression. This is confirmed by the finding that different clinical variables are associated with a different rate of phenoconversion, suggesting a relatively fixed temporal progression of symptom occurrence. Subsequently, additional stratification based on clinical variables might further decrease variability in phenoconversion rates. Third, IRBD participants will not yet use any dopaminergic medication. Therefore, dopaminergic medication would not interfere with motor symptoms, if these would be part of the primary outcome, for instance as part of the clinical diagnosis of PD. One of the largest multi-center trials assessing conversion rates of IRBD to a synucleinopathy, found a 73.3% conversion rate after twelve years follow-up, with an average conversion rate of 6.3% per year. The authors provided sample size calculations stratified for associated symptomatology for a two-year trial with a power of 80% and alpha set at 0.05, assuming a therapy with a hazard ratio of 80% and alpha set at 0.05, assuming a therapy with a hazard ratio of 0.5. Sample size estimates ranged from 157 to 366 per group. The combination of elevated UPDRS scores and hyposmia resulted in one of the lowest required sample sizes of 157 subjects per group, with an annual conversion rate of 15.7%. However, only 29% of the included IRBD subjects would still be eligible. Without stratification for other symptoms, 366 IRBD subjects per group would be required. Treatment duration was nearly proportional to reduced sample size requirements, with a one-year trial requiring 709 participants per group when only selecting IRBD subjects without further stratification. Two
phase III trials are registered at ClinicalTrials.gov aimed at detecting disease-modifying effects in P5G-proven iRBD, with the diagnosis of a synucleinopathy, or PD in particular, as primary outcomes. Differential exposure to the intervention is planned to last two years in one study and half a year in the other study, with the latter study having a delayed start design with follow-up up to three years after treatment initiation. Sample sizes are 90 and 366 subjects per treatment arm, respectively. Based on the above sample size calculation, both trials would be underpowered when assuming a 50% reduction in phenoconversion due to differential exposure. Nonetheless, selecting iRBD subjects, preferably stratified according to co-symptomatology, seems a viable strategy towards establishing a disease-modifying effect, in particular of interventions along the microbiota-gut-brain axis in PD.

Conclusion

In this thesis, the relevance of the gut microbiome in PD and the possibility of a body-first versus brain-first classification of PD were investigated. Gut microbiome studies in PD were characterized by a large heterogeneity in outcomes and methodologies. Moreover, hardly any treatment-naïve de novo PD subjects were included. The objectives of the Dutch Parkinson Cohort (DUPARC) study, a prospective cohort study of treatment-naïve de novo PD subjects, included the assessment of gut microbiome perturbations in de novo PD before and after treatment initiation. We found gut microbiome composition changes in both the DUPARC cohort and a Finnish cohort of treatment-naïve de novo PD subjects compared to HC, mainly related to reduced levels of taxa belonging to the family Lachnospiraceae and other SCFA producing taxa. Initiation of dopaminergic medication seems to aggravate the loss of Lachnospiraceae, but was also associated with increased levels of taxa belonging to Bifidobacteriaceae in a paired analysis of DUPARC PD participants before and after dopaminergic treatment initiation. In addition, we showed that PD subjects with low levels of asymmetry of the dopaminergic defect have more non-motor symptoms with a probable neurological substrate below the substantia nigra, suggestive of an overrepresentation of probably body-first PD subjects. No individual brain regions showed differential degeneration suggestive of probable body-first and probable brain-first PD after correction for multiple testing. However, the revealed trends were largely in line with the SOC-model. Different imaging modalities and longitudinal follow-up might reveal differential degeneration suggestive for the two subtypes. Translation of our microbiome findings to the clinic requires an assessment of disease modifying properties in preclinical models suitable for measuring progression of already established pathology. However, translation of preclinical findings to the clinic is also hampered by the clinical heterogeneity of PD and the subsequent sample size requirements. Sample size requirements might be reduced by using more objective markers of symptomatology and subtyping to enrich study populations with participants with a relatively homogenous disease progression. In particular, iRBD subjects might represent the prodromal stages of the body-first subtype and therefore seem to be suitable candidates to assess interventions along the microbiota-gut-brain axis in PD.

References


75. Holtermann, F. et al. Convergent patterns of structural brain changes in rapid eye movement sleep behavior disorder and Parkinson's disease on behalf of the German rapid eye movement sleep behavior disorder study group. Sleep 44, (2021).