1 GENERAL INTRODUCTION
PARKINSON’S DISEASE

In his ‘Essay on the Shaking Palsy (1817) [1], the English surgeon James Parkinson was the first to extensively describe the clinical syndrome of ‘paralysis agitans’, which was later to bear his name. Parkinson’s disease (PD) is a progressive neurodegenerative disorder affecting more than 5 million individuals worldwide [2]. In the Dutch population, PD has a prevalence of approximately 0.3 percent among persons aged 55 to 64, rising to 4.3% in the age range of 85 to 94 [3]. Although aging is a major risk factor for PD, the disease can already start before the age of 21 [4], and ten percent of patients is younger than 45 years [5].

Bradykinesia, muscle rigidity, resting tremor and postural instability are the cardinal motor symptoms that clinically characterize PD [6]. As we have come to appreciate, PD is not merely a movement disorder, since most PD patients also suffer from a variety of non-motor symptoms. These non-motor symptoms can already appear years before the onset of the motor symptoms and include hyposmia, autonomic dysfunction, sleep disorders, depression, anxiety, psychosis and cognitive dysfunction [7].

PD is characterized neuropathologically by the region-specific loss of neurons, including the dopaminergic neurons in the substantia nigra pars compacta (SNpc) and noradrenergic neurons in the locus ceruleus. Besides neuronal loss, inclusions of aggregated proteins, called Lewy neurites and Lewy bodies, are observed. Of these pathological structures the protein alpha-synuclein is the primary component [8]. With progression of the disease, alpha-synuclein pathology affects specific brain regions in a predictive caudal to rostral order [9]. The pathology begins in lower brain stem nuclei, first affecting the dorsal motor nucleus of the vagal nerve. The locus ceruleus is another brain stem region that is involved in the early pathological stages of PD. At these stages, the olfactory system and the peripheral autonomic nervous system may already be affected [9-11]. In later pathological stages, alpha-synuclein pathology ascends to the substantia nigra in the midbrain and, subsequently, via the amygdala and entorhinal cortex, towards neocortical areas.

The cardinal motor symptoms of PD result from the loss of dopaminergic neurons in the SNpc that project to the striatum. Cell counts in quantitative post-mortem studies of the nigral dopaminergic neurons indicate that at the onset of the classical motor symptoms at least 48% of the dopaminergic neurons are already lost [12]. The non-motor symptoms are to a large extent associated with extranigral Lewy body pathology involving non-dopaminergic neurotransmitter systems [13]. Olfactory dysfunction in PD, for example, appears to be related to Lewy body pathology in the olfactory bulb [13] whereas autonomic dysfunction could be associated with pathology in the central and peripheral autonomic nervous system [13,14]. Cholinergic and noradrenergic deficits probably contribute to cognitive impairment in PD [15].
It is unlikely that a single cause for PD will ever be found [16]. Although one in seven PD patients has a first-degree relative with parkinsonism, less than 10% of familial disease is currently ascribed to a single gene mutation [17] and most patients suffer from the sporadic form. Sporadic PD most likely has a multifactorial etiology, including genetic as well as environmental risk factors. Research over the past decades has revealed various mechanisms at the molecular level that may play a role in the pathogenesis of PD. A central pathogenetic role is currently attributed to alterations in protein handling in the form of either increased production of unwanted proteins or their impaired clearance by the ubiquitin proteasome system or the endolysosomal pathway [16,18]. Of note, alpha-synuclein is considered a key player in these pathogenetic processes. Other major processes that are supposed to contribute to neuronal death in PD include oxidative stress and mitochondrial dysfunction [16].

The current treatment for PD is symptomatic, and primarily focuses on the substitution of nigrostriatal dopamine [5]. Levodopa, an intermediate in the pathway of dopamine synthesis [19], in combination with a peripheral dopa decarboxylase inhibitor (e.g. benserazide or carbidopa), is presently the most effective symptomatic treatment [5,20]. Dopamine agonists (e.g. pramipexole, ropinorole, rotigotine) are other efficacious dopaminergic drugs, while also selective type B monoamine oxidase inhibitors (e.g. selegiline and rasagiline) and amantadine have antiparkinsonian effects. The existing treatments effectively alleviate bradykinesia and muscle rigidity, in particular early in the disease course [19]. However, with progression of the disease, higher doses are required and side-effects emerge in many patients, in particular motor response fluctuations and levodopa-induced involuntary movements (dyskinesias) [19]. Furthermore, many of the non-motor symptoms cannot be treated adequately [21].

**DIAGNOSTIC ACCURACY**

Currently, a diagnosis of PD essentially relies on clinical features: the presence of bradykinesia in combination with muscle rigidity, resting tremor and/or postural instability [6]. Signs and symptoms indicative of atypