Overview of Parkinson's Disease: Examining the Role for the Gut-Brain-Gut Axis and the Use of Nutrients Based on Preclinical and Clinical Evidence

June 2011-March 2012

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General Introduction

Parkinson's disease (PD) is first described in 1817 by James Parkinson. The monograph "An essay on shaking palsy" provided an extensive description of the clinical characterization of the disease. It is a common neurodegenerative disease and it affects approximately 0.3% of the global population and 1 to 2% of the people over 60. Globally, around 5 million people are affected by the disease and only in Europe and the United Stated 1 million people are living with the disorder. The average age of onset is 60 years and the risk of suffering substantially increases with ageing. It is estimated that the prevalence of the disease will largely augment in the coming decades.

The most common clinical symptoms of the disorder are resting tremor, bradykinesia, rigidity and gait dysfunction but recently, other non-motor features associated with the disease have been described. These features are autonomic dysfunction, mood disorder, pain and sensory disturbances, sleep impairment and dementia. It is known that clinical symptoms become present after the degeneration of at least 50-70% of the dopaminergic neurons in the Substantia Nigra pars compacta. In the last decade it has been suggested that the neurodegenerative process of PD may not start in the brain but in the periphery. This may explain why many non-motor symptoms such as constipation, anosmia and depression often precede the onset of motor symptoms by many years and their occurrence in otherwise healthy people has been associated with an increased risk of PD.

The mechanism of development of the disease remains unclear. Several environmental factors are associated with the development of the disorder in epidemiological studies. These factors can cause mutations in genes, mitochondrial dysfunction with accumulation of misfolded proteins and generation of oxidative stress. These mechanisms together produce dopaminergic cell loss in the Substantia Nigra pars compacta and formation of α-synuclein containing lewy bodies in the brain. Lewy bodies are citoplasmatic inclusions made by several proteins, misfolded α-synuclein is one of the major proteins present. Importantly, mitochondrial dysfunction, oxidative stress and genetic mutations influence the functions of each other. So if a drug prevents oxidative stress, dysfunction of the mitochondria can still occur, still promoting progression of the disease. Because of this complicated disease mechanism, only symptomatic treatment is available by administration of levodopa, often in combination with catechol-O-methyl transferase and amino acid decarboxylase inhibitors, which prevent the peripheral degradation of levodopa. Monoamine oxidase inhibitors and N-Methyl-D-Aspartate antagonists are also used in the treatment of the disease.

Secondary effects have been found when using these drugs, for this reason it would be interesting to examine other compounds and nutrients. Especially nutrients could be an interesting opportunity because of their pleiotropic effects. The compounds have multiple targets while synthetic drugs have one target. Compounds with pleiotropic effects have small influence on different targets while synthetic drugs have a large effect on one target.

An important role of gut-brain-gut axis has been shown in PD development. Pesticide rotenone, when administered intragastrically produces a Parkinson disease like syndrome in rodents, but the occurrence of α-synuclein lesions starts first in the periphery and later in the brain. This supports the hypothesis mentioned above that the disease does not start in the brain but in the periphery. Interestingly, in rotenone treated rodents the pesticide was not present in the periphery, indicating that a different signaling pathway is involved between the gut, enteric nervous system and the brain.

To examine the effects of nutrients in PD, animal models in which five nutritional substances have been evaluated. These nutrients are creatine, coenzyme Q10, vitamin D, ω-3 fatty acids and nicotine. There is a lack of information concerning these substances effects except for nicotine. Based on the failures of many neuroprotective agents in clinical trials the evidence available at the start of the clinical trials is examined. The results of this analysis are striking. From the 12 clinical trials that should have been completed, 7 have failed. These results lead to a success rate of approximately 42% for the five nutrients in clinical trials. The examination of the preclinical data showed that few animal studies were performed and in general not all the animal models and species were used. It has become clear that additional studies need to be done before a nutrient is clinically tested.

Introduction to Parkinson's Disease: Pathophysiology, Genetics, Environmental Factors and Mechanisms of Neuronal Cell Death

Parkinson Disease: Pathology, clinical symptoms and current treatment

Different forms of Parkinsonism can be distinguished based on their pathology and clinical symptoms. Figure 1 describes the most important forms as diagnosed by the physician. Parkinson's Disease (PD) is accounting for 75% and is the most common form of Parkinsonism seen in the clinic.

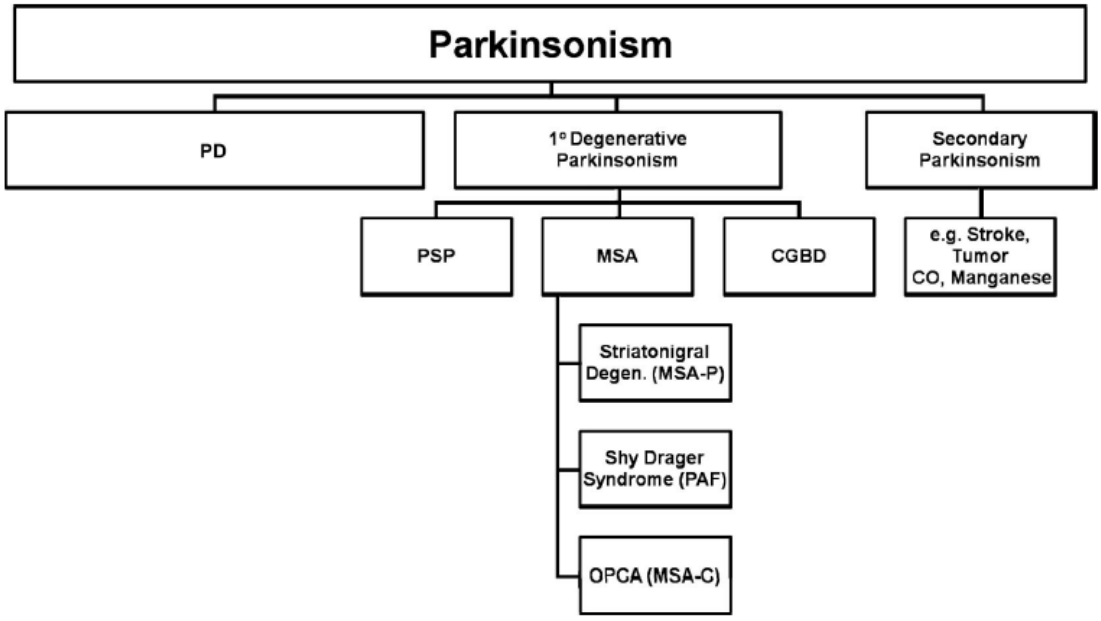


Figure 1: Overview of different forms of Parkinsonism.
As seen in the figure several forms of Parkinsonism have been described, the major form is Parkinson Disease (PD) and accounts for 75% of the patients seen at the physician. PSP=Progressive Supranuclear Palsy, MSA=Multiple System Atrophy (P=Parkinsonean, C=Cerebellar) CBGD= Cortical-basal ganglionic degeneration (1)

Historically, the diagnosis of the disorder was confirmed in the presence of two of three cardinal features. These features include tremor, bradykinesia and rigidity. Examining brain pathology was important because, degeneration of dopaminergic neurons in the Substantia Nigra pars compacta and Lewy Bodies' presence could be observed (1). The diagnosis was not adequate because the presence of Lewy bodies was not confirmed in 24% of the patients (2). This population was retrospectively analyzed and different clinical features were most likely to predict pathology. The frequent found symptoms such as resting tremor or prominent asymmetry and a good response to levodopa (L-Dopa) were the best predictors for the disease (3). This was confirmed in a study with 73 patients by Hughes et al in 2002(4).

The degeneration of the dopaminergic neurons is severe and located in the Substantia Nigra pars compacta. It seems to be that dopaminergic neuronal cells in this area are more vulnerable to metabolic dysfunction, oxidative stress and exitotoxicity. The exact mechanism of this vulnerability is yet to be elucidated (5). Furthermore, not only dopaminergic neurons are involved. PD is now associated with an extensive non-dopaminergic pathology as well. This pathology involves the norepinephrine neurons of the locus coerulus. In the nucleus basalis of meynert, the cholinergic neurons seem to be affected and in several areas, including the middle line raphe, cerebral cortex, brainstem, spinal cord and periphery nervous system, alterations in serotonergic neurons are observed (6).

Note that not all patients have all clinical symptoms and this could be associated with the progression of the disease. Around 30% of the patients have no resting tremor. Patients with an akinetic rigid form of the disease tend to have a more rapid progression in contrast to patients with tremor dominant form of PD. The akinetic-rigid form of the disorder is often misdiagnosed as a case of atypical Parkinsonism. Recently, the disease has been associated with a variety of non-motor features which pathologically differs from classic diagnosis of the disease. It has been suggested that individuals with constipation, REM behavior disorder and anosmia have not only an increased risk

for developing the disease but could have an early form (7). These non-motor symptoms will be discussed further in this thesis.
The less common forms of Parkinsonism are described as atypical Parkinsonism, multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and secondary forms of Parkinsonism are these forms.
Atypical forms of Parkinsonism can be predicted by several clinical features. Including; early onset of speech and gait dysfunction, postural instability, absence of resting tremor, axial rigidity and poor response to L-Dopa. Clinical features of MSA include the presence of prominent and symptomatic orthostatic hypotension or concomitant cerebellar signs. MSA can be divided in predominant Parkinsonism or cerebellar features. Pathologically, the characterization of MSA is by striatal and/or cerebellar degeneration of neurons caused by deposits of α-synuclein in glial cells (8). PSP is characterized by the clinical features as stated above and additionally an impairment of vertical eye movements, early falling and hyperextension of the neck. Degeneration in the Substantia Nigra pars compacta and other brain regions combined with prominent tau-positive neurofibrillary tangles are observed after pathologic examination (9).
Secondary forms of PD can occur but they are usually easier to diagnose after the examination of the patient's history. The most common secondary cause of secondary Parkinsonism is drug-induced Parkinsonism. This can be caused by neuroleptic agents used in the management of psychiatric disorders. The disease is observed as an adverse event during the use of neuroleptic agent as well as after drug withdrawal. Toxins such as manganese and carbon monoxide and other health problems such infarcts, tumors in the basal ganglia, hemorrhages and hydrocephalus can cause secondary forms of Parkinsonism. Additionally, infections such as HIV disease and influenza can cause secondary Parkinsonism (1).
All these secondary forms are very similar to each other, making difficult to diagnose the exact form of Parkinson in the patients. All groups of patients with different forms of PD make it more difficult in clinical trials which are conducted, with neuroprotective agents and nutrients. Until now, most trials with these agents fail, showing a lack of effects (8).

Currently, the only symptomatic treatment for the disease is L-Dopa, the compound needs to cross the blood brain barrier and not to be metabolized in the periphery. This metabolism, caused by L-Amino Acid Decarboxylase (AAD)), to dopamine and dopamine is further metabolized by Catechol O-Methyl transferase (COMT) or mono amino oxidase B. The metabolism of L-dopa, and drugs that inhibit peripheral metabolism, is shown in figure 2. Dopamine can induce severe adverse events like-dyskinesias and schizophrenic symptoms. The latter because the balance shifts from under stimulation in the brain to overstimulation of dopaminergic areas in the prefrontal cortex, which are not severely damaged in PD. To reduce the adverse events caused by metabolism in the periphery, an AAD inhibitor is usually added to the treatment when L-Dopa is administered. In general, carbidopa or benserazide are used. Sometimes, entacapone, which is a COMT inhibitor, is added to the treatment of patients which are severely suffering from adverse events of dopamine. This promotes entrance of more L-Dopa in the brain ameliorating the treatment. An overview of dopamine metabolism is shown in figure 2 (10).
Apomorphine is used to improve the uptake of L-Dopa into the brain and to create higher affinity of the receptors to L-Dopa. The exact mechanism of action is still unknown. This compound is frequently used when the patient suffers from"off" symptoms in disease progression and alleviates this symptom. Dopamine agonists, such as pramipexol and ropinirol, could be used as substitutive treatment of L-dopa when the effect of it is unpredictable or not there at all. This is called "on/off" fluctuations. Symptoms as rigidity and decreased motor function can be alleviated by these substances. This medication is used at the early stage, after diagnosis of the disease (11). As seen in figure 2, Mono-Amine Oxidase B (MAO-B) inhibitors can be used to prevent the dopamine to 3,4-dihydroxyphenyl acetic acid (DOPAC) and in this way increasing the levels of dopamine in the brain and decreasing the symptoms (11).

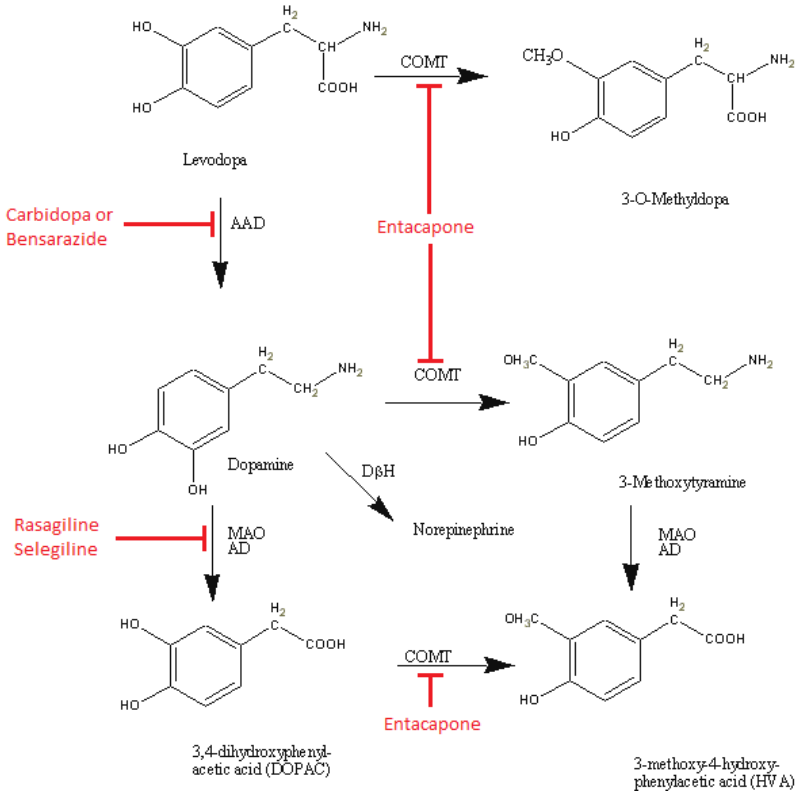


Figure 2: Overview Levodopa metabolism, Levodopa is metabolized in the periphery by Catechol-O-Methyl Transferase (COMT) and Amino Acid Decarboxylase(AAD) to dopamine or 3-O-methyldopa, further metabolism of dopamine by Mono Amine Oxydase B (MAO-B) and COMT leads to the formation of DOPAC and 3-methoxytyramine which both can be metabolized to HVA as final step. In the treatment of PD COMT, AAD and MAO-B inhibitors (marked in red) are often used to increase the amount of L-Dopa crossing the Blood Brain Barrier and maintain the Dopamine levels. Adapted from (10)

Amantadine is a NMDA antagonist which is the only substance reported to reduce L-dopa induced dyskinesias without severing disease symptoms, and could therefore be added to the therapy of a patient. The effect of amantadine for PD patients seems to be time limited and lasting for eight months. A major drawback of this drug is its potential to induce psychosis and other cognitive symptoms as well as edema and livedo reticularis, which all could lead to discontinuation of the therapy (12).

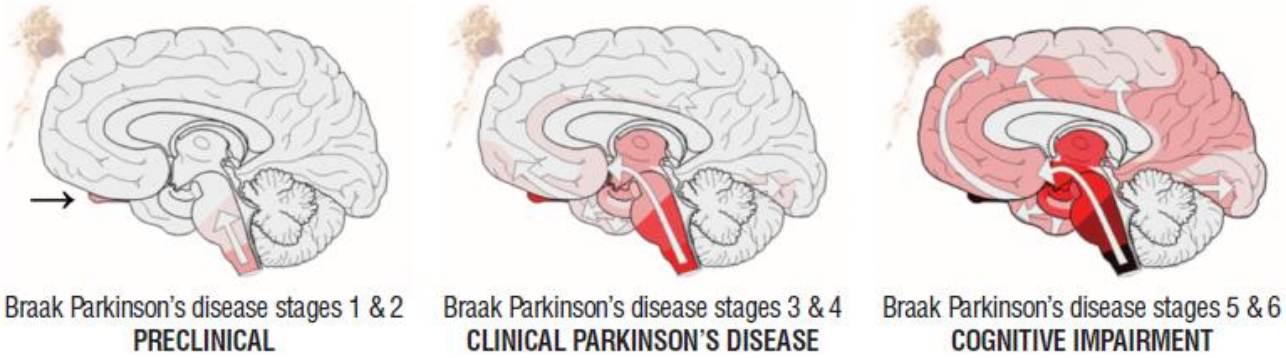
Another drug which could be added to the therapy of PD is clozapine. Clozapine decreases the severity and duration of dyskinesias. A 30% reduction in duration was observed in patients. A disadvantage of the use of clozapine is the weekly control of blood parameters to monitor for agranulocytosis. Furthermore the symptoms of resting dyskinesias are alleviated but not the dyskinesias which are action-induced, these peak dose dyskinesias could be more disabling (13).

Preclinical diagnosis of PD: Early phase PD or predictors for developing the disease

Pathological and imaging studies suggest that clinical features such as motor signs in PD develop when 50-70% of the neurons in the Substantia Nigra pars compacta have degenerated. Since it is a progressive disease, stages before the clinical onset of the disease should be detectible in the Substantia Nigra. Therefore dopaminergic imaging and quantitative motor could be used as predictors of the disease.

The most important principle in predicting PD is that the disease may not start in the Substantia Nigra (14). Braak et al.(15) proposed a staging system for disease progression, as seen in figure 3. The first stage involves the deposition of a-synuclein in the anterior olfactory bulb and dorsal motor nucleus of the vagus. Other areas such the peripheral autonomic ganglia and unmyelinated lamina-1 spinal cord could be involved in stage 1 PD. Medulary and pontine involvement in the lower raphae, reticular formation and the coeruleus/subcoeruleus complex may be involved in stage 2. In Stage 3 the midbrain is affected including the Substantia Nigra pars compacta. At stage 4-6 cortical structures in the brain are affected. In general these stages are confirmed by investigators from other groups (16,17), but there could be some modifications and exceptions.

It is not likely that the same pathological process is seen universally. Various progression patterns are seen with the onset of the disease. These are limitations of the stage system (14).



Figuur 3: Staging system in PD as suggested by Braak et al, at the left side the first two stages are shown with no alterations in the brain, but alteration in the Olfactory Bulb (Arrow) and Dorsal Motor Vagus. In the middle, the cells of the Substantia Nigra pars compacta, lower raphe and coeruleus/subcoeruleus complex are harmed. The right figure shows the parts involved in severe PD. (14)

Two markers for the first stage of PD are impaired olfaction and autonomic dysfunction. The first, impaired olfaction is caused by α-synuclein deposits in the anterior olfactory nucleus and, to lesser extent in the olfactory bulb. Dystrophic neurites are found in 2/3 of the patient' olfactory epithelium and a large majority of them have severe olfactory loss at disease onset. Usually olfaction is normal or mildly impaired in other forms of Parkinsonism (18). Additionally, olfactory loss could be an important marker in other neurodegenerative diseases such as dementia and Alzheimer disease (19).

Several studies suggest that olfactory loss is a good predictor in the development of the disease. In one study, the function of olfaction was determined in 400 first degree relatives from PD patients. A dopaminergic denervation was observed in relatives who had loss of olfaction. Within two years 10% of these hyposmics developed PD compared with 0% of the subjects with a normal olfactory function at the base line. After 5 years the patients showed further loss of olfaction (20). The Honolulu-Asia Ageing Study (HAA study) shows the strongest evidence between olfaction and PD prediction. The investigators in this study correlated loss of olfaction with pathologically confirmed PD. After 4 years of follow up, the odds ratio (OR) in the lowest quartile of olfactory function was 5.2 for developing the disorder and in the second lowest quartile the OR was 3.1 for developing PD compared to subject without loss of olfaction. Specificity of this predictor is low. 2% of the people in the lowest quartile in the HAA study developed the disorder (21). So testing for olfactory loss is insufficient for prediction of the disease.

The second stage 1 marker is autonomic dysfunction. A prominent staining of α-synuclein of unmyelinated projection neurons of the dorsal motor vagus is observed (15). Peripheral sympathetic denervation is suggested to occur even earlier. Many PD patients experience autonomic dysfunction, with a prevalence of 40-70% (22). Two studies (23,24) suggested that constipation could predict PD. This indicates that the gut-brain-gut axis is involved. In the HAA study a single question about bowel movement frequency was asked at the baseline. Infrequent bowel movement <1 per day, showed an OR of 2.3, compared to 1 bowel movement per day, to develop PD. A comparison with an even higher frequency of bowel movement showed an OR of 4.8 (23). In a second study a 2.5 fold increased risk of Parkinson was demonstrated after diagnosis of constipation. The association was evident already 10 to 20 years before the onset of the disease (24).
Measurement of the postganglionic sympathetic cardiac innervation could be a predictor of PD. This function can be measured with metaiodobenzylguanidine (MIBG) scintigraphy. Abnormal MIBG scintigraphy is present in a majority of PD patients and it seems to be present at the earliest phase of the disease (25).
Autonomic dysfunction is not universally present in the disease and due to progression it is hard to detect PD in an early phase. This indicates a low sensitivity. Specificity is probably low because the prevalence of constipation is approximately 30% and the prevalence of PD is 2%. MIBG scintigraphy is more expensive and time consuming (14).

Depression and REM sleep behavior disorder are two stage 2 markers. Many patients have a history of depression and the disease is often present in PD patients, already in an early disease stage. The pathophysiology of depression in PD is complex and several structures are involved. The serotonin neurons of dorsal raphae, the dorsal motor nucleus of the vagus, Substantia Nigra and the catecholaminergic neurons of local ceruleus are linked to depression (26). Some of these structures are involved in the second stage of the disease as suggested by Braak et al. Patients with a history of depression have a 2.4 fold increased risk of developing PD. Many studies have shown an increased risk of developing PD in patients with a history of depression (27). Additionally, patients with depression could have abnormalities on the Substantia Nigra pars compacta transcranial ultrasound similar to what is observed in PD patients. The specificity of depression as predictor for the disorder is low because not every person with depression develops PD (28).

REM (rapid eye movement) sleep behavior disorder (RBD) is typified by a loss of normal atonia during REM sleep. Patients with RBD, kick, trash or cry out in association with a dream content. One third of the PD patients have burden of RBD. It has a male predominance, which is unexplained. RBD could be explained by the appearance of lesions in the brainstem, especially in pontine areas which are involved in stage 2 of the disease (29). In patients diagnosed with idiopathic RBD the risk of developing PD is elevated. After 5 years follow up 9.5% to 19% of them develop PD and after 10 years these rates increase from 20 to 32.5%. RBD is an ideal marker for PD prediction due to the high risk and long latency of the disorder (30). Recently, Postuma et al have suggested that the risk of PD correlates with the severity of the RBD (31).
One of the disadvantages of using RBD as a predictor, is that not all patients develop clear symptoms while the progression of RBD is ongoing. Because RBD occurs when the patient is asleep clinical diagnosis has to be performed with questionnaires. The problem with those questionnaires is, is that they are not specific enough for clinical diagnosis. Currently, no neuroprotective agent is available to cure the disease. In clinical trials for testing new compounds as treatment of PD, RBD patients could be useful (14).
In the HAA study one question was asked about excessive daytime sleepiness. 9 patients developed PD of the 244 who answered positive. The OR for development of PD was 2.8 compared to persons without excessive daytime sleepiness (32).

Stage 3 markers contain several diagnostic methods to distinguish PD from other neurodegenerative disorders. These markers include dopaminergic PET and SPECT imaging, transcranial ultrasound and quantitative motor measures. In dopaminergic imaging radiolabeled ligands are used to measure

dopaminergic innervation from the Substantia Nigra pars compacta. This method is very sensitive and specific for Parkinsonism. However distinguishing between forms of Parkinson's disease is difficult. A decreased dopaminergic innervation is observed in hypo osmic relatives from PD patients. This method directly measures the function of the Substantia Nigra pars compacta, however it is not often used because it is expensive and/or patients might refuse the radiolabeled injection. Therefore dopaminergic imaging can be used as secondary screen after diagnosis of stage 1 and/or 2 markers and risk groups can be screened. The accuracy of diagnosis is not established for premotor phases of PD (14).
Transcranial ultrasound (TCS) imaging is a laboratory method for imaging the Substantia Nigra. It is a non invasive and inexpensive neuroimaging method. Abnormal hyperechogenicity occurs in 80 to 90% of the PD patients. In MSA and PSP patient the TCS is normal (33). One study found that 60% of normal persons with hyperechogenicity have reduced uptake of dopamine in the Substantia Nigra. Abnormalities in TCS are observed in approximately 40% of the RBD patients (34). The degrees of hyperechogenicity and dopaminergic innervation do not correlate. Hyperechogenicity does not progress during PD development. This would be expected if abnormalities in TCS were a direct marker for development of the disease (14).
Currently, the diagnosis of PD is based on clinical symptoms and is subjective. The use of detecting subtle changes in quantitative motor functions is now investigated to perform objective diagnosis of the disease. However, identification of changes in a preclinical disease state has never been studied. Limitation to quantitative motor measurements is that subtle slowing of the motor occurs in approximately 40% of the elderly persons (14).

It could be interesting to combine several predictors of PD in patients based on the history of the patient and the clinical symptoms such as impaired olfaction, autonomic dysfunction and RBD. Positive symptoms could contribute the screening of the patient for dopaminergic activity, TCS and quantitative motor measures. This can lead to the diagnosis of preclinical PD without clinical motor symptoms. Preclinical disease could be treated with neuroprotective agents to slow the progression of the disease. Currently, no neuroprotective agent is available for treatment but it is possible to monitor patients for years observing signs of PD. For our work we are especially interested in autonomic dysfunction because they would imply the involvement of gut brain axis in PD.

Genetics in PD

A familial pattern of inheritance is observed in approximately 5 to 10% of patients with PD. Several mutations in 11 known genes linked with familial PD development are indentified and the role of 4 others remains unclear. The Mendelian genes with their location and inheritance are stated in table 1 (35). Gene mutations are observed in a small number of PD patients, in most of them the disease occurs sporadically (36). This thesis will describe the 5 most important genes involved in the pathogenesis of PD.

Table 1: Overview of the different genes which are involved in PD disease mechanism (35)

Locus	Chromosome	Gene	Inheritance
PARK 1/4	4q21.3	α-*Synuclein*	Autosomal dominant
PARK 2	6q25.2-27	*Parkin*	Autosomal recessive
PARK 3	2p13	Unknown	Autosomal dominant
PARK 5	4p14	*UCHL-1*	Autosomal dominant
PARK 6	1p35-p36	*PINK1*	Autosomal recessive
PARK 7	1p36	*DJ1*	Autosomal recessive
PARK 8	12q12-q13.1	*LRRK2*	Autosomal dominant
PARK 9	1p36	*ATP13A2*	Autosomal recessive
PARK 10	1p32	Unknown	Susceptibility locus
PARK 11	2q36-37	*GIGYF2*	Autosomal dominant
PARK 12	Xq21-25	Unknown	X-Linked
PARK 13	2p13.1	*HTRA2/Omi*	Autosomal dominant
PARK 14	22q13.1	*PLA2G6*	Autosomal recessive
PARK 15	22q11.2-qter	*FBXO7*	Autosomal recessive
PARK 16	1q32	Unknown	Unclear

Leucine Rich Repeated Kinase 2 (LRRK2) mutations occur with an autosomal dominant inheritance. Mutations in this gene have been reported to be the most prevalent in sporadic as well as familiar disease (37). One LRRK2 mutation, the G2019S mutation, is present in 0.4- 1.6% of the patients with idiopathic PD in white population (38,39). These rates increase in patients with the familial form 2.8-6.6%. Two ethnic subgroups, the North African Arabs and Ashkenazi Jews, have a significant higher prevalence with a frequency up to 39% (35). The exact function of the LRRK2 protein remains to be elucidated. The LRRK2 gene codes for a large multidomain protein which exist of a leucine repeated rich domain, a Ras of complex (ROC), the c-terminal of the ROC (COR), mixed lineage kinase domains and WD proteins. The most observed mutations in the LRRK2 gene are stated in figure 4.

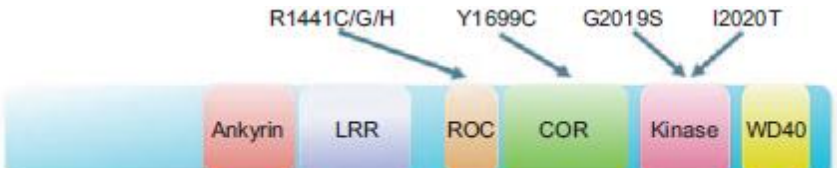


Figure 4: The LRRK2 gene domain which codes for Ras of complex (ROC), the C-terminal of the Ras of complex (COR), mixed lineage kinase domains and WD40 proteins (35)

Smith et al. and West et al. showed that kinase activity of LRRK2 mutant proteins strongly correlates with neuronal cell death (40,41). This indicates that the kinase activity of LRRK2 proteins is altered and causes PD (35).

Mutations in α-synuclein were the first mutations identified. Several missense mutations are identified and duplication and triplication of the wildtype α-synuclein gene are observed in PD patients. This indicates that over expression of the protein could produce the disease. The inheritance of the mutations is autosomal dominant. α-Synuclein is a soluble, unfolded, protein involved in synaptic plasticity, dopaminergic neurotransmission and the turnover of synaptic vesicles (42). It tends to oligomerize and form insoluble aggregates. Therefore the possibility raises that mutations or over expression of the protein cause accumulation in the cell, inducing cell death. α-Synuclein is a key component of Lewy bodies in patients with sporadic form of the disorder. In 1997 the A53T replacement was identified as the first mutation which causes familiar form of the disease (43). In 1998, the A30P replacement was described by Kruger et al.(44) and the existence of the

mutation was confirmed by imaging in 2001(45).Transgenic mice with overexpressing A53T mutation develop interneural inclusion as well as degeneration and mtDNA damage (46). Both A30P and A53T α-synuclein are poorly degraded by chaperone mediated autophagy. Both mutants bind the lysomal membrane with high affinity but do not enter the lyosomes, inhibiting their own degradation in this way (47).

Mutations in the Parkin gene are described to cause PD and more than 70 of them have been identified. Patients with mutations in the Parkin gene have an autosomal recessive inheritance and cause young onset form of the disease. The disease has its onset before 40 years of age (36). E3 ubiquitin ligase is the protein product of Parkin. Ubiquitin is one of the most abundant proteins in the brain and it contributes to the formation of Lewy bodies. Ubiquitin ligases attach ubiquitin to misfolded proteins. This signal promotes transport of misfolded proteins to the proteasome for degradation (48). Furthermore, Parkin gene plays a role in mitochondrial biogenesis by regulating and initiating transcription of mt DNA (49). α-Synuclein has been identified as a substrate for Parkin, this links the two genes to the ubiquitinuous system. Mutations in the Parkin gene causes impaired clearance of unwanted proteins leading to an accumulation of misfolded proteins and neuronal cell death. Another interesting fact is that the protein is influenced negatively by oxidative stress and mitochondrial dysfunction. The E3 ubiquitin ligase loses its protective function (50)Combining a heterozygous mutation with oxidative stress could be one of the key factors in the pathogenesis of PD (42).

DJ-1 is identified as a gene involved in the pathogenesis of PD. It is linked with autosomal recessive pattern in 1% of the early onset Parkinsonism (42). DJ-1 is a H_2O_2 responsive protein, indicating a role as antioxidant. DJ-1 seems to act as a redox sensor within the cells. Acidification of the 106cysteine residue seems to have an important signaling function, in response to oxidant as well as for relocalization of DJ-1 to the mitochondria. This cysteine residue has an important double function (51). DJ-1 Parkinsonism is characterized with slow disease progression of symptoms and variable severity of disease. Mutations in the DJ-1 gene limit its function in protecting the cell for oxidative damage. Mutated DJ-1 causes pathogenic loss of cytoplasmic activity in the mitochondria (36).

Phosphatase and tensin homologue-(PTEN) induced kinase 1(PINK1) is an ubiquitinously expressed protein with an N-terminal mitochondrial targeting motif. It contains a highly conserved serine-threonine kinase domain and an autoregulatory domain at the c-terminal (52). The mechanism of action of PINK1 remains still unclear, but it has an important role in mitochondria maintenance. It is a kinase, which can phosphorylate specific mitochondrial proteins and modulate their function. However, the substrates are unknown (42l). Mutations in PINK1 are rare in pathogenesis of PD (36). In vitro work with the silencing the PINK1 gene has been shown to decrease cell viability and a progressive loss of dopaminergic neurons was observed in drosophila, these effects could be attenuated by a human antioxidant super oxide dismutase 1(SOD1). These results indicate that loss of function mutations in the PINK1 gene could induce neuronal death via the oxidative stress pathway (53). Interestingly, the phenotype of a PINK1 knockout in Drosophila share many similarities with the Drosophila Parkin knock out model suggesting that Parkin and PINK1 have their mechanism of action in the same pathway, with PINK1 functioning upstream of Parkin (54,55).
The most interesting part is the cooperation of the different proteins and their influence on each other. Most of them seem to act in the same pathway up or downstream one from the other. When a mutation occurs in a gene, a pathway is initiated causing oxidative stress and/or metabolic dysfunction and influencing the transcription, translation and functioning of the other genes.

Environmental factors and PD

In 1984 a method to measure the incidence of PD and the role of MPTP was published. From then, approximately 40 epidemiological studies about the association between pesticides and the disorder have been conducted. It was suggested that it was unlikely that MPTP was playing a major role in PD, but that other environmental factors could be associated with the prevalence and incidence of the disease (56). This suggestion led probably to all the epidemiological studies performed until now. Table 2, obtained from Wirdefeldt et al, shows the epidemiological evidences after the exposure to different environmental factors and their association with PD. Pesticides, herbicides, insecticides and nicotine are examined in detail. In general, the evidence for association is limited or inadequate and could be positive as well as negative (57).

Table 2: Epidemiologic evidence and the exposure to environmental factors (57)

Exposure	Epidemiologic evidence	Direction of association
Pesticides	Limited	Positive
Metals	Inadequate	
Organic solvents	Inadequate	
Magnetic fields	Inadequate	
Smoking	Sufficient	Negative
Alcohol	Limited	Negative
Physical activity	Limited	Negative
Adiposity	Inadequate	
Coffee	Sufficient	Negative
Intake of dairy products	Limited	Positive
Intake of macronutrients	Inadequate	
Dietary intake of antioxidants	Limited	Negative
Dietary intake of minerals	Inadequate	

Li et al. reviewed in 2005, using 27 studies, the association between pesticides, herbicides and insecticides with PD. They showed a significant increased risk for pesticides in 7 studies, 8 studies showed a positive but not significant association and 6 studies showed a non significant negative association between the exposure to pesticides and the prevalence of the disease. For herbicides similar results were obtained although fewer studies were used. In total 9 studies were examined, four studies showed a significant positive association between the exposure of herbicides and the prevalence of PD, while 3 studies showed a positive, but not significant association. Two studies showed a negative association. Finally Li et al evaluated insecticides exposure and its consequences. Eight studies were found and analyzed, in 3 studies a significant positive association was found, in two studies a non-significant, positive association was found and in 3 studies a non-significant, negative association was found. The evidence for association of pesticides, herbicides and insecticides, seems to be directing into a positive trend but the data available was limited to make a clear conclusion (58).

Smoking has been inversely associated with PD for a long time. Wirdefeldt et al evaluated 8 studies in which the association of smoking with the incidence of PD was examined. Data from current smokers and past smokers were compared with controls which never had smoked. The relative risk factor for the incidence and prevalence of PD for current smokers vs control was between 0.29-0.56 and the relative risk factor for past smokers versus controls was in between 0.5-1.15 (57). The positive association was found in one study (59), the second highest relative risk factor found, was 0,78. Based on the amount of subjects examined in the 7 other studies and the population used in this particular study it was not considered as relevant. Wirdefeldt et al concluded that an inverse association exists between smoking and PD (57).

Concluding, environmental factors are associated with the incidence and prevalence of PD. Other environmental factors such as the intake of caffeine, exposure to bacterial infections, rural living,

wood pulp mills in the area and the availability of good quality could be associated, in a correlating or inverse way, with the incidence and prevalence of PD. Interestingly, paraquat (herbicide) and rotenone (pesticide) have been recently described as compounds used in Parkinson's disease animal models. Nicotine is currently tested in animal models and in clinical trials to examine the positive effects, and the influence of nicotinergic receptors in PD. Unfortunately, all the associations between environmental factors and the incidence and prevalence of PD could be biased because exposure never occurs to one single substance. This could be positive as well as negative, controls could be exposed to other environmental factors, giving rise to smaller relative risks but results on a specific environmental compound could be misunderstood. Furthermore, most studies on environmental factors used questionnaires to examine the exposure, which could produce a recall bias.

Neuronal cell death in PD

Several processes are involved in neuronal cell death in PD. These processes include oxidative stress, mitochondrial dysfunction, protein aggregation, inflammation and excitotoxicity. Al these processes are involved in a different way and can influence each other. Figure 5 shows a brief overview of the mechanisms involved in neuronal cell death (60). This chapter will discuss the different mechanisms.

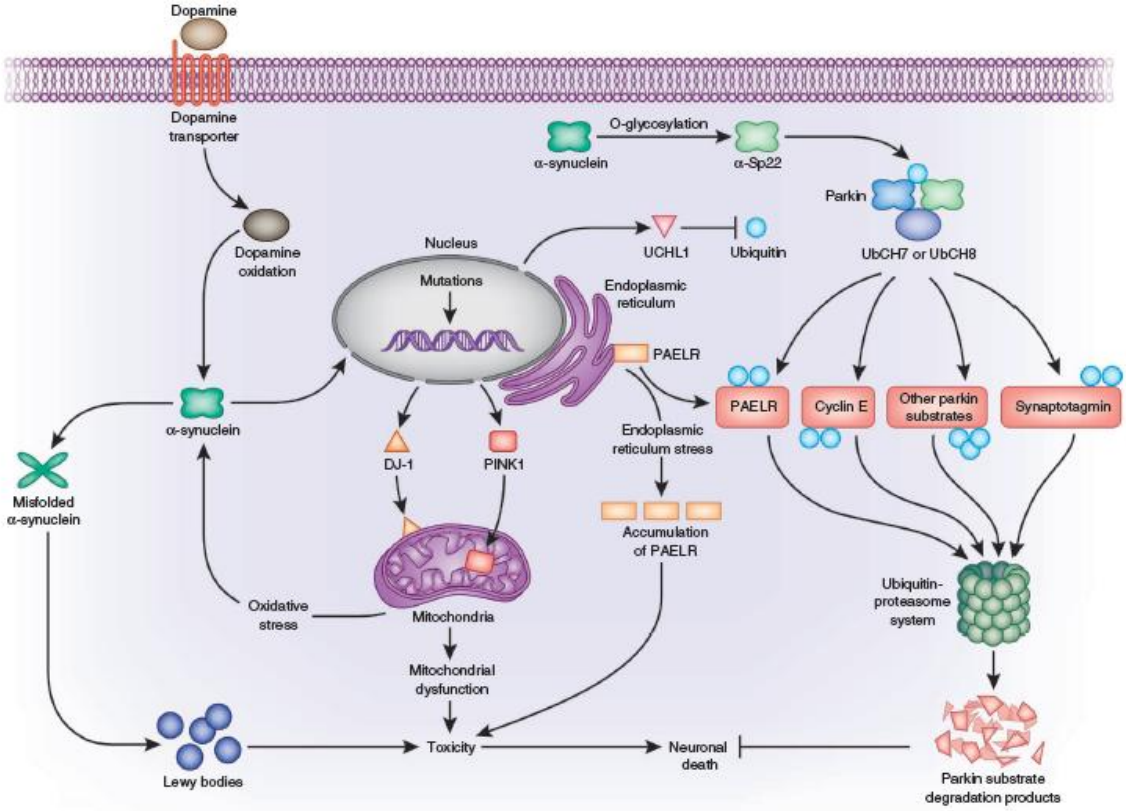
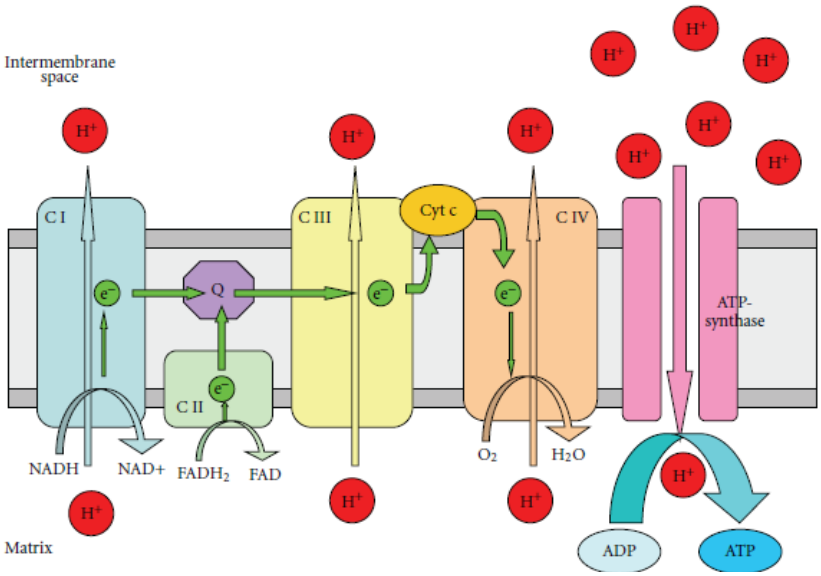


Figure 5: Schematic overview of all the factors involved in neuronal cell death, and the interactions between mitochondrial dysfunction, oxidative stress en genes which lead to neurotoxicity and neuronal cell death. (60)

Mitochondrial dysfunction

Mitochondria are present in almost every eukaryotic cell and their function is to generate cellular energy as adenosine tri-phosphate (ATP) by oxidative phosphorylation of adenosine di-phosphate (ADP). Mitochondria are involved in the regulation of apopthosis, heam biosynthesis, calcium homeostasis and function in the control of cell division and growth. Mitochondria consist of a lipid double bilayer surrounding the intra compartmental matrix which contains the major units used for oxidative phosphorylation. A unique feature is the presence of circular, double stranded, DNA (mtDNA) which codes for unique mitochondrion related proteins. An important feature of the mitochondria is the electron transport chain, which consist of five complexes (I-IV, ATP synthase) and include ATP synthase, as shown in figure 6 (61). The function of this chain is to generate ATP used as cellular energy. The electron transport is a highly efficient process but during oxidative phospholylation electrons can leak at complex I and complex III and react with oxygen to form superoxide. The production of this superoxide occurs in low levels by healthy mitochondria and it is removed by manganese superoxide dismutase (mnSOD) (62). It is thought that dysfunction in the electron transport chain or mnSOD of could promote an increase of intracellular $\cdot O_2^-$ and this could cause neuronal cell death in the Subtstantia Nigra pars compacta in PD (63).

Complex I and III are the most important complexes involved in mitochondrial dysfunction. Oxidative stress can cause dysfunction of complex I, which is a nicotinamide adenine dinucleotide phosphate (NADH) ubiquinone oxidoreductase. This dysfunction produces increase of cytosolic



Figuur 6: The mitochondrial respiratory electron chain: A schematic representation of mitochondrial electron transport chain involved in oxidative phosphorylation. Complex I and I transport electrons to Coenzyme Q10, which transports the electrons to Complex III and via Cytochrome c the electrons are transported to Complex IV, Complex IV arranges transformation of O_2 to H_2O. During these steps protons are transported from the matrix to the intermembrane space. These protons are used by ATP synthase to phosphorylate ADP to ATP (61)

cytochrome c,which is a crucial initiator of caspase apoptotic signaling cascade, trough peroxidation of cardiolipin (64). Furthermore, dysfunction of complex I lead to lowering in the threshold of to Bax transcription. Bax transcription initiates mitochondrial-dependent apopthosis, which is as crucial event in the degeneration of dopaminergic neurons in the Substantia Nigra pars compacta (65). Dysfunction of complex I can decrease proteosomal activity, which makes dopaminergic neurons more vulnerable to damage by neurotoxins (66).

Oxidative stress

There are three major oxidation routes which cause oxidative stress and protein misfolding : enzymatic oxidation of the dopamine and the neuromelanin route, non enzymatic oxidation by reactive oxygen species and enzymatic oxidation of dopamine by MAO-dopamin,aldehydes and H_2O_2.

Different enzymes, such as tyroxinase, COX, lipoxygenase and xanthine oxidase, can oxidate dopamine to dopamine quinones. These quinones can activate the neuromelanin pathway and are involved in the pathway. The pathway produces dopamine quinine neurotoxic metabolites, such as o-semiquinones and benzothiazolines, which can inhibit mitochondrial pyruvate dehydrogenase (67). Additionally, these quinones are initiators of α-synuclein plagues formation. Oxidized dopamine can react with thiols which can react further producing proteine misfolding and aggregation. Activation of the neuromelanin pathway causes not only neurodegeneration but also to α-synuclein aggregation, oxidative stress, Lewy body formation, inflammation, depletion of the antioxidant reduced glutathione and functional loss of dopamine transporters (68). Neuromelanin has a natural protective function but due to massive oxidation, cell death and release of Neuromelanin from dying cells in PD, a final lose occurs. This makes dopaminergic cells vulnerable to free radicals and toxic quinones (5).

Non enzymatic dopamine oxidation is caused by reactive oxygen species and metals. Auto-oxidation of dopamine promotes the formation of 6-hydroxydopamine and superoxide O_2^-. This superoxide can react with nitric oxide to form $ONOO^-$. Peroxynitrite can re-oxidize dopamine and depletes the reduced glutathione peroxidase and thereby inhibits the function of reduced glutathione as antioxidant (69). The presence of 6-hydroxidopamine can trigger neurodegeneration by reduction of striatal zinc and metallothione. Normally these structures are antioxidant or metal detoxifiers.

Reduction of the structures promotes the release free iron from ferritin (70). Increased free iron deposits are found in neurodegenerative regions. In PD patients the release of free iron could be increased by mutations in iron regulating proteins, iron storage and iron transporter proteins. Accumulation of Iron could be increased by heme oxygenase. This enzyme is expressed in dopaminergic neurons in the Substantia Nigra, the nigral neurophils, reactive astrocytes and Lewy bodies (5).

Oxidation of dopamine by MAO A or B is the third route. Activity of MAO increases with ageing and can give rise to toxic products such as hydrogen peroxide, aldehydes, ammonia and reactive oxygen species. Especially two aldehydes (3,4 dihydroxyphenlacetaldehyde and 3,4-dihydroxyphenylglycolaldehyde) are important because they react with H_2O_2 forming neurotoxic radicals. In catecholamine neurons, dopamine can react with H_2O_2 creating 6-hydroxydopamine neurotoxin. Further condensation of dopamine with acetaldehyde is possible, producing toxic endogenous precursor of N-methylated pyridines. The structure of this toxin is similar to the MPTP structure (71).

Neuronal inflammation

Neuronal inflammation is one of the factors that contribute to PD's development. Increased expression, proinflammatory cytokines release and complement activation are observed PD patients' brains. Microglia are phagocytic and playing an important role as a part of innate immune system in the brain. They act as scavenger cells during degeneration, inflammation, ischemia and infections. Major histo-compatibility complex and glycol proteins are up regulated by microglia. Additionally, several inflammatory cytokines, such as adhesion molecules, tumor necrosis factor alpha, interleukin 1β -6, cycloxygenase-2, nitric oxide and inducible nitric oxide synthase are expressed by microglia. These molecules can mediate and amplify irreversible destruction or dopaminergic neurons in the Substantia Nigra. T-cell activation and infiltration in the Substantia Nigra and $CD4^+$-cell involvement have been reported in PD (72).

Dying neurons and aggregated α-synuclein can trigger local gliosis, microglial activation of T-cell infiltration and increase expression and release of immunological molecules. The major regulators of the inflammation response are tyrosine kinase, phosphatidylinositol 3-kinase/akt (PI3/akt) and mitogen activated protein kinase (MAPK) signaling pathways. Important pathways are the c-jun NH_2 –terminal kinase (JNK), extracellular signal-regulatad kinases (ERK) and p38 MAPK.

Co-expression of iNOS and COX-2 have a detrimental function because the product formed by NO and O_2^- plays a relevant role in the pathological process of PD. Removal of one or both radicals can promote the prevention of neuronal cell death in PD.

Excitotoxicity

The theory for the role of excitotoxicity in PD pathology is consistent and is one of the mechanisms which could attribute to disease progression. It involves depolarization of the plasma membrane causing over-activation of the N-methyl-D-aspartate (NMDA) receptors in the brain. This causes a fast inward of Ca^{2+} and can cause excitotoxicity. Excitotoxicity can develop in three ways. Mg^+ can block NMDA at presynaptic receptors in a voltage dependent way. Deficiency in Mg^+ or ATP causes a failure in the regulatory control of Ca^{2+}. In response to glutamate activation on NMDA/AMPA/kainate receptor, increasing Ca^{2+} level inward at the post-synaps occurs. Loss in GABA metabotrobic inward of ions occurs, causing decreased inhibitory effect of these neurons upon receptor activation (5).

In PD the over activation of NDMA receptor could cause neurodegeneration, the increased levels of Ca^{2+} could activate neuronal nNOS causing nitrosative stress and, modifying proteins that could ended misfolded and aggregated. Accumulation of α-synuclein activates nNOS and contributes to neuronal toxicity. This effect can be blocked with NMDA receptor antagonists.

Mitochondrial excitotoxicity creates an accumulation of glutamate in the Substantia Nigra pars compacta increasing Ca^{2+} influx, calpain activation in the cytosol and causing toxic effects. The

balance between over and under activation of glutaminergic receptors is very important because glutamate is required for synaptic plasticity in neurotransmission (5).
The role for NDMA receptor seems to be important in the pathogenesis of PD , over activation of the receptors creates misfolded and aggregated proteins. But, if under activation occurs the synaptic plasticity could be affected (73).

Summarizing, PD is a very interesting but very complicated disease. Genetics, neuronal cell death and exposure to environmental factors are linked with development of the pathology. An overview is shown in figure 7.

The exact cause of PD will probably never be found in the future, because many different mechanisms are involved. But knowledge about all the factors involved could contribute new insights in the mechanisms of the disease and new pharmacological markers which could be used for drug targeting. Nutritional intervention could be here very interesting because of their pleiotropic effects. In the second part the gut-brain-gut axis, enteric nervous system and the evidence of nutrients' role in animal models for PD as well as the role of nutrients in the clinic will be discussed.

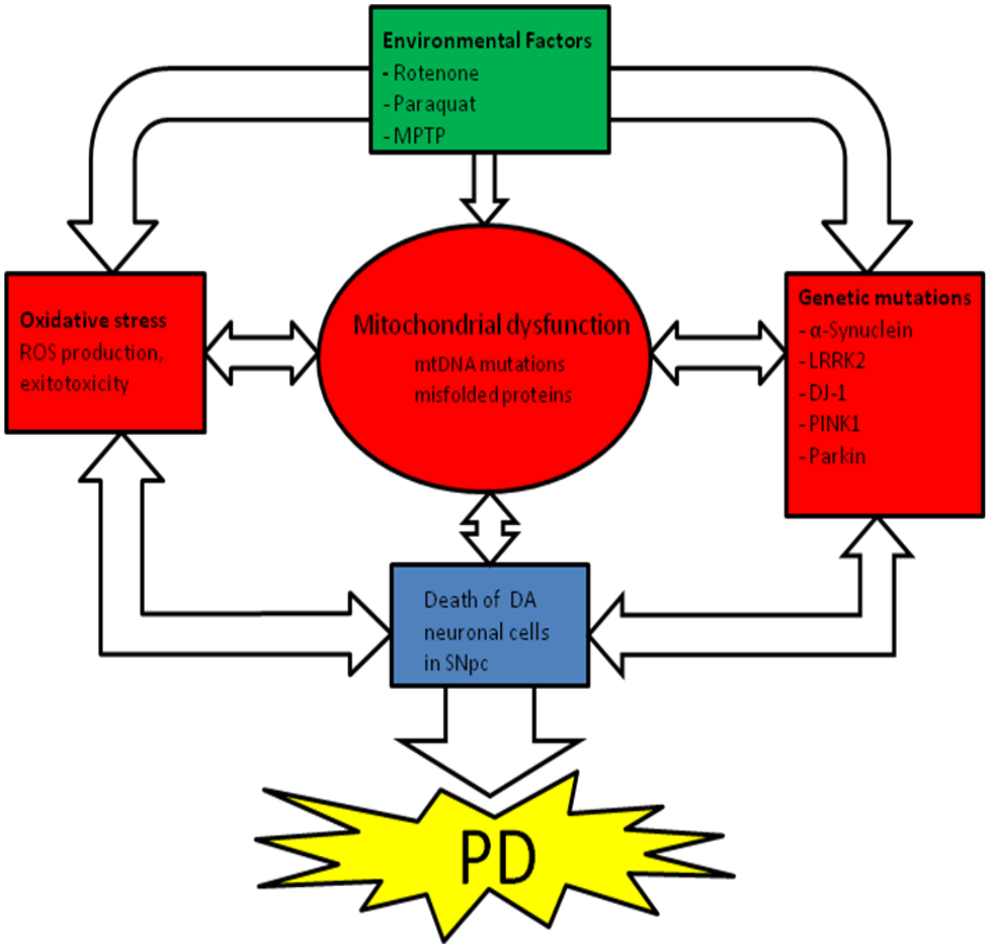


Figure 7: Difficulties in finding a cause for PD: Overview of the role of environmental factors which influence, genes, Mitochondrial dysfunction, oxidative stress, exitotoxicity. Which leads to dopaminergic neuronal cell loss in the Substantia Nigra pars compacta and lead eventually to PD.

The role of the Gut-Brain-Gut Axis in Parkinson's Disease: Preclinical and Clinical Evidence for the Use of Nutrients in PD

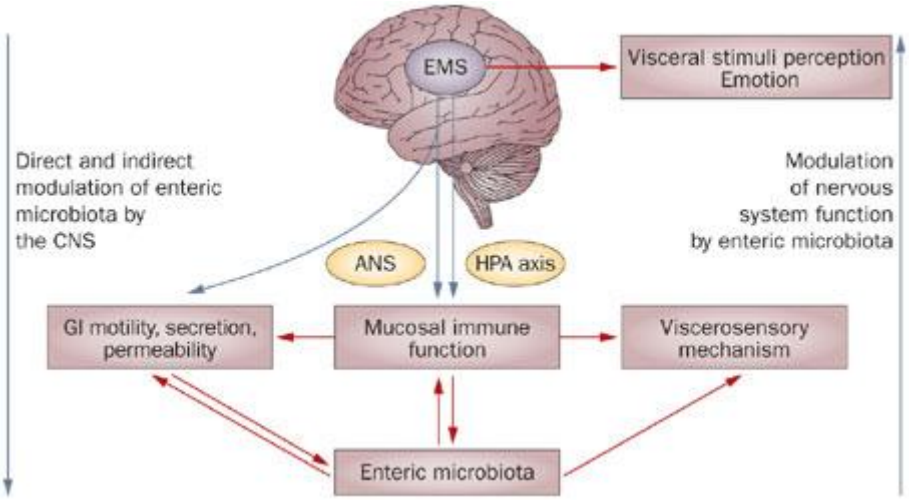
A role for the gut-brain-gut axis: Gastrointestinal tract disturbances and systemic problems in PD

Gastrointestinal tract disturbances are typical non-motor symptoms affecting PD patients. Until now, they were described as late phase symptoms of the disease. Recently, it has become clear that the gastrointestinal motility is already disordered in an early phase of the disease. Gastrointestinal disturbances include not only problems with constipation which is often diagnosed in elderly patient in late stage of the disease but also problems with esophagus, stomach and the whole bowel. However, those problems are largely under diagnosed due to patients' avoidance to tell their physicist. The incidence of gut problems is increasing, because of recognition in a targeted study. In a targeted study, PD patients were especially asked for problems with their gut by their physicist. The incidence can be overestimated in those trials because of recall bias, so every small disorder will associated with early phase of PD. During the progression of the disease the symptoms will invariably worsen (74).

Relation CNS and gut

The whole gut is innervated by the CNS. The vagal nerve is responsible of the motility of the stomach and the esophagus. The parasympathetic nucleus of the spinal cord innervates the remaining colon. Sympathetic innervation of the small and large bowel seems to be less important in the regulation of the gut motility. Colonic motility is predominantly regulated by intramural ganglia from the enteric nervous system (ENS). The ENS is mostly independent of extrinsic innervation and controls intestinal motility. The Vagal nerve innervates the ENS to integrate impulses from the CNS when necessary. The ENS mainly consists of two plexuses, which are ganglionated. The myenteric plexus is located between the longitudinal and circular smooth muscles layers, and the submucosal plexus in the submucosal tela. The ambiguous nucleus of the upper gastrointestinal tract innervates the striated muscles extrinsic. The neurons of Onuf's innervate the voluntary muscles, for example the anal sphincter (75).

Micro-organisms play an important role in the activation of the gut-brain axis. Animal models have provided evidences showing that micro-organisms in the gastrointestinal tract can activate neural pathways directly. Orally administered, subclinical doses of Campylobacter jejuni does not initiate a immunological response in mice but still results in anxiety provoking effects (76). This has been confirmed by Gaykema et al. C. jejuni can activate the visceral sensory nuclei in the brainstem, even when a subclinical dose is administered, thus provoking no immune response (77). Interestingly, the parts in the brainstem that are activated, the nucleus of the solitary tract and the lateral parabrachial nucleus, are involved in neuronal information processing. This indicates that activation of this area by bacteria could promote autonomic, neuroendocrine and behavioral responses. In figure 8 a schematic representation of the bidirectional brain-gut-microbiota interaction is shown (75).



Figuur 8: Schematic representation of the bidirectional gut-brain-gut interactions with the enteric microbiota. The ENS can interact directly with the microbiota and is innervated by the CNS. ANS=Autonomic Nervous system, GI=Gastrointestinal, HPA=Hypothalamus-Pituitary-Adrenal, EMS=Emotional Motor System (75)

Gut-brain-gut axis and PD

According to the model of disease stages from Braak et al, PD is not initiated in the brain (15). This is observed in the animal study from Pan-Montojo et al (78).The group administered rotenone intragastrically to mice inducing Parkinson's like disease, the brain was affected but there was no Rotenone observed in the periphery. Furthermore, α-synuclein lesions were found in the ENS. Additionally, Braak and his colleagues suggested that two specific sites are affected by α-synuclein plagues, before the Substantia Nigra is involved. These two sites are the dorsal motor nucleus of the vagal nerve and the anterior olfactory bulb as described above. Since the vagal nerve innervates the whole gastrointestinal tract, autonomic dysfunction could be expected from esophagus to the colon. And because the dorsal motor nucleus is affected before the rest of the brain, it is expected that gut problems could be observed before the clinical diagnosis of PD. This will be further explained in the chapter: "Animal models for PD and GI disturbances".

It has been known for a long time that neuronal degeneration also occurs in the intramural ganglia throughout the gastrointestinal tract, next to the central degeneration. The exact start location of PD remains unclear because α-synuclein lesions or Lewy bodies seem to appear at different locations the same time. This animal model is a confirmation of the Braak theory that PD could start in the peripheral nervous system. This justifies the use of MBIG for screening patients without clinical symptoms of PD but with gut problems, to indentify the disease in an early phase and start treatment.

Problems with the gut and other systems in PD

Patient with PD can experience several problems with their gut. These disturbances can occur in the upper and lower gastrointestinal tract. As mentioned earlier, gastrointestinal problems in PD are largely underestimated. In the upper gastrointestinal tract, patients suffer from delayed gastric motility causing swallowing problems and delay of gastric emptying. The sphincters in the esophagus are disturbed, which produces dyssynergia of the propulsion. This means there is incomplete relaxation of the sphincters (74).

Non specific symptoms become apparent by impaired gastric emptying, such as belching, heartburn and sensation of fullness. These symptoms are present in early phase PD already and are variable per individual. Impaired gastric emptying causes reduce or delay of L-Dopa absorption. A significant correlation between gastric motility and L-Dopa uptake is shown by Goetze et al (79). When L-Dopa is administered in a solution there are less problems with the uptake, solution seems to be absorbed normally. It is not clear what exactly causes the impaired gastric emptying in the gut, there are conflicting reports about dopamine levels in the gut. Zeng et al (80) showed an upregulation of dopamine whereas Tian et al 2008 showed an increase in expression of the proteins tyrosine hydroxylase and dopaminergic transporters (DATs) (81). However this has never been reproduced again. Till now only gross pathologic changes have been described in the upper gastrointestinal tract such as dilation of the small bowel.

Constipation has been identified as a common symptom in PD. Braak et al. suggests it could be a marker for developing PD. Constipation is one of the most frequent complications of the disease, with a prevalence of 70-80%. Interestingly Abbot et al followed almost 6800 men from the Honolulu heart program, with a follow up of 24 years. They showed that men with an infrequent bowel movement <1/day had an increased risk of PD of 4.5 compared to men with frequent bowel movement, >2/day p=0.025. But there is even an increased risk of PD comparing the infrequent bowel movement with bowel movement 1/day, relative risk 2.7 p=0.007 (23). This study could be a bit confounded because of the use of interviews, which can produce a recall bias. A recall bias could exist because persons with a disease remember more complications then healthy persons and could lead to biased results. All the results were corrected with consumption of coffee, cigarettes and fruit and for exercise. Because of the possible existence of a recall bias, Savica et al. examined the medical history of PD patients matched with a control and they showed as well an increased risk of 2.48 for developing PD, and constipation already was present 20 years before the onset of PD (24). The results have to be confirmed in trials with women and other ethnic groups. Severe constipation is associated with megacolon, pseudo-obstruction and volvulus in PD. These morphologic changes in

the colon often cause no more symptoms than constipation but occasionally perforation of the ileus and colon are described (82).
Another complication that many patients with PD experience is weight loss. One of the possible mechanisms of action was recently revealed by Kuranuki et al (83). In this study rats were fed with a high fat diet for 2 weeks. The treated rats were unilaterally injected with OHDA while the controls were operated but nothing was administered to the brain. After two weeks there was no difference in the body weight in both groups. After sacrifice, abdominal body fat was obtained and weighted. A significant reduce in abdominal fat was observed in the group treated with 6 OHDA. After that, mRNA levels of several transporters and enzymes involved in glucose and fat metabolism were examined, but no significant differences were obtained. Therefore glucose and insulin levels were measured. A large, significant, increase in glucose level was observed and a significant decrease of available insulin was seen in the treated group. This indicates that the brain sends altered signals to the pancreas, to produce less insulin, or there is pathology of the pancreas caused by the disease. This shows the importance of controlling insulin levels in humans with PD. PD patients may lose their appetite because of GI disturbances with a consequent loss of weight. Surprisingly, with a normal food intake some individuals lose much weight. Insulin levels may here be examined due to the possibility of developing diabetes-like syndrome associated to PD.

Role of gut-brain axis in other psychiatrical diseases
In other psychiatric diseases positive the effects of nutrients has been described in humans. Here, three examples will be examined, probably there are several other examples confirming the role for gut-brain axis. Bacterial interactions are studied in major depressive disorders and the effects of it on mood and memory, as well as the role of stress in inflammatory bowel diseases. The last one is an example of brain-gut interaction showing that the interaction can be bidirectional.

The first example of an interaction between the gut and brain is a study from Benton et al (84). In this short double-blind placebo-controlled trial, 132 healthy volunteers with mean age of 61.8 were included. At the base line there were no differences in the parameters. The subjects filled in several forms about their behavior and had to describe their mood, furthermore they performed a memory test at the baseline. They were supplied with Yakult (Lactobacillus casei Shirota) or milk with the same taste daily. After 10 and 20 days the results were examined by repeating the tests for mood and memory. The drink was well tolerated in this study. Based on their mood at the base line the subjects were assigned into a group, bad, middle, good. Intake of the probiotic resulted in a significant increase in mood in the first group comparing to the controls. An increase in mood was observed in both middle and good mood groups as well but this was in favor of the placebo. This indicates that there could be a benefit to use a probiotic for persons with bad mood. Unfortunately the study showed a decrease in memory, which was obtained subjectively, in the probiotic group comparing it with the controls.
Concluding, an increase in mood, which always fluctuates and a significant decrease in memory, was observed. But, treated subjects lost 1 item comparing to controls so the question remains if this is clinically relevant. Unfortunately, the drink with the bacteria did not improve frequency of defaecation in the subject.

The second example is the administration of probiotics as adjuvant therapy in MDD. Microflora in the gut consists of at least 500 species from 200 genera. In healthy persons there is a balance between pathogenic bacteria and bacteria which are beneficial for health. The balance favors to the beneficial bacteria. In case of MDD, an unbalance is created in the gut which causes Irritable Bowel Syndrome (IBS) in 30% of the patients (85). Another brain-gut connection is MDD with atopy. There is an increase risk, 2.7 fold of developing MDD or Attention Deficit Disorder, when the subject has atopic allergy. In atopic state, the amount of Bifidobacteria is significantly lower (86).
Patients with chronic fatigue syndrome (CFS) often show depression symptoms. It has been shown that administration of non-digestible oligosaccharides increases the availability of nutrients including Zinc. These nutrients are intended to increase the amount of Bifidobacteria in the gut. During CFS

small intestinal bacterial overgrowth can occur. When treated, an improvement in depression occurred but also improvements in memory and concentration were observed (87).
Already in 2002, Horrobin et al showed a beneficial effect of ω-3 fatty acids in various disorders (88). This is interesting because there is an inverse correlation between the intake of ω-3 fatty acids and the incidence of MDD in the western world. Furthermore, polyunsaturated fatty acids seem to have an effect on the adhesion of probiotics to cells of the intestine. Administration of eicosapentaenoic acid (EPA) increases adhesion of Lactobacillus paracasei to intestinal cells with 12% in the gut of piglets (89). The fatty acids are taken up by the bacteria, causing the formation of altered structures of the bacteria and eventually give rise to increased adhesion. This interaction works bidirectional, because in atopic babies of seven months old, the combination of Bifidobacterium Bb-12 and flaxseed oil showed a larger increase of linolenic acid in the plasma compared to flaxseed oil alone (90).

A very interesting article was published about brain to gut to ENS interaction by JD Wood(91). The effects of bacteria on the ENS was described but it starts with the influence of stress on the mucosal barrier, making the barrier more leaky, which in turn promotes the infiltration of pathogenic bacteria into the system. Pathogenic bacteria activate mast cells, which start to produce histamine and activate the ENS. Indirect activation of the ENS by pathogenic bacteria ultimately could give rise to IBS-like syndromes such as; IBS, Crohn's disease, Ulcerative Collitis, Gastro esophageal reflux disease and peptic ulcer disease.
This is one way to describe the brain gut interaction because in this model the stressed brain influences the mucosal barrier directly. Another interesting hypothesis is the inflammation of the gut is initiated after interaction between the stressed brain and mast cells in the gut. Mast cells get activated and start to produce histamine, which in turn activates the sensory neurons, interneurons and motor neurons in the ENS. As described earlier, the ENS is the little brain of the gut which can work independent, but in this case it gets innervated by the brains in an indirect way. After this, the neurons activate muscles, glands and blood vessels, which cause hyper secretion and power propulsion in the gut. Ultimately this causes the symptoms of the several disease as mentioned above. But, the gut wall could get inflamed, as well give rise to a leaky barrier and infiltration of several pathogens. To clarify the differences between both hypotheses, an overview is shown in figure 9.

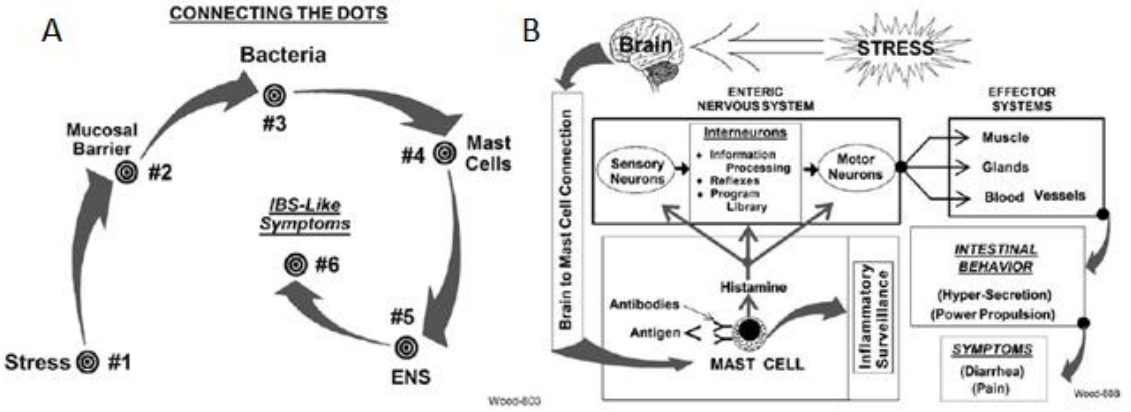


Figure 9: Schematic overview of two different mechanisms how stress could lead to alterations in the gut. A) Shows direct reaction of stress in the brain on the mucosal barrier which promotes leakiness and translocation of bacteria to the periphery, activating mast cells. Mast cells release histamine which activates the enteric nervous system (ENS) and cause Irritable Bowel syndrome-symptoms. B) Shows another mechanism of initiating an immune response in the gut by stress in the brain. Mast cells are activated and produce histamine. Histamine activates the ENS, which in case activate effector cells in the mucosal barrier. This causes hyper-secretion of mucus, diarrhea and pain. (91)

Animal models for PD and GI disturbances

To investigate Parkinson's disease and especially gastrointestinal functions, good animal models need to be available. Most animal models show an interesting pathology in the brain but fail to show changes in gastrointestinal function, unfortunately. This chapter will summarize animal models for Parkinson disease and examine if they are useful to use in research relating Parkinson disease with gastrointestinal disturbances. The most important animal models for the pathology in the brain are administration of MPTP, 6-OHDA, rotenone, paraquat and there are genetic models available. In figure 10 a simplified overview is shown of the different toxic substances, which can enter in the dopaminergic cells and exhibit their toxic actions there. Most of the animal models are developed to investigate symptomatic treatment. But, some are used to research the possibilities for neuroprotecting agents.

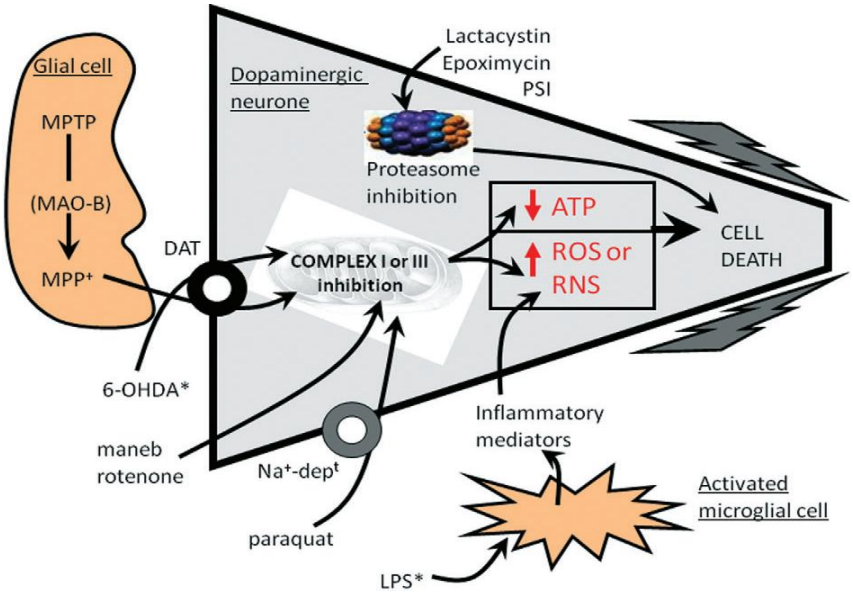


Figure 10: Toxins and dopaminergic cells in the Substantia Nigra pars compacta: An overview of different ways of entering the dopaminergic neuronal cells, initiating mitochondrial dysfunction, oxidative stress, neuroinflammation and proteasome inhibition. MPTP=1-Methyl-4-Phenyl-1,2,3,6-TetrahydroxyPyridine, MAO-B=MonoAmine Oxidase B, MPP=1-methyl-4-phenylpyridinium DAT=Dopamine Transporter 6-OHDA= 6-O-Hydroxy Dopamine, LPS=LipoPolySacharide ATP=Adenosine Tri-Phosphate, ROS=Reactive Oxygen Species, RNS= Reactive Nitrate Species (92)

MPTP model

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine) is a substance with neurotoxic action, it is a lipophilic protoxin which crossed the blood brain barrier rapidly. After crossing the barrier it is converted by MAO-B into an intermediary called $MPDP^+$. This intermediary undergoes rapid and spontaneous oxidation to MPP^+. This is the toxic moiety. After this, MPP^+ is released into the extracellular space and taken up by via DAT in the dopaminergic neurons. When MPP^+ is taken up it triggers the production of ROS which can contribute to neurotoxicity (92). Another mechanism of action of MPP^+ is disturbing the mitochondrial electron transport chain. This produces reduced availability of cellular ATP and a raise in ROS activity. Both mechanisms, inhibition of complex I and elevated ROS levels, promote activation of cell death pathways such as the p38 mitogen-activated kinase pathway, the c-jun N-terminal kinase (JNK) pathway and the bax pathway. MPTP is often used studies which study the central motor symptoms in PD (92).

MPTP was discovered by accident. Langston et al showed that MPTP causes marked Parkinsonism in 4 subjects after intravenous injection of MPPP. Unfortunately, no opoid effect was observed as intended but Parkinsonism was the result. After analysis of the substance, it became clear that MPTP was manufactured by a failure in the production mechanism instead of the synthetic opoid (93). The first hint however was obtained years earlier when a student in Maryland was synthesizing MPPP incorrectly and injecting himself with the compound. Probably the MPPP was contaminated with

MPTP. The student was successfully treated with Levodopa. On autotopsy the brain was examined and destruction of the dopaminergic neurons was observed.
Both failures in science show the validity of MPTP to use the substance in animal models to induce PD. However it has been shown that mice are more resistant to MPTP than humans. There is a general problem in science which is translation of results of animal models to humans and vice-versa. The second problem with this model, in contrast to central motor functions, is that MPTP causes no typical gastrointestinal disturbances associated with PD, in rodents (94,95). Instead of causing a decrease in gastric motility, hyper motility of the gut is observed. Interestingly, a decrease of 40% in dopaminergic myenteric neurons is observed (96).
MPTP rodent model is often used to study central motor effects in PD, but it is not useful to study the gastrointestinal disturbances in the disease. This could be due to the fact that mice and rats seem to be more resistant to the compound. This resistance could be caused by the rapid metabolism of MPP^+ in the body of the animals.

6-OHDA model
Before MPTP was discovered as a PD initiator, and studied extensively in PD research, 6-OHDA was used in animal models. This analogue of dopamine is a hydroxylated version of dopamine. It was observed as a toxin, inducing degeneration of the dopaminergic neurons in the nigra-striatal tract, by the group of Ungerstedt in 1968 (97). In contrast to MPTP, 6 OHDA is not efficient in crossing the blood-brain barrier. Therefore it needs to be injected straight into the brain. Because of the difficult way of administration, other mechanism such as an AAV viral vector, or liposomes could be examined in the future (92).
After injection, 6-OHDA is taken up with high affinity by DATs on the dopaminergic neurons. However 6-OHDA shows also a high affinity for nor-adrenergic transporter (NET). Addition of MAO-B inhibitors and NET inhibitors could be useful for more effective take up of 6-OHDA in the dopaminergic neurons. The exact mechanism of degeneration, by 6-OHDA, remains unclear. The current view is that 6-OHDA triggers several events in the dopaminergic neurons. 6-OHDA is known to oxidize very rapidly to form reactive oxygen species, for example H_2O_2. Additionally, a depletion of the total glutathione levels and superoxide dismutase levels is observed in the striatum. Levels of iron are elevated in the Substantia Nigra, due to 6-OHDA. Interactions with complexes I and IV of the mitochondrial respiratory chain are reported frequently. This promotes energy depletion in the neuron and further oxidative stress and upregulation of ROS species (98).
Because of the difficult way of administration of 6-OHDA to brain, a large variety in effects can be observed. Partial as well as full lesions can occur. To create the most effective model, 6-OHDA has to be injected in three different sites: the Substantia Nigra pars compacta, median forebrain bundle and the striatum (99).
This model has many similar features to the observed in Parkinson patients' brains, the reduced uptake of dopamine and degeneration of the dopaminergic nerves. But it does not follow the stage system as described by Braak et al. 6-OHDA works locally and cause no Lewy bodies (92). Furthermore, the administration is difficult and there is a large variety causing the lesions in the brain of the animals.
Based on the pathology that is there, 6-OHDA is used a lot for drug research for the central motor functions but many drug fail to succeed in clinical trials.

Recently, groups have been investigating the interactions between PD and the gut, and the problems that occur in this model. Tian et al. investigated two markers in the gut which could influence its functionality and emptying (81). They show a significant increase of tyrosine hydroxylase and DAT protein expression but a decrease in mRNA expression, suggesting a raise in dopamine expression in epithelial cells as well as cells from the enteric nervous system. This also could suggest that there is an increase in dopaminergic neurons in the gut. This is totally in contrast with the findings in the brain. Indeed Zheng et al showed that there is a significant upregulation of dopamine in the gut of 6-OHDA treated rat. Furthermore, not only the dopaminergic neurons are influenced in the gut, when 6-OHDA is administrated, the amount of acetylcholine is significantly

lower in treated rats comparing to controls, $p<0.05$. Additionally, Zheng et al studied food intake and bowel emptying in rats after 1 hour of fasting. They showed an increase of food uptake but a decrease in gastric emptying in 6-OHDA treated rats (80). Both groups used different ways of administrating 6-OHDA, Tian et al. used bilateral infusions and Zeng et al. used intraperitoneal injections. In 17 of 30 animals lesions in the brain where observed after 6-OHDA administration. These 17 rats were used in further experiments (80,81).
Zheng et al, used an uncommon way of administration and there could be a conflict in the results, because 6-OHDA does not pass the blood brain barrier, the delay in gastric emptying could be caused by the substance (80). Blandini et al showed a delayed gastric transition, and decrease stool output after administration of 6-OHDA but the exact mechanism remains to be elucidated (100).

Rotenone model
Rotenone is a pesticide which has been reported more often recently. It is a toxic pesticide and has been used because of the epidemiological association between pesticides and PD. Furthermore, an interaction is observed in PD patients predisposed to pesticide and complex I function in the mitochondria. Rotenone is, as MPTP, a highly lipophilic substance and crosses the blood brain barrier diffusing to the neurons in the brain, similar as MPTP, it accumulates in the mitochondria. Rotenone has been shown to inhibit this complex and causes oxidative phosphorylation by binding NAPH dehydrogenase (101).
Inhibition of complex I in the respiratory chain is not the important feature of Rotenone. The combination of inducing ROS activity and depletion of glutathione causes oxidative stress. Interestingly, carbonyl formation has been found in many areas in the brain including, midbrain, olfactory bulb, striatum and the cortex of the rats (92). This shows that PD involves more areas in the brain as suggested by Braak et al. Additionally, extensive microglial activity is observed after Rotenone administration, indicating that neuronal inflammation is involved, consistent with idiopathic PD in humans. Recently, Wang et al. observed that Rotenone inhibits proteosomal activity which is also implicated in the pathogenesis of PD(102).
Betarbet et al.(103) discovered this model but there are a couple of disadvantages. Rotenone is very toxic when administrated systematically. In this study almost 30% of the mice died, independent of the way of administration. But for the mice that survived Rotenone was not really effective. Almost 50% of the mice developed neurodegeneration. In the mice which developed neurodegeneration, GI disturbances were observed. Many other groups challenge the model. Especially Hoglinger et al showed interesting results. They showed that not only dopaminergic nerves are destroyed by Rotenone but also 5-HT fibres, cholinergic neurons, DARP32 projecting neurons as well as noradrenergic neurons in the locus coeruleus and Substantia Nigra pars compacta are for 20-30% destroyed(104). However, reduced locomotor activity and presence of Lewy bodies were observed in this study. No difference was observed in variability between subcutaneous and intravenous administration in the obtained results.
Drolet et al published an article about i.p. injection of Rotenone and the effects on gut motility as shown in figure 11. The figure describes an increase of transit. After 6 months there is a significant difference between the control group which received vehicle and the treated group which received rotenone I.P. in the transit time of the food (105).

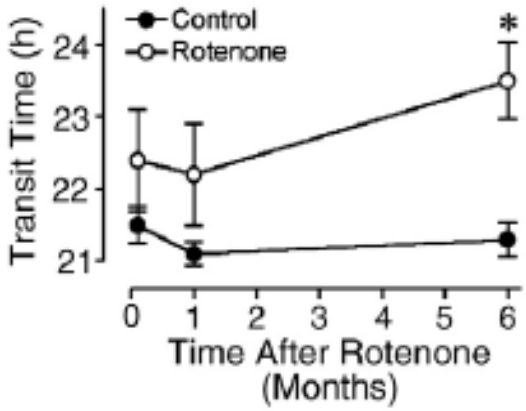


Figure 11: I.p. injection of rotenone leads to a delayed transition time of food in mice. (105)

Figure 11 shows that there is an effect of the rotenone on gut motility. However it could be confounding because the rotenone is administered systemically. The intraperitoneal administration showed to be more effective, so lower doses could be used. And reducing the amount of mice needed. The group of Cannon et al. used a novel fatty acid-based vehicle which reduces the death rates even more, without loss of the α-synuclein inclusions in the gut and the brain, and with at least 45% cell loss after 30 days of administration. The problem is that it has not been reproduced yet by other laboratories (106).

In the model an unnatural way of pesticide exposure is used. Therefore, Pan-Montojo et al. proposed an intra-gastric way of administration(78). In this study, rotenone was not present systemically. Inclusions of α-synuclein were observed in the enteric nervous system but, importantly also degeneration of the dopaminergic neurons in the Substantia Nigra pars compacta with α-synuclein inclusion bodies were present. Mice also showed a reduced motor function activity on the rotarod. Inclusions in the enteric nervous system and dorsal motor nucleus of the vagus were earlier present than in the brain. This indicates that another mechanism is involved, which could be the gut-brain axis. This study also confirms the suggestions that Braak et al. made that PD may not start in the Substantia Nigra pars compacta but in the periphery, in this case the enteric nervous system. This confirms the existence of the gut brain axis. Without entering the systemic system Rotenone was able to induce the pathology of PD in the brain (78).

This is a striking finding. However the main concern is really the rotenone causing the PD pathology here? Does it works directly on the enteric nervous system or via epithelial cells, or even enterochromafins? Is the transfer of α-synuclein to the brain that causes the pathology, or are other factors involved?

One downfall of the model is that there is nothing published yet about the motility of the gut, or if delayed emptying of the gut is present. Probably if studies try to publish this information, it will be difficult to distinguish the cause of the problems with the gut. This could be the result of the model or the direct impact of rotenone on the gut wall. It has to be made clear somehow, if delayed emptying of the gut or decreased motility of the gut is caused by rotenone or by the alterations in the enteric nervous system and the brain.

Existing drugs, except L-Dopa and Apomorphine, do not seem to be effective to treat the symptoms in this model. On the other hand, pramipexole(107) and seligiline(108), two neuroprotective agents which failed clinical trials, are effective in treating the symptoms of PD in the rotenone induced animal model, and could be of importance for a certain group of patients with early PD and are exposed to rotenone. There is certainly an interesting opportunity there for this model to use in further gut-brain-gut axis investigations and the influence on PD. Maybe, with this model it is possible to find new markers for drug in the future, after the model is optimized and the mechanism of action and alternating gut motility is confirmed in other papers.

Paraquat and Maneb model

The herbicide paraquat (1,1'-dimethyl 4,4'bipyridinium) and the fungicide maneb (manganese ethylene-bis-dithiocarbamate) are used as single substances as well as in combination to cause a Parkinson like disease model in rodents. Both can enter in a dopaminerigic neuronal cell via a Na^+ transporter. In the cell paraquat causes mitochondrial dysfunction by redox cycling and direct

inhibition of complex I while maneb inhibits complex III of the mitochondrial respiratory chain (92). When administered in combination a synergistic effect is observed because maneb is inhibiting the degradation of paraquat. The rationale for combining the two substances to generate a PD model is that they are used in the same geographic regions (109). Especially paraquat is gaining more interest recently, because epidemiological studies show more and more associations between paraquat and the prevalence and incidence of PD in the geographical regions where it is used. In a study in which the combination of these substances was injected i.p., another interesting fact was discovered. Nitric Oxide Syntase (iNOS) was significantly upregulated and give rise to significant increased levels of nitrite in the striatum of the mice. The amount of TH^+ cells in the Substantia Nigra pars compacta was 50% reduced. This indicates that besides a role in mitochondrial dysfunction, paraquat and maneb are also involved in generating oxidative stress in the dopaminergic cells (110). A major drawback of using this model is the suffering of the animals. Several animals died during the studies which are performed until now and this should be taken into account when this model is used in the future (111). Unfortunately, an unnatural way of administration is used in the model until now. It should be possible to mimic the human situation better by adding the substances to a diet or drinking water. Currently, nothing is published about alterations in gut so the model cannot be used to study problems in the gut.

Transgenic models for gastrointestinal disturbances in PD
Currently, there are two transgenic animal models which display disturbances in gut motility. These are a knockout model for the vesicular monoamine transporter 2 (VMAT2) and a model in which a gene combination of thy and α-synuclein is inserted, promoting over expression of α-synuclein in mice when Thy is injected, or ingested.
A disadvantage of transgenic models is that an artificial situation is created, which is not comparable to the situation in humans.

VMAT2 is responsible of packaging dopamine, noradrenaline and serotonine into synaptic vesicles. The first identification of a possible mouse model was described by Mooslehner et al (112). This is not a robust model because delayed gastric emptying is reported as well as enhanced gastric emptying, the same accounts for stool frequencies. Furthermore, it is very variable. Different statistical tests make it significant or not. For example it is significant using a student's t-test but when a two way ANOVA with Bonferroni post hoc analysis is used, it is not. This creates a non-robust model for gastric disturbances. Also, delayed effect where observed between 2-12 months but not after 18 months. With these conflicting results it is not possible to obtain confident results at this moment.

As described earlier α-synuclein plays an important role in the pathogenesis of PD. The Thy- α-synuclein over expressing model shows a delayed transit time of food proven by the use of a dye. Transit time was 2.2 fold higher in the transgenic mice compared to the control group. Furthermore the amount of colonic fecal content was 2.9 times higher in the transgenic mouse group. These results were obtained when the animals were placed in a familiar environment continuously. When mice were placed in a new environment, the amount of fecal content went down and frequency of stool increased leading to a shorter transit time. This fact indicates that stress plays a significant role in this model (113).
Recently, a new model was produced with a human mutant of α-synuclein inserted in the genome, A53T or A30P. Both mutants where inserted on a P1 artificial chromosome. Mice expressing both genes showed high levels of α-synuclein in the brain and the gut. They showed an increased transit time in the gut and the distal colon. A53T mice were more severely affected than A30P mice. Surprisingly, Kuo et al, the creators of the model, described that male mice are far more affected than female mice. This is not comparable with the human situation in which males as well as female are equally affected. Staining of the human α-synuclein revealed a co-localization of α-synuclein and nitric oxide synthase in sub mucosal and myentheric plexuses but, the majority was located in the synapses or near neural processes. The increased transit time of α-synuclein transgenic mice make

them an interesting model. Downfall is the unexplained difference between the grade of illness between male and female mice (114).

Concluding, at this moment there is not a specific mouse model mimicking the gastrointestinal problems described in human PD. Or the specific pathology in the brain is lacking or, in case of paraquat and the intragastrically administered rotenone, the gastrointestinal problems have not been evaluated yet. Furthermore, unexplainable differences occur between males and females, as in the case of the α-synuclein mutation models. In the future these models have to be optimized to obtain a useful general model, or the different models have to be improved for different specific purposes. This means that rotenone model could be used to test neuroprotective agents which failed in clinical trials. This model can be useful to test substances in specific clinical patient groups. The same accounts for agents which reduce or at least are involved in α-synuclein aggregation. The first, two older models could be used to research problems with central motor function, but they are not suitable to for studying gastrointestinal disturbances. The same problems occur with the VMAT KO model. Another interesting idea is to create a model with a pesticide or herbicide administered orally in a very low concentration in mice which have a mutation in one of the different genes. Especially LRRK2 is interesting because the mutant occurs in sporadic cases as well as in familiar cases of PD in humans. A confirmation of the exact mechanism of action of intragastric administration of Rotenone is necessary in the future because it could reveal several new markers and possible mechanism to inhibit the progression of the disease. Difficulties with translation of the results to human will be one of the most important things that have to be solved to obtain better results in clinical trials.

A role for nutrition for Parkinson or gastrointestinal disturbances in PD

At this moment there is still only symptomatic treatment available for patient with PD. With the recent developments and knowledge, it is maybe is possible to look into different approaches to try to inhibit the progression in an early phase of PD. One of the options is taking advantage of the properties of different nutrients. Due to the several failures of neuroprotective agents in clinical trials another approach could be useful. In this chapter caffeine, nutrients and nicotine will be discussed. Currently, several different nutrients have shown to be effective for symptomatic treatment and inhibiting the progression of PD in mouse models. Compounds which showed slightly positive results in some clinical trials include creatine, ubiquinone, vitamin D, ω-3 fatty acid and nicotine (115).

Consumption of coffee and tea

Caffeine is an antagonist of the adenosine 2A receptor, which seems to be involved in the improvement of motor function. The action of caffeine is not present in adenosine 2A receptor KO mice. Caffeine can bind in the striatum producing changes in the dopamine affinity of the receptors and in the signaling pathway (115). Recently, a novel mechanism of action was proposed by Jones et al. Caffeine would stimulate cytochrome oxidase activity in a sexually dimorphic manner, especially Cox7c is upregulated in male mice but not in females. The primary metabolite of caffeine, paraxanthine, showed protection against loss of synaptic function and dopaminergic cell death in a culture model. However, the exact mechanism remains unknown (116).

Several epidemiological studies have shown an association between a significant reduced incidence of PD and the coffee intake in males. The risk of PD in man declined from 10.4 per 10.000 person years, in men who drank no coffee, to 1.9 per 10000 person years ,in men who drank coffee, $p<0.01$. Data, after 6 years of follow up, from 8004 men was obtained from the Honolulu Heart Program (117). However, there is no association for women between coffee intake and PD risk. Estrogens may play an important role; It has been shown that in postmenopausal women, coffee intake reduce the risk of developing PD and increases it in women who take hormones and caffeine (118). In the MPTP animal model caffeine decreased the induced striatal dopamine loss in males, but not in females. In the males the effect was alleviated when estrogen was administered. Obviously, there is an interaction between estrogen and caffeine (119) . Currently, the hypothesis is that estrogen modulates the effect of caffeine.

Epidemiological evidence showed that tea could have a positive effect of the prevalence of PD. Two papers have shown that the incidence of PD in China is lower than in Caucasian cultures. Consumption of tea, even green tea, clearly lowers the risk of PD. Difficulties with tea are present because the effect on the prevalence cannot clearly be ascribed to caffeine, because tea contains several possible neuroprotective phenolic pleiotropic compounds. Most of the phenolic compounds have antioxidant and radical scavenger properties (120,121). In the future, it should be possible to find the compounds in tea which reduce the risk of PD. The neuroprotective properties of those agents could be an interesting topic.

Diets fatty acids, carbohydrates and proteins

The Mediterranean diet appears to be the preferable diet in protecting against several diseases. In PD, it has been observed that mortality lowers and that there is a protective effect against cognitive loss in an aging population. Trans-fatty acids intake should be as low as possible. The American Heart Association has made a recommendation for daily saturated fat intake to a maximum of 7% of the total calories. For PD however there is some doubt to this percentage because the risk on developing PD increases with low serum cholesterol (115). The effects of poly-unsaturated fatty acids will be discussed below.

The same accounts for carbohydrates, a healthy diet is necessary to keep the progression of the disease as slow as possible. It has been indicated that elevated glucose levels correlate with increased production of ROS. A linear correlation has been showed between elevated glucose levels

and the excretion of 8-iso prostaglandine F2 reflecting oxidative stress (122). A glucose challenge in healthy individuals stimulates superoxide production in mononuclear cells, activates Nuclear Factor κB (NF-κB) and inhibits endothelium-dependent vasodilation. In non-diabetic mice, epigenetic changes occur in the promoter for NF-κB. The changes are accompanied with an increase of adhesion and chemo attractant molecules levels. These changes can be reduced by reducing superoxide levels in the body (115). As mentioned earlier, PD seems to cause a diabetic like syndrome in mice, with lower levels of available insulin. This results in a decrease of fat tissue in mice. Concerning the average age of PD patients and the knowledge that is revealed by Karunuki et al 2011 on the diabetic syndrome occurring in mice which received 6-OHDA to induce PD, it is very important to reduce the amount of sugars used by the general population as well as PD patients (83).

Creatine

Creatine or α-methyl-guanidineacetic acid is synthesized from glycine, methionine and arginine. The protein is synthesized in the liver and pancreas and appears to have a mitochondrial –enhancing anti oxidative function. In healthy persons creatine is used to improve muscle performance during exercise and as an energy reserve (123). In the mitochondria, creatine is converted to phosphocreatine by creatine kinase and after the conversion it functions as a high energy buffer for conversion of ADP into ATP. The ratio between creatine and phosphocreatine balances the ratio between ADP and ATP. Furthermore, creatine shuttles high energy phosphate from the mitochondrial sites to cytosolic sites for ATP utilization. It has been suggested that creatine acts as an indirect antioxidant, by promoting energy transduction and stabilizing reactants inhibiting mitochondrial permeability transition (124)

Lawler et al. showed that creatine not only acts as an indirect antioxidant but also as a direct one against aqueous radicals and other reactive radicals such as peroxyl nitrite and superoxide (125). In animal models for neurodegenerative disorders the direct anti oxidative effects of creatine have been considered as a possible mechanism of action (126).

Creatine seems to have a number of side effects which are not admirable in PD including nausea, abdominal and muscle cramps, loss of appetite, diarrhea, weight gain and redistribution of body fluids. However a 10 gram daily administration of creatine is well tolerated (127).

An interesting fact about creatine is that 90% is found in skeletal muscle, but creatine deficiency contributes to mental retardation, seizures and speech delay (128).

In PD the function of the mitochondrial compartments is altered. The activity of the mitochondrial creatine kinase is induced causing the maintenance of high levels of ADP and an inhibition of the mitochondrial transition pore opening (129). This could be caused by several signals: mitochondrial complex 1 dysfunction, slowing of oxidative phosporylation, mitochondrial membrane depolarization, loss of coenzyme Q10, oxidative stress, calcium increase in the cell, DNA destruction and leakage of cytochrome c can all promote cell death via caspase 3 signal activation of members of the Bcl-2 family. Local rephosphorylation of ADP causes adequate functioning of the calcium pump and prevents calcium accumulation, which is a well-known promoter of apoptosis via exitotoxicity (130). In the MPTP mouse model, administered doses of 1-2% creatine by weight led to protection of cell death in this model. The animals used were male Swiss Webster mice, an outbred strain. In which normally at least 30% of the nigral cells die because of MPTP (131,132).

In a phase 2 clinical trial, creatine, 5 grams daily, was evaluated for alteration of PD development. 12 months after the beginning of the experiment the Unified Parkinson's Disease Rating Scale (UPDRS) was measured. 30% difference should be obtained to consider the drug an improvement, else it was rejected. Unfortunately, creatine did not reach the 30% improvement but based on the slow progression of PD with the component the authors suggested that it should be tested in a Phase III trial (133).

In 2007, U.S. National Institute of Health announced that a phase III study was started including 1720 early PD patients and it was expected to take 5-7 years. Creatine is compared with placebo. Unfortunately there are no preliminary results available of this study currently (134).

Ubiquinone

Coenzyme Q10 (CoQ10) is a conditionally essential lipid mobile benzoquinone which contains ten isoprenyl groups. In a reduced form, called ubiquinol, the molecule shuttles electrons along complexes I and III in the mitochondrial electron transport chain. From the perspective of the whole body, electrons are moved from metabolites of food to oxygen. This occurs during the process of storing energy in ATP, due to oxidative phosphorylation. CoQ10 is an effective free radical scavenger in human microsomal membranes and in low density proteins (135).

In PD patients, a 33% decrease of levels of CoQ10 in blood and platelet mitochondria has been observed, compared to age-matched and gender-matched controls. Additionally, levels of CoQ10 levels decrease faster in PD patients than in controls (136).

In several preclinical studies the role of CoQ10 was examined, *in vitro* as well as *in vivo* models for PD were used. In these models protection of CoQ10 against nigrostriatal dopiminergic damage was observed. In a MPTP induced PD model with C57BL/6, 1 year old mice (National Institute of Ageing), CoQ10 partially reversed the loss of dopaminergic nerves in the striatum (137). Moreover, CoQ10 decreases markers for neuronal death reduces dopaminergic cells degeneration induced by rotenone and a decrease in H_2O_2 in *in vitro* models and showed neuroprotective effects in rodent models (115). The exact mechanism of protection against neuronal cell death remains unclear. In a primate PD model it was shown that activation of the uncoupling protein 2 (UCP-2) was responsible for the neuroprotective effects of CoQ10. Over expression of the protein reduced the MPTP induced dopaminergic loss in those mice. The mice that were used were transgenic for UCP-2 over expression in a C57BL/6J background (138).

Clinically, the effect of CoQ10 was first tested in a small pilot study, a trend to normalization of mitochondrial complex 1 activity was observed but did not reach statistical significance due to a small sample size (139). But the same group continued with these results in a phase II clinical with 80 patients. A significant, clinically relevant, difference was observed in UDPRS scores, suggesting that CoQ10 may slow progression of PD. The low functional decline was observed in all three parts of the UDPRS (140). In 2007 another randomized double blind clinical trial was performed by The NINDS NET PD investigators (141). No clinical response difference was found between 2400 mg administration and placebo, but they concluded that further studies with a larger time scale and more power could be done. So the same group started a phase III study with 600 participants which were untreated for PD. Two dosages of CoQ10 were examined, 1200 mg and 2400 mg daily. The UDPRS was scored at 1, 8, 16, months. In April 2011, the study is terminated after 3 years because no effect of CoQ10 was observed compared to placebo. This failure could be due to misdiagnosing in the screening, the variety in subtypes of the disease or something else in involved which has to be elucidated (link: Appendix B). Currently, a new trial is set up which examines the effect of CoQ10 in Progressive Supranuclear Palsy (PSP) which is a specific atypical form of PD (link: Appendix B).

A significant issue with CoQ10 and several other endogenous enzymes and nutrients is that the substances are not regulated by the FDA. There is a lack of information about the quality and content of the preparations. In several report details on the preparation are missing which causes difficulties in setting up a clinical trial. Nowadays, not ubiquinone is used but its reduced form ubiquinol is used, because the absorption is better.

Vitamin D

Vitamin D (Calcitriol; 1,25-dihydroxyvitamin D) is a secosteroid hormone, which together with the vitamin D receptor is involved in genomic and non genomic signaling. Vitamin D plays an important role in health and in a wide spectrum of diseases. Currently, a difference of recommended amounts as stated by the FDA and the amounts needed for health benefit are very large. The amounts needed for health benefits are much higher than the dose stated by the FDA. Deficiencies are more often recognized and the importance of vitamin D in maintaining health is clarified (115).

The factor vitamin D is important for neutrophins, glutathione and iNOS monoamines synthesis regulation, as well as in suppressing apoptosis of neuronal cells. In PD this mechanism is altered, 55% of the PD patients from a neurological database showed low levels of vitamin D compared to 36% of healthy controls. PD patients were more severe vitamin D deficient than non-PD patients (142). In 2011 the same group published an article in which they analyzed the controls, 159 subjects, from the DATACOP (the Parkinson study group) trial. They observed that 69.4% of all the subjects in the control group had a vitamin D insufficiency and 26.1% had a deficiency for vitamin D. Surprisingly, the subject showed higher levels in vitamin D 18 months after the baseline measurement. This could be explained by natural fluctuations and by the effect of the sun in that period. Sunlight is known to enhance the production of vitamin D in the skin (143). Several studies show that low serum vitamin D levels are associated with poor cognition performance in older men and lower bone mass (144). The latter is interesting because vitamin D deficiency lowers proximal muscle strength and postural sway. Clinical trials have shown that vitamin D at a dose of 5000 international units increase lumbar bone mass with 28% when supplied with calcium. This combination prevents osteoporotic fractures (145). Currently, three new clinical trials are recruiting patients for the examination of the role of vitamin 3 in PD and loss of bone mass (links: Appendix B).Unfortunately the trials have not started yet, so there are no preliminary results.

In PD, falls, postural instability, frailty and poor muscle strength are common features. Vitamin D could be an interesting substance to add to the diet of PD patients and maybe even when people seem to have early PD without clinical symptoms, as suggested by Braak et al. Furthermore it is a cheap and simple way to intervene in this disease.

ω-3 fatty acids

The ratio between in ω-3 fatty acids and ω6 fatty acids in a typical American diet is about 1:20. In ancient times this was 1:1-2, and nowadays is has been suggested that the optimum ratio is 1:4. This ratio leads to an intake of ω-3 fatty acids that is approximately 3 times lower than the recommended amounts. All-cause mortality rate decreases with increasing intake of ω-3 fatty acids (146). Approximately 60% of the brain weight is fat, in which ω-3 fatty acids are dominant. In the nerve membrane phospholipids around 60% is Docosahexaenoic acid (DHA). Increasing intake of ω-3 fatty acids from several sources enhances cognitive function in healthy older adults (147). ω-3 fatty acids become part of the cell membranes in the brain and alter several parts of the cells were lipids are accumulated. Changes in raft structure, composition control and protein and lipid signaling occur (148). Additionally, ω-3 fatty acids are precursors for prostaglandins which have a very strong effect on inflammation. ω-3 fatty acids can activate the NF-κB pathway and influence oxidative stress, partly by inhibition of NOX .

In a primate animal model using MPTP, DHA was used as intervention therapy before and after L-Dopa therapy. DHA reduced LD-induced dyskinesias in both groups. This suggests that DHA could reduce the severity of motor symptoms or even delay the presence of those symptoms. Bousquet et al. showed that ω-3 fatty acids prevented dopaminergic cell death and increased the availability of dopamine in the striatum after MPTP injection, while a decrease of 50% in the availability of dopamine and a reduction of 31% of dopaminergic cells was observed after administration of ω-6 fatty acids. Several other mechanisms of action could play a role in the prevention of cell death. As MPTP alleviates oxidative stress, an antioxidant role could be there for ω-3 fatty acids, alternatively it could be that ω-3 fatty acids inactivate the caspase mediated apoptosis (149). Two important molecules can be derived from DHA, neuroprotectin 1 and sAPPα. The first one exerts several prosurvival and beneficial neurotrophic actions. These actions include upregulation of members

from the anti-apoptotic BCL-2 gene family, decrease of inflammatory mediators and maintenance of functional integrity of membranes. Furthermore neuroprotectin D1 can alternate signaling involving protein misfolding, which is an interesting new target (150). The latter substance, sAPPα, is involved in nerve growth and survival (151).
The studies on ω-3 fatty acids intake and the risk on PD show conflicting results. The Rotterdam study showed that ω-3 fatty acids significantly reduced the risk on PD, but not the ω-6 fatty acids (147). But, Chen et al. showed no correlation between the intake of ω-3 fatty acids and the decreased risk of developing PD, however this study could be biased because of the use of food questionnaires(152). An even more recent study on dietary intake of fish fruit and vegetables revealed an inverse association with the incidence of PD (115). All these results make it clear that there could be a function for ω-3 fatty acids in protecting the dopaminergic neurons in early PD.

Nicotine
There is consistent evidence from epidemiological studies that smoking can delay the onset of PD symptoms (57). This suggests that cigarette smoke may contain substances which are potentially neuroprotective (153). Below a large overview will be presented about all the preclinical evidence for the use of nicotine in clinical trials. Several actions of nicotine have been observed in animal models. At least 68 papers are published about the use of nicotine in animal models. In several species, neuroprotective effect of nicotine was observed in rescuing the TH^+ dopaminergic neuronal cells and dopamine levels after administration of MPTP, 6-OHDA and environmental toxins. Nicotine reverses the inhibiting effects of the toxins on several proteins. These proteins include complement component 1, heat shock protein 1, cathepsin B, caspase 9, interleukin 4 receptor, nuclear protein 1 and Toll-interleuking 1 receptor domain containing protein (119). The levels of FGF2 and BDNF mRNA are inhibited by MPTP and this effect is also reversed by nicotine (154). Furthermore nicotine reverses the effect of toxins on CYP2D22 (155) and HLA DR^+ microglia (156). Furthermore nicotine attenuates the dyskinesias caused by L-Dopa in different models in which 6-OHDA was used to create lesions in the brain (157). MPP^+, which is the toxic metabolite of MPTP, has been used together with nicotine, nicotine showed anti oxidative effects in reducing the amount of MPP^+ in the brain of rats (158). Unfortunately, the first clinical trial which was completed was not published indicating no positive result for the use of nicotine in PD.

Evaluating the different animal models of PD used to examine different nutrients in preclinical phase: Is the inadequate use of animal models the reason for failure in clinical trials?

Many different compounds, especially neuroprotective agents, which gave positive results in the pre-clinical trials, fail to show positive results in clinical trials. The question remains: What could be going on? There are a lot of downfalls in the studies that are performed in the pre-clinical as well as in the clinical phase. First of all, PD is not one disease, it consists of several subtypes and the genome of individuals is not comparable with others. Furthermore, different environmental factors are involved and many factors are probably not known at the moment. All of these facts lead to many different genotypes.

Secondly, the patients which enter clinical trials are not screened sufficient for all the different subtypes. A substance could be beneficial for a certain small group of PD patients, but is rejected because it shows no beneficial effect in the total study. In this field, it has been realized that this is not a good development. For example, pramipexole and seligiline failed to show neuroprotective effects in a clinical trial but the substances have shown to be effective in the rotenone animal model (107,108). The animal models that have been used to mimic the human form of PD are not sufficient. As it is not possible to test agents directly in humans, nutrients or compounds extracted from nutrients are an interesting opportunity to test. This chapter will evaluate preclinical evidence of the nutrients; Creatine, Coenzyme Q10, Vitamin D, ω-3 fatty acids and nicotine and the association between preclinical evidence and failures of substances in the clinic is examined.

For the nutrients, stated above, several clinical trials have been conducted. The failure of Coenzyme Q10, and several other failures in clinical trials with neuroprotective agents, led to the idea that an insufficient amount of data from animal models was available at the time of the start of the clinical trials. This is an important issue because a lot of money is involved in these clinical trials as stated in figure 12. After the preclinical phase, the costs are going exponentially up. So adequate examining of animal models is very important. Especially because the animal models available do not resemble the human form of PD.

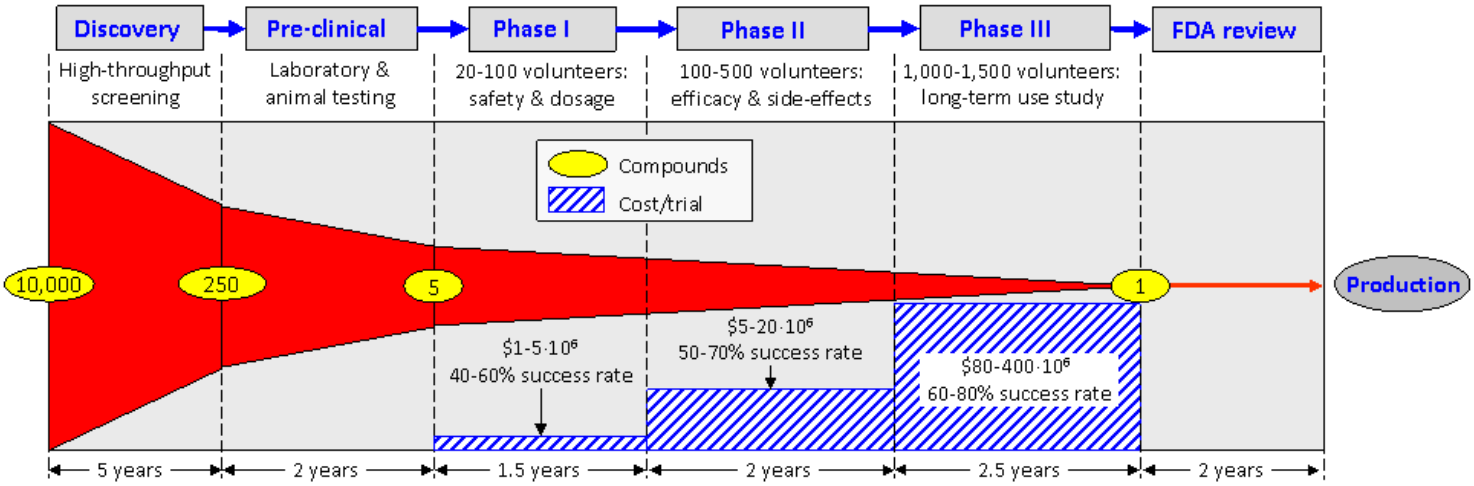


Figure 12: Overview of the time and costs from discovery of a target to bring a drug to the market. How further the drug gets in the pipeline, how less drugs stay effective, and the costs going up exponentially after entering the clinical phase. (http://maravelias.che.wisc.edu/?page_id=23)

To examine the animal trials in PD for creatine, coenzyme Q10, vitamin D, ω-3 fatty acids and nicotine, a search in the MEDLINE database was performed. The following queries were used, *Parkinson, Creatine, Coenzyme Q10, Vitamin D, Calcitriol, ω-3 fatty acids and Nicotine* in December 2011. This resulted in 1356 papers which were available for further examination. An overview of the results per compound and which species of animals were used is shown in table 3 and in figure 13. Detailed information is provided in the appendix A.

Table 3: Analysis of animal studies for five different nutrients in PD

Nutrient	Nr of studies found	Nr of studies with animal models	Mouse	Rat	Monkey	Cats	Drosphilia	First published study with supplementation
Creatine	452	**9**	6	2		1		Mattews et al 1999 (131)
Coenzyme Q10	113	**8**	4	2	1		1	Beal et al 1998 (137)
Vitamin D	42	**6**	5					Wang et al 2001 (159)
ω-3 Fatty acids	74	**10**	6	2	2			Grataroli et al 1988 (160)
Nicotine	675	**65**	20	31	14			Janson et al 1988 (161)
Total	1356	**98**	37	42	17	1	1	

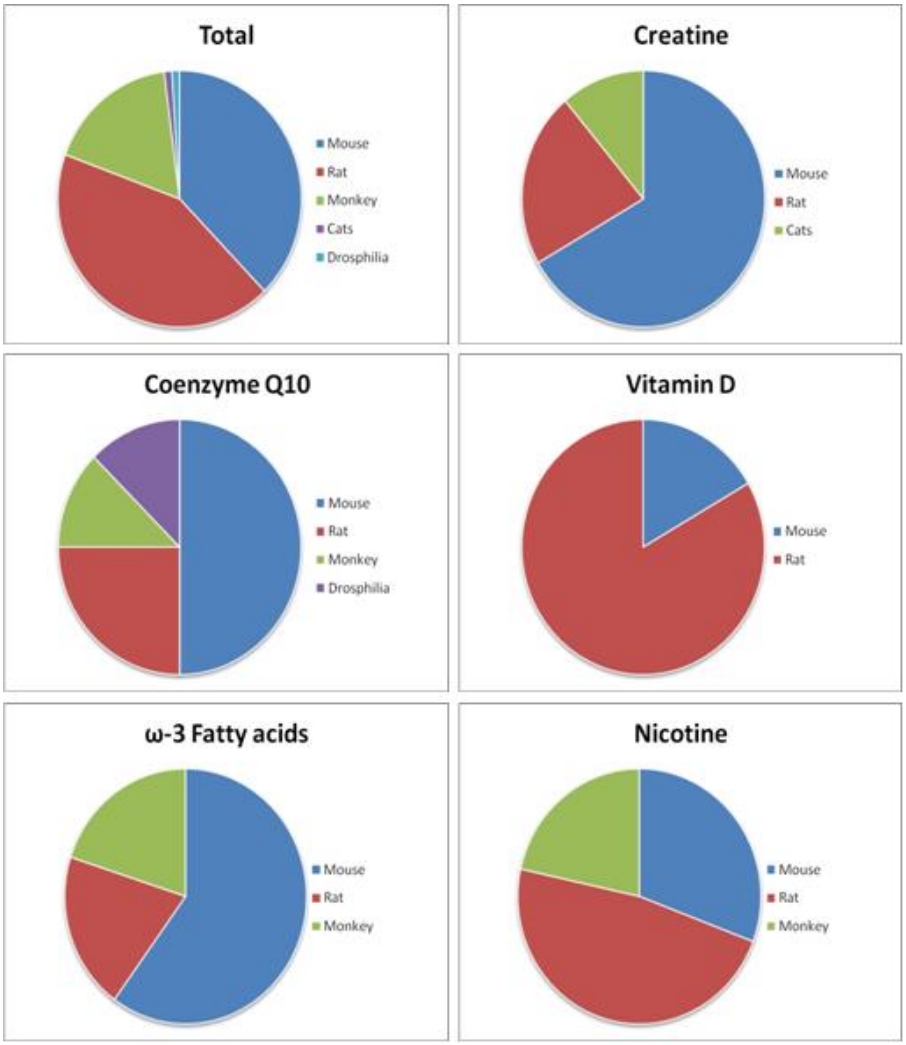


Figure 13: Overview of different animal models used per compound

In table 4 a short overview of the clinical trials for several compounds is given with the year in which they started. Additionally their current status will be examined. The website: http://clinicaltrials.gov/ct2/home was used with the following search terms: *Parkinson or Dyskinesia or Motor function AND Creatine or Coenzyme Q10 or Vitamin D or Omega fatty acids or Nicotine* examined in December 2011. This resulted in 22 clinical trials which had several statuses: Completed, Completed and no publication, Recruiting, Terminated and Unknown. The details are shown in Appendix B

Table 4: Overview of the status of the clinical trials on 5 nutrients, and the animal studies used before starting the clinical trials.

Nutrient	Number of clinical trials found	Completed, Published	Completed but un-published	Recruiting	Unknown	Terminated	Start year of first success-full of recruiting trial	Number of Preclinical trials performed before starting the clinical trial	Animal species used in pre clinical phase	Models used in pre-clinical phase
Creatine	5	3 (60%)	-	1 (20%)	1 (2004) (20%)	-	2003	2	1	1
Coenzyme Q10	7	2 (28.6%)	2 (1998,2006) (28.6%)	2 (28.6%)	-	1 (in 2011) (13,6%)	2003	2 (1 in 1998)	2	1
Vitamin D	3	-	-	3 (100%)	-	-	2007	4	1	3
ω-3 Fatty acids	4	-	1 (2005) (25%)	2 (50%)	1 (2008) (25%)	-	2009	7 (2 in 2005)	3	2
Nicotine	3	-	1 (2009) (33.3%)	2 (66,7%)	-	-	2009	58	3	10
Total	22	5 (22.7%)	4 (18.2%)	10 (45.5%)	2 (9.1%)	1 (4,5%)				

The amount of preclinical work which is performed before starting a clinical trial is very little. At the start of the clinical trials with creatine, coenzyme Q10, vitamin D and ω-3 fatty acids, a minimal of results was obtained from animal models. Furthermore, for all compounds few different animal species or models were used. The year of start taken for analysis of the preclinical evidence was based on completed or "recruiting patients for trial". In these cases the trials were successive or did not fail yet. Moreover, the initiators of the trials could have learned from failures of earlier clinical trials which failed. The amount preclinical evidence which was available at the start of the failures for coenzyme Q10 (started in 1998) and ω-3 Fatty acids (started in 2005) was even lower compared to this analysis, as one paper for coenzyme Q10 and two papers for ω-3 Fatty acids were available. For nicotine several papers were available at the start time of the clinical trial but 1 was completed and failed. Trials were considered as failures if: 1) The study was completed but not published, or 2) The status of the study was not known, and it should have finished at this time point (December 2011), or 3) When the study was terminated because no difference of the treatment compared to placebo was observed in preliminary studies. Based on this data the success rates of clinical trial were examined and those are shown in table 5.

Table 5: Success rates of clinical trials

Nutrient	Number of clinical trials	Completed, Published	Completed Unpublished	Unknown	Terminated	Succes rate (%)
Creatine	4	3		1		**75**
Coenzyme Q10	5	2	2		1	**40**
ω-3 Fatty acids	2		1	1		**0**
Nicotine	1		1			**0**
Total	12	5	4	2	1	**41,67**

In table 5, the results of studies which still recruited patients were not considered to be relevant, this led to no examination of vitamin D because the three trials are still recruiting patients. It is striking to see that the overall success rate is only 41,67% at this moment for 12 clinical trials which were completed, terminated or the status was unknown.

In summary, these results show the importance of adequate use of animal models. For all compounds, except nicotine, no more than 4 papers on the compound in animal models were available when a clinical trial was initiated. Furthermore, differences are observed in the species and models used for examining each different compounds. For these five compounds inadequate evaluation of the substance in different species and animal models could have led to the high rate of 58,33% of failures in the clinical trials. Other factors which could induce failures in clinical trials is the fact that many patients are misdiagnosed with the different forms of PD. Patient selection is important because some compounds could have positive effects in selective patient groups.

Conclusion

This thesis describes PD as a complicated disease in which environmental factors, genetics and mitochondrial dysfunction are involved. Several mechanisms, such as oxidative stress, exitotoxicity, mutations in several genes and environmental factors cause an impaired functioning of the mitochondria. This causes neuronal cell death in dopaminergic cells of the Substantia Nigra which eventually leads to the clinical symptoms of PD. Currently, only symptomatic treatment is available for PD patients. The combination of susceptibility to genetic mutations and environmental factors could be an interesting topic to investigate. This may reveal new markers for the treatment of PD. The gut-brain-gut axis is involved in PD as constipation is shown to be associated with the development of PD. This gastrointestinal problem seems to be present already 20 years before PD symptoms become present. Other predictors for developing PD in the future are anosmia, history of depression and REM sleep disorders. Indicating that evaluation of medical and disease history seems to be very important. In an animal model, in which the pesticide rotenone is administered intragastrically, PD pathology was observed without rotenone entering the periphery indicating that other mechanisms are involved which are currently unknown.
Animal models for PD and its progression do not reproduce all the clinical symptoms as in human disease. This, in combination with few preclinical animal studies which are done, contributes to failures in clinical trials. It is important to choose the correct model and use different models to confirm results. In the future it is possible to combine transgenic and toxin models to evaluate the interaction between these factors in the animals. These kinds of models should resemble the disease better in contrast to the human condition. In this thesis, the preclinical evidence for the use of five nutrients in clinical trials was analyzed. The results are striking, very few animal studies have been performed before the compounds enter the clinical phase. The overall success rate in the clinical trials of the 5 nutrients was approximately 42%. The poor use and examination of animal models could be one of the reasons for the failures in the clinical trials. The correct evaluation of nutrients, neuroprotective agents and symptomatic drugs in the preclinical phase could give rise to many more rejections in this phase with the consequent decrease of the economical burden for companies. When a compound is tested clinically the costs of the trials increase exponentially with the years tested.

Concluding, Parkinson's disease is a complicated disease, which is suggested to start in the periphery and not in the brain. The gut-brain-gut axis plays an important role in PD. Nutrients could be an interesting intervention but they have to be tested extensively in different animal models. Furthermore, a lot of effort has to be done on creating animal models which mimic the human situation as adequately as possible and the selection of patients for clinical trials should be more critical, with a good diagnosis of the disease. This could lead to fewer compounds tested in clinical trials and to fewer failures as well.

Appendix A: Overview of animal models performed for five different compounds

Table 6: Overview of creatine in animal models

Creatine								
Study	Animal model	Route of administration	Gender	Strain	Intervention	Number of animals	Results	Reference
1	MPTP 15mg/kg	I.P. 5 times, every 2H	Male	Swiss webster mice	0.25%-3% creatine, p.o. , 5 concentrations	7 groups of 10 mice	MPTP only causes a 70% reduce of dopamine levels, 1% creatine caused increase of 104% in dopamine levels vs MPTP ($p<0.01$)	Mattews et al 1999 (131)
1	MPTP 15mg/kg	I.P. 6 times, every 2H	Male	Swiss webster mice	1% creatine p.o.	4 groups of 12 mice	MPTP only causes a 66% reduce of dopamine levels, 1% creatine caused increase of 165% in dopamine levels vs MPTP ($p<0.01$)	Mattews et al 1999 (131)
2	MPTP 20mg/kg	I.P. 4 times, every 2H	Male	Swiss webster mice	2% creatine p.o.	3 groups of 12 mice	MPTP only causes a 69% reduce of dopamine levels, 2% creatine caused increase of 80% in dopamine levels vs MPTP ($p<0.05$)	Klivenya et al 2003 (132)
3	MPTP 5mg/kg	S.C. Daily for 10 consecutive days	Male	Cats	No supplementation of creatine but results were based on the the ratio N-actylaspartate, choline, myoinositol and glutamate with Creatine after MPTP intervention with or with an MAO inhibitor	3 groups of 3 cats	MPTP reduces the production of N-acetylaspartate/creatine ratio, but not the other ratios significantly compared with the MAO inhibitor	Podell et al 2003 (162)
4	Middle cerebral artery occlusion		Female	C57BL/6 mice	Prophylactic, 2% diet, ad libitum feeding	2 groups of 5 mice	The MCA causes severe loss of ATP in the brain, which leads to neurological symptoms, Creatine atenuates the drop in ATP and improves the neurological scores significant ($p<0.01$)	Zhu et al 2004 (163)
5	MPTP 20mg/kg	I.P. 4 times, every 2H	Male	C57BL/6 mice Charles river	No supplementation of creatine but results were based on the Lactate/Creatine ratio	25 mice	An significant decrease of dopamine levels of 90% was observed after the animals were treated with MPTP, the Lac/Cre ratio in these animals increased due severe increase of Lactate (baseline 0, after MPTP treatment 0.5)	Koga et al 2006 (164)
6	6-OHDA 1µg/µl	10µl, Unilateral injection in the medial forebrain bundle	Female	Sprague Dawley Rats Harlan	21 days post injection of 6-OHDA the creatine supplementation (2% diet) was started and after one month the L-dopa (6 mg/kg) intervention was started to induce dyskinesias.	2 groups of 9 rats for biochemical analysis, 2 groups of 12 animals for behavioral studies	A significant decrease in abnormal involuntary movements was observed in the group treated with creatine compared with the control group. In the biochemical analysis no interesting results were obtained	Valastro et al 2009 (165)
7	VMAT2	Low expression of the Vesicle Mono Amine Transporter 2, heterozygious for an insertion that disrupts the Slc18a2 gene	C= 6M, 3F KO M 3F	C57BL/6 Harlan background	No intervention, observing changes in several endogenous substances in the brain including Creatine/phosphocreatine	Wildtype 9 and VMAT KO 6 mice	A decrease in dopamine levels of 50% was observed in the cortex, hippocampus, striatum and 40% in the substantia nigra was observed. Creatine levels were significantly lower in the Striatum (after 12 and 24 months), Substantia Nigra (after 6,12 and 24 months), Hippocampus (after 24 months) and Cerebellum (after 6 and 12 months)In the VMAT2 KO mice compared to the wildtype	Salek et al 2008 (166)
8	MPTP 40 mg/kg/day	S.C. osmotic pump, 28 days	Male	C57BL/6J mice	2% diet, ad libitum feeding	3 groups of 15 mice	MPTP only causes a 56% reduce of dopamine levels, 2% creatine caused increase of 67% in dopamine levels vs MPTP ($p<0.05$)	Yang et al 2009 (167)
9	6-OHDA 2µg/µl	Injection of 5µl in the right part of the Substantia Nigra	Male	Wistar rats	Apomorhine was used to detect the rats with denervation in the brain	For imaging PD (n=10) C (n=6) and for behavioral studies PD (N=8) and C(N=6)	The number of TH^+ dopaminergic neurons in the right part Substantia nigra decreased significantly after 6-OHDA treatment. Furthermore the N-acetyl aspartate/creatine ratio was significant decreased compared to the controls. A dramatic, significant decrease in neurofilament protein and synaptosin was observed in the PD model compared with the controls ($p<0.01$)	Hou et al 2010 (168)

Table 7: Overview of Coenzyme Q10 in animal models

CoQ10								
Study	Animal model	Route of administration	Gender	Strain	Intervention	Number of animals	Results	Reference
1	MPTP 15 mg/kg	I.p. 5*15 mg/kg every 2hr	Male	C57BL/6 National Institute of ageing	CoQ10 200 mg/kg/day, food	3 groups of 11 one of 12 mice	MPTP reduced the amount of dopamine in the brain with 73%, CoQ10 attenuated this significantly compared to the MPTP controls with an increase of 37% ($p<0.01$) The TH-IR density was significantly reduced by MPTP but a significant increase of 62% was observed in the CoQ10 group ($p<0.01$)	Beal et al 1998 (137)
2	MPTP 1.5mg/kg	I.p. at Day 10	Male	Cercopithecus aethiops sabeus	CoQ10 100 mg/day longitudinally spread	4 groups of 6 primates	MPTP caused a decline in Dopaminergic neuronal cells of 70% ($p<0.01$) CoQ10 significantly attenuates the loss of Dopamine cells compared to the control MPTP group, the levels of recovery were not significantly different from both control groups	Horvath et al 2003 (169)
3	Rotenone 2,5 or 10mg/day	I.p. once daily for 60 days	?	Albino rats	CoQ10 200 mg/kg/day or 600 mg/kg/day, with or without additional L-dopa	7 groups of 10 rats	Rotenone decreased the levels of striatal dopamine, ATP, coenzyme ($p<0.01$) severely, Coenzyme Q10 induced the levels of striatal ATP and striatal coenzyme (both $p<0.001$) also the complex I activity was increased ($p<0.05$), but no increase was observed in striatal dopamine	Abdin et al 2008 (170)
4	MPTP 10 mg/kg	Acute: I.p 3 doses every 2 hrs, semi chronic: 10 mg/kg each 24 hours for 4 day, Chronic: S.c implanted osmotic minipump Alzet, 40 mg/kg/day for 28 days	Male	Swiss webster mice Charles River	CoQ10 1600 mg/kg in the (semi-) chronic models was the most effective concentration	4 groups 9-12 mice per group	A 60% decrease on striatal dopamine levels was observed after the MPTP treatment, CoQ10 increased the level of striatal dopamine significant ($p<0.05$)	Cleren et al 2008 (171)
5	MPTP 40 mg/kg/day	S.c. osmotic pump, 28 days	Male	C57BL/6J mice	1% diet, ad libitum feeding, start one week before MPTP	3 groups of 15 mice	MPTP only causes a 56% reduce of dopamine levels, 1% CoQ10 caused increase of 100% in dopamine levels vs MPTP ($p<0.01$), Further more an significant increase in TH+ neurons and dopamine was observed in the groups which received MPTP chronically and receive CoQ10 ($p<0.01$)	Yang et al 2009 (167)
6	DJ-1A	Transgenic model		Drosphilia	CoQ10 100 mg/ml	4 groups 25 heads per group	In this model no significant improvement on dopamine levels was observed, furthermore an increase was not shown in TH^+ cells for the treated group but not significant	Faust et al 2009 (172)
7	Paraquat 10 mg/kg	I.p. Injection 3 weeks post intervention, weekly injection for 3 weeks total	Male	Long evan hooded rats, Charles river	CoQ10 100 µg/ml +Polyoxyethany α-tocopheryl sebacate 150 µg/m, administered in drinking water	8 groups of rats, Biochemical analysis (C)+ (-PQ)N=3, (C)+ (+PQ) N=4,(Q10) + (-PQ) N=4, (Q10)+(+PQ)N=4, Behavior (C)+ (-PQ)N=6, (C)+ (+PQ) N=6,(Q10) + (-PQ) N=6, (Q10)+(+PQ)N=7	A significant decrease of TH+ neurons was observed after PQ treatment($p<0.001$) but coenzyme Q10 reversed the effect significantly ($p<0.001$), The behavioral test, walking backwards on the Rotarod, showed that animals treated with PQ and normal drinking water reduced their movements, indicating decline of motor functions, this was significantly different comparing with the other 3 groups ($p<0.01$)	Somayajulu et al 2009 (173)
8	MPTP 60 mg/kg	I.p. 2*30 mg/kg 24hr difference	Male	C57BL/6N	CoQ10 PO 1 day before every MPTP injection, 10% in emulsion of 50 mm particles	?	CoQ10 significantly attenuates the loss of Dopamine transporter in Dopaminergic synaptosomes and in crude synaptosome fraction ($p<0.05$) intrestingly the DAT Protein corrolated with the actin and synaptophysin protein levels	Kobayashi et al 2011 (174)

Table 8: Overview of vitamin D in animal models

Vitamin D								
Study number	Animal model	Route of administration	Gender	Strain	Intervention	Number of animals	Results	Reference
1	6-OHDA 8µg/3µL	Unilaterally, day 7, Median forebrain bundle	Male	Sprague Dawley	Vitamin D3, 7 days pre 6-OHDA, 1 µg/1 ml/kg	2 groups of 16 rats	A significant decrease of dopamine levels was observed after injection of 6-OHDA but there was a significant increase in the treated group with vitamin D3 compared to the 6-OHDA control group. Furthermore the TH+ cells were significantly protected and there was a significant increase in locomotor activity	Wang et al 2001 (159)
2	Zinc 0.2, 2.and 20 nM	Intranigral, unilateral day 7	Male	Sprague Dawley	I.p. vitamin D3, 13 days total,7 pre 6-OHDA, 1 µg/1 ml/kg	?	Zinc causes a decrease in striatal dopamine levels by 65% ($p<0.05$) Vitamin D3 diminished the increased levels of cytochrome c, Vitamin D3 increased the dopamine levels restoring it to almost normal, still significantly lower ($p<0.05$)	Lin et al 2003 (175)
3	Photothrombotic lesions	Catheterized femoral vein, rose bengal	Male	Wister rats	Vitamin D3, 4 µg/kg bodyweight	control 3 groups of 3 rates, 5 intervention groups of 6 rats	Vitamin D3 protect HO-1 positive glial cells in two different fields, near to the lesion and one remote region ($p<0.05$)	Oermann et al 2004 (176)
4	6-OHDA 100µg/ 10µL	Lateral Ventricle, day 7, together with protector of noradrenergic receptors	Male	Fischer 344 and Sprague Dawley (Harlan)	injection vitamin D3 1.0 µg/kg/day, short term 8 days, long term 3,5-4 weeks	4 groups of 7-9 animals	No difference was observed between but species of rats, A non significant trend was observed in increase of dopamine in both short and long term treatment, significant increases were seen in levels of DOPAC, HVA, potassium evoke overflow of Da, Amphetamine evoked overflow of DA and the striatal content of Da was increased	Smith et al 2006 (177)
5	6-OHDA 8µg/2µL	Unilaterally left median forebrain bundle, one group(gr 4) 6-OHDA at day 1,another group(gr3) 7 days post intervention	Male	Sprague Dawley	Intraperitoneal injection of 1 µg/ml/kg per day, group 3 7 day pre lesions, group 4 21 days after lesions	4 groups of 30 animals	The group that only received 6-OHDA showed a significant decrease in TH^+ dopaminergic neurons after 21 day ($p<0.001$) Both pre as post treatment showed the same results, an increase of TH+ neurons was observed comparing to the untreated group and an increase in the GDNF/tubulin ratio was observed, however it seems that the short term administration of vitamin D3 works better than long term treatment.	Sanchez et al 2009 (178)
6	Trangenic Klotho mice	Heterozygous$^{(kl/+)}$ mice were crossed with wildtype$^{(+/+)}$ or mutant$^{(kl/kl)}$ the obtained F1 mice were intercrossed to obtain wildtype, heterozygious and homozygious F2 mice	Male	C3HxC57 BL6 F2 Clea Japan Inc.	1.5 IU/g 1,25(OH)2D3 in diet ad libitum	4 time points, 2 groups per time point (n=6)	Only after 9,5 weeks a significant decrease in mecenphalic dopaminergic neurons was observed, at the other time points not. Indicating that vitamin D3 could influence the viability of the neurons in these transgenic mice.	Kosakai et al 2011 (179)

Table 9: Overview of ω-3-fatty acids in animal models

ω-3-fatty acids								
Study	Animal model	Route of administration	Gender	Strain	Intervention	Number of animals	Results	Reference
1	Wild type	-	Male	Wister rats, IFFA Credo	3 different diets: Low fat diet (4.4% fat), corn-oil enriched diet(17%) and salmon-oil enriched diet(12,5% supplemented with 4.5% corn-oil)	3 groups of 10 animals	In the Salmon-oil group a significant decrease was observed in the ω-6/ω-3 ratio in the gastric mucosa compared to the controls and corn-oil diet group	Grataroli et al 1988 (160)
2	Transgenic KO α-synuclein	*Sncα*-ablated inbred 129S6/SvEv	Sex matched	Mice	The diet contained 5.5% fat including 2.5% PUFA with an ω-6 to ω-3 ratio 4:1	2 groups of 6 mice	The composition of N-PUFAs was variable for different phospholipid in both models, for some phospholipids the Ko mice were in favor while for others the wildtypes were in favor. A significant decrease in complex I and III activity was observed in the KO model	Ellis et al 2005 (180)
3	MPTP	Repeated until bilateral parkinson syndrome was present, 5-6 months	Female	Drug naıve ovariectomized monkeys (Macaca fascicularis) (3–5 kg)	P.O. or S.C. L-Dopa, Bensserazide, DHA 100/25/100 mg/kg daily or L-Dopa/benserazide alone 100/25 mg/kg daily, emulsified in 8% PEG 600 and sterile water,	2 groups of monkeys	At the baseline the dyskinetic score was equal in both groups, after 4 week a significant difference was observed in the reducing dyskinesias for the group which received DHA treatment ($p<0.05$). Furthermore, a decrease in parkinsonian scores was observed already after 1 week and maintained significant after 4 weeks of L-Dopa administration and a significant increase is locomotor activity was observed($p<0.001$)	Samadi et al 2006 (181)
4	MPTP 20 mg/kg	I.p. 7 injections, 2 days twice daily, 3 days once daily	Male	C57BL/6 Charles River	DHA microencapsulated, in specialized diet 424 mg/kg	2 groups of 19 mice	The MPTP intervention caused a 31% decrease in the number of dopaminergic cells. The DHA intervention increased the amount of TH^+ positive cells significant ($p<0.01$). Nurr1, a protein involved in DA neuron survival, was significantly increased after DHA intervention	Bousquet et al 2008 (149)
5	Human α-synuclein transgenic	Human α-synuclein with the A53T mutation under the control of mouse PrP promoter	?	B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J)		3 groups	A 4 fold increase of human α-synuclein was observed in the transgenic mice, For several oxidative stress markers a significant decrease was observed in the n-3 fatty acid diet compared to the controls, Addition of DHA did not make a difference	Muntane et al 2010 (182)
6	MPTP 20 mg/kg	I.p. 7 injections, 2 days twice daily, 3 days once daily	Male	C57BL/6 Charles River	DHA microencapsulated, in specialized diet 425 mg/kg, months	2 groups of 19 mice	MPTP leads to a decrease of dopamine in the striatum of 53% ($p<0.01$) and the PUFA diet protects against loss of dopamine partly but significant comparing to the controls with MPTP administration ($p<0.01$) In the substantia nigra no loss of Dopaminergic neurons was observed compared to the control without MPTP administration. Interesting a trend was observed between the PUFA diet and increased numbers of dopaminergic neurons	Bousquet et al 2009 (183)
7	MPTP 0.5 mg/24hr	S.c. Alzet minipums, repeated until bilateral parkinson syndrome was present, 5-6 months	Female	Drug naıve ovariectomized monkeys (Macaca fascicularis) (2.5–4.3 kg)	P.O. L-Dopa, Bensserazide, DHA 100/100/25 mg/kg daily, emulsified in 8% PEG 600 and sterile water,	4 groups of 4-5 monkeys,	MPTP in this model cause a 99% decrease in dopamine levels in the striatum, L-Dopa induced dyskinesia were observed in all the groups which were treated with MPTP. However the score in the group which received DHA was lower (3.43 vs 4.05) compared to the controls. L-Dopa induces the production of preproencephalin mRNA in the caudate nucleus and putamen, addition of DHA in the diet reduces this upregulation op preproencephalin in the ventro medial as well as lateral parts of the caudate nucleus significantly.	Tamim et al 2010 (184)
8	6-OHDA	4 µg, middle forebrain, uni or bi lateral	Male	Wistar rats	4 gr/kg fish oil daily, containing 120 mg EPA and 180 mg DHA	2 groups of 64 rats	In this model a decrease of 54% in DA levels, Dopac 30% and HVA 38% (All,$p<0.0001$) was observed when 6-OHDA was administered, The fish oil did not alter the dopamine levels but the ratio over DOPAC/DA and HVA/DA were significantly increased comparing the treated animal with the controls (for both $p<0.05$)	Delattre et al 2010 (185)
9	Wildtype	-	Male	C57BL/6 Harlan	4 different diets, control (young and aged mice), ω-3-fatty acids (3 and 12%),	4 groups of 4-6 mice	In this study the brain was examined using MEMRI, Several significant alterations in the anteruir Pituary, arcuate nucleus, ventromedial hypothalamus, paraventricular nucleus were observed when the ω-3-fatty acids diet was used in low or high percentages,	Kou et al 2010 (186)

					saturated acids (3 and 12%), and combination of ω-3-fatty acids and saturated acids		interestingly for the buttermilk diet accounts the same	
10	MPTP 25 mg/kg	S.C. injection 10 times, in 3.5 days. The drug is combined with probecid which decrease renal excretion of MPTP	Male	C57BL/6 Charles River	Treated group received 0.8% E-EPA, Controls received palm oil, 5 week pre administration of MPTP and continued there diet after administration of MPTP	2 groups of 20 mice	Dopamine levels in al the group decreased significant, 80%. This was observed in striatum, frontal cortex and Hippocampus. There was no clear effect seen of both diets on behavior, Palm oil as well as E-EPA showed positive effects in the different tests, In this study proinflammatory effect of MPTP were examined, increase of striatal IFNγ and TNFα and Midbrain IL-10 was observed after MPTP administration in the control groups but not in the EPA groups so EPA attenuates the inflammatory response in the brain. Interestingly, COX-2 mRNA was upregulated in the brain of the mice treated with palm oil and no MPTP, decreased when MPTP was added, but in EPA it was significantly lower in the controls without MPTP but increased significant when MPTP was injected	Luchtman et al 2012 (187)

Table 10: Overview of nicotine in animal models

Nicotine								
Study	Animal model	Route of administration	Gender	Strain	Intervention	Number of animals	Results	Reference
	MPTP mice							
1	MPTP 36 mg/kg	i.p 4 injection, first time 36 mg/kg and 3 times 5 mg/kg with an interval of 2 hours	Male	C57BL/6	Pretreatment with 3 times 1 mg/kg, 30 minutes interval with the last dosis 30 minutes before administration of Diethyldithiocarbamate		A decrease in dopamine levels was observed when MPTP was administered in all the groups, it is observed that DDC and nicotine even cause a lower dopamine level in the mice. A significant increase was observed in FGF2 and BDNF mRNA levels which indicates a neuroprotective role	Maggio et al 1998 (154)
2	MPTP 15 mg/kg	4 injections in 1 day with 2 hour intervals	Male	C57BL/6 CERJ or Harlan France	Pretreatment with 0.2 or 2 mg nicotine, 5 times daily for 4 weeks	9 groups of mice	A significant decrease in TH^+ positive cells in the substantia nigra was observed compared to control ($p<0.001$), this decrease was partly reversed with the administration of nicotine, $p=0.024$ compared to MPTP treated group for the 2.0mg/kg and $p=0.044$ for the 0,2mg/kg group. Interestingly the loss of TH^+ is better protected by low exposure to nicotine than the high exposure group and the placebo group $p<0.037$	Parrain et al 2003 (188)
3	MPTP 20 mg/kg	Injection daily for 4 weeks	Male	Swiss albino mice IITR lucknow	8 weeks treatment, daily i.p. Injection of 1 mg/kg nicotine, pre MPTP treatment	2-3 mice per experimental group, 4 groups per experiment and 3-5 separate experiments	A decrease in TH^+ positive dopaminergic neurons and dopamine levels was observed after administration of MPTP ($p<0.001$), this was reversed by nicotine ($p<0.01$) This was observed pretreatment as well as post-treatment. CYP2D22 was lowered by MPTP but nicotine reversed this, a trend was observed but there was no significance	S Singh et al 2008 (155)
4	MPTP 20 mg/kg	Injection daily for 4 weeks	Male	Swiss albino mice IITR lucknow	8 weeks treatment, daily i.p. Injection of 1 mg/kg nicotine, pre MPTP treatment	2-3 mice per experimental group, 4 groups per experiment and 3-5 separate experiments	A significant decrease in TH^+ positive neurons was observed compared to control ($p<0.001$), this decrease was partly reversed with the administration of nicotine, $p<0.01$ compared to MPTP treated group. The effect of MPTP on several brain protein, such as complement component 1, heat shock protein 1, cathepsin B, caspase 9, interleukin 4 receptor, nuclear protein 1 and Toll-interleukin 1 receptor domain containing protein, are reversed by nicotine significantly	K Singh et al 2010 (119)
	Pesticides and herbicides							
5	Paraquat 10 mg/kg	Injection weekly 3 times	Male	C57BL/6 Charles River	6 times, orally, in 2% sacharine drinking water, first week, 3 times second week 3 times, administration with increasing concentrations, 25,50 100,200,300 and 400 µg/ml continued until 18 hours pre-sacrifice at week 6		Paraquat causes a significant decrease in TH^+ dopaminergic neurons in the substantia nigra ($P<0.001$) and this is partly reversed by nicotine($p<0.001$)	Khwaja et al 2007 (189)
6	Rotenone 30mg/kg	Administered orally dialy for 28 days by gavage with a catheter	Male	C57BL/6J SLC Japan	Nicotine was injected s.c. Daily 30 minutes for rotenone administration in 0.21 or 0.42 mg.kg	4 groups, 6-12 mice per group	Behavior was significantly altered by examining the latency to fall at the rotarod, a significant decrease was observed in the rotenone group which was not treated with nicotine ($p<0.001$), in the low as well as the high group this decrease was reversed significantly ($p<0.001$) and showed the same results as the control. Additionally a partly reversal was observed in the amount of TH^+ positive dopaminergic neurons ($p<0.05$)	Takeuchi et al 2009 (190)
	Transgenic models							
7	Point mutation in a4 nicotinic receptor	129ySvJ a4 genomic clone containing exon 5 and the L99S mutation was inserted into pKO Scrambler V907 which was after several steps introduced to embryonic stem cells, these stem cells		C57BL/6	0.02 mg/kg nicotine equivalent of nicotine hydrogen tartrate salt dissolved in 0.9% saline was injected 10 minutes post control measurement and 30 minutes before second behavioral test	18 mice	A severe decrease TH^+ dopaminergic neurons in the substantia nigra was observed, in heterozygotes without the NEO cassete deleted no effect was observed but after deletion the decrease was significant. Injection of nicotine significantly reduced the locomotor activity in the mutated mice, indication a neuroprotective role for nicotine	Labarca et al 2001 (191)

		were injected in oocytes from the mice						
8	Mutations in subunits of nicotinergic receptor	$\alpha6^{-/-}$ $\beta2^{-/-}\alpha4^{-/-}$ knockout mice were obtained as described earlier by Picciotto et al., 1995 Marubio et al., 1999 Champtiaux et al.,2002 m which were eventuely backcrossed with wildtype C57BL/6 to obtain heterozygotes	Male and Female	C57BL/6j	6-OHDA 3µg was injected stereotaxically to cause lesions, and cause abnormal involuntary movements wildtypes to examine the subtypes of the subunits of the nicotinergic receptor,a concentration range on nicotine was used to examine the dopamine release in striatal synaptosomes	60 animals for evaluations subunits	In the wildtype animals a significant decrease of α6, α5, α4, β3, β2 was observed ($p<0.05$) indicating these subunits are involved in PD, The assay in which the effect of increasing concentrations of nicotine on striatal dopamine release was examined showed a significant decrease in striatal dopamine release in the $\alpha4^{-/-}$ KO model but not in $\alpha6^{-/-}$, indicating that the α4 subunit is particularly important in PD	Champtiaux et al 2003 (192)
9	KO model for $\beta2^{-/-}$ subunit of nicotinergic receptor	$\beta2^{-/-}$ KO mice were crossed with wildtype C57BL/6 mice to obtain heterozygotes	Male and Female	C57BL/6 Charles River background	6-OHDA 3µg was injected stereotaxically to cause lesions, and cause abnormal involuntary movements, in heterozygotes to examine their locomotor function, Nicotine was started at 25 µg/ml and titrated up to 300 µg/ml in 10 days, Levodopa (3 mg/kg) was used to initiate dyskinesias		In wildtype control animal, a significant decrease in abnormal involuntary movements was observed when nicotine was added to the treatment compared with controls ($p<0.05$), after 15 weeks of levodopa administration a significant decrease was observed in the KO model when nicotine treatment was initiated compared with control ($p<0.01$)	Huang et al 2009 (193)
	Rats							
10	Hemitransection of the meso-diencephalic junction of the right hemispere	The lesion causes extensive axotomy of both ascending and descending pathway including the mesostriatal dopamine system	Male	Sprague Dawley	Immediately after the lesion 0,5 mg/kg nicotine was administered i.p. For 4 consecutive times with 30 minutes interval	2 groups control (n=4) and treated (n=6)	A significant increase in right striatal dopamine levels was observed in the group treated with intrastriatal nicotine pretreatment	Janson et al 1997 (194)
11	MPP^+ 75 nMol	Injected via microdialysis probe in the brain	Male	Wistar	Nicotine or saline was added to the MPP^+ administration and the amount of active DHBA was examined, Three concentrations of iron(II) were added to avoid oxidation of MPP^+	2 groups of 6 animals	Adding of nicotine with the MPP reduce the amount of the toxic metabolite from MPTP in the brain, indicating a protective effect for nicotine	Obata et al 2002 (158)
12	6-OHDA 12.5 µg/µL	Injected unilaterally in the right side of de median fore bundle	Male	Sprague Dawley Harlan	Intrastriatal pretreatment with nicotine before 6-OHDA administration, and post treatment	2 groups of 6 animals	A significant increase in striatal dopamine levels were observed in the group treated with intrastriatal nicotine pretreatment 4 as well as 8 days	Visanji et al 2006 (195)
13	6-OHDA 3 µg/µL	Injected 2 µL,twice unilaterally in the right side of de median fore bundle	Male	Sprague Dawley	L-dopa 8 mg/kg in combination with 15 mg/kg benzarazide was administered to induce dyskinesias. For nicotine 2 different techniques were used, normal injection of nicotine (0.01-0.75 mg/kg) 10 and 30 minutes pre-treatment of L-dopa or via an Alzet osmotic pump (0.1-2 mg/kg/day)	6 groups of 10 animals	A significant decrease in abnormal involuntary movements was observed after an injection with 0.1 mg/kg of nicotine compared to the controls ($p<0.01$), the same is shown for the use of the osmotic pump. A significant decrease of 99% loss of striatal dopamine transporter was observed after administration of 6-OHDA	Bordia et al 2010 (157)
	Monkeys							
14	Nicotine	I.m. Administration nicotine bitartrate (3.2-56 µg/kg) 15 prior each session.	Male	Macaca Mulatta,6 years old	The effect of nicotine is examined in cognitive tests for the animal	6 animals	A trend of improvement in the delayed match to sample test, self ordered spatial search test and paired associate learning test compared with the controls was observed in this model but it is not significant	Katner et al 2004 (196)
15	MPTP 0.025-0.10 mg/kg	I.v. Injection 3 times a week, during 98-198 days	Male	Macaca Mulatta,8-9 years old	Levodopa(20mg/kg) was injected 60 minutes before the performance task, Nicotine ditartrate (0.5 mg/kg) was injected 20 min before the performance task. A wash out period of 4 days was used to perform the control tests	3 animals	A significant effect of nicotine in the modified delayed response task with cue durations of 2,4,6-8 seconds compared to untreated animals ($p<0.01$). The effect nicotine on levodopa induced deficits on the modified delayed response task was significant compared to levodopa alone ($p<0.05$)	Decamp et al 2009 (197)

16	MPTP 2 mg/kg	S.c. One injection, control saline	Female	Saimiri sciureus, squirrel monkey	One month after the MPTP treatment was started with Tang in drinking water, then was started at Day 10 with 10 µg/mL nicotine and this was titrated up to 650 µg/mL in 3 weeks and maintained after, 24 hours before sacrifice the animals were withdrawn	11 animal, Control (N=3), Nicotine (N=4), MPTP (n=4) and MPTP+nicotine (n=4)	Slight improvement was observed in monkey receiving nicotine but it was significant ($p<0.05$) previous work has shown that no increasement of TH^+ positive dopaminergic neuron occurred after treatment with nicotine.	Huang et al 2009 Quik et al 2006 (193,198)
17	MPTP 1,5 mg/kg	S.c. One injection, control saline once every 2 months	Female	Saimiri sciureus, squirrel monkey	6 months before the MPTP treatment was started, the titration period for nicotine was initiated with 10 µg/mL nicotin in 1% sacharin drinking water and this was titrated up to 650 µg/mL in 3 months and maintained after, 24 hours before sacrifice the animals were withdrawn		A significant decrease in dopamine transporter level was observed after the MPTP administration. Interestingly an increase in expression of α3/α6β2* and α4β2 nicotinergic, receptor subunits was observed in the group of animals which received Nicotine and were lesioned by MPTP. An increase of HLA DR^+ microglia was observed in both groups which received nicotine, but the increase in the group with the MPTP lesions was significantly higher ($p<0.05$)	Quik et al 2010 (156)

Appendix B: Overview of clinical trials in which the five compounds are evaluated

Treatment	Study	(Estimated) enrollment of patients	Phase	Datum initiated	Status	Reference
Creatine						
	National Institute of Neurological Disorders and Stroke (NINDS) Parkinson's Disease Neuroprotection Trial	195	II	May 2003	Completed	Bavina et al. 2006 (133)
	Creatine supplementation in Parkinson disease: A placebo-controlled, randomized pilot trial	60	II	October 2000 and May 2003	Completed	Bender et al 2008 (127)
	Resistance training with creatine monohydrate improves upper-body strength in patients with Parkinson disease: A randomized trial	20	II		Completed	Hass et al 2007 (199)
	NET-PD LS-1 Creatine in Parkinson's Disease	1741	III	20 March 2007	Ongoing, not recruiting, estimated finish May 2015	Bloom et al 2007, Parashos et al 2009, Schneider et al 2010 (134,200,201)
	A Pilot Clinical Trial of Pyruvate, Creatine, and Niacinamide in Progressive Supranuclear Palsy.	20	I	April 2004	**Unknown**, not been verified recently, estimated finish December 2009	http://clinicaltrials.gov/ct2/show/NCT00605930?term=motor+function&intr=creatine&rank=2
Coenzyme Q10						
	Mono-Center, Prospective, Double-Blind, Placebo-Controlled, Randomized Clinical Phase IIa Trial to Assess the Safety, Tolerability, and Immediate Biological Effects of **Coenzyme Q10** - nanoQuinon® in Progressive Supranuclear Palsy	20	I and II	May 2006	Completed, **no publication**	http://clinicaltrials.gov/ct2/show/NCT00328874?term=Parkinson%27s+Disease&intr=coenzyme+q10&rank=8
	Effects of Coenzyme Q10 in Progressive Supranuclear Palsy (PSP)	60	II and III	September 2006	Recruiting, preliminary results in January 2012	http://clinicaltrials.gov/ct2/show/NCT00382824?term=Parkinson%27s+Disease&intr=coenzyme+q10&rank=5
	NINDS Parkinson's Disease Neuroprotection Trial of CoQ10 and GPI 1485	195	II	January 2004	Completed	NINDS NET PD et al 2007 (141)
	Effects of Coenzyme Q10 in Early Parkinson's Disease	80	II	September 1998	Completed, **no publication**	http://clinicaltrials.gov/ct2/show/NCT00004731?term=Parkinson%27s+Disease&intr=coenzyme+q10&rank=3
	Randomized, double-blind, placebo-controlled trial on symptomatic effects of coenzyme Q(10) in Parkinson disease.	131		September 2003	Completed	Storch et al 2007(202)
	Effects of Coenzyme Q10 (CoQ) in Parkinson Disease (QE3)	600	III	December 2008	**Terminated August 2011**	http://clinicaltrials.gov/ct2/show/NCT00740714?term=Parkinson%27s+Disease&intr=coenzyme+q10&rank=1
	Phase III Trial of Coenzyme Q10 in Mitochondrial Diseases	50	III	January 2007	Recruiting, estimated finish May 2012	http://clinicaltrials.gov/ct2/show/NCT00432744?term=motor+function&intr=coenzyme+q10&rank=1
Vitamin D						
	Clinical Effects of Vitamin D Repletion in Patients With Parkinson's Disease (VIDIP PILOT)	150	IV	May 2007	Recruiting, estimated finish December 2011 (last update: January 2011)	http://clinicaltrials.gov/ct2/show/NCT00571285?term=Parkinson%27s+Disease&intr=vitamin+D&rank=2
	The Effects of Vitamin D and Bone Loss in Parkinson's Disease (PDVD3	40	II	September 2009	Recruiting, estimated finish May 2011, no preliminary data available (last uptdate:	http://clinicaltrials.gov/ct2/show/NCT00907972?term=Parkinson%27s+Disease&intr=vitamin+D&rank=1

					June 2011)	
	Effects of Vitamin D in Parkinson's Disease (PD)	140	II	May 2011	Recruiting, estimated finish June 2015, no preliminary data available (last uptdate: June 2011)	http://clinicaltrials.gov/ct2/show/NCT01119131?term=Parkinson%27s+Disease&intr=vitamin+D&rank=3
ω-3 fatty acids						
	Efficacy of Docosahexaenoic Acid on Tardive Dyskinesia	40	II	February 2008	**Unknown**, not been verified recently, estimated finish June 2011	http://clinicaltrials.gov/ct2/show/NCT00621634?intr=fish+oil&rank=89
	The Effects of Efalex Active 50+ on Cognitive Performance, Well-being and Cerebral Hemodynamics in Healthy Older Adults	250		July 2010	Recruiting, estimated finish May 2012	http://clinicaltrials.gov/ct2/show/NCT01185379?intr=fish+oil&rank=98
	Effects of Dietary Interventions on the Aging Brain		IV	November 2009	Recruiting, estimated finish November 2012	http://clinicaltrials.gov/ct2/show/NCT00996229?intr=fish+oil&rank=136
	The Efficacy of Omega-3 Fatty Acids in Maintaining Optimal Mental Health in Elderly People	302		October 2005	Completed, **no publication**	http://clinicaltrials.gov/ct2/show/NCT00124852?intr=fish+oil&rank=200
Nicotine						
	Efficacy of Transdermal Nicotine, on Motor Symptoms in Advanced Parkinson's Disease (NICOPARK2)	40	I and II	February 2009	Recruiting, estimated finish May 2012	http://clinicaltrials.gov/ct2/show/NCT00873392?term=motor+function&intr=nicotine&rank=1
	Randomized, Double-Blind, Parallel Group, Placebo Controlled Safety, Tolerability and Efficacy Study of NP002 in Subjects With Idiopathic Parkinson's Disease With Dyskinesias Due to Levodopa Therapy	65	I and II	October 2009	Completed, **no publication**	http://clinicaltrials.gov/ct2/show/NCT00957918?term=parkinson&intr=nicotine&rank=3
	Nicotine Treatment of Impulsivity in Parkinson's Disease: A Pilot Study	20		October 2010	Recruiting, estimated finish December 2011, no preliminary results available	http://clinicaltrials.gov/ct2/show/NCT01216904?term=parkinson&intr=nicotine&rank=2

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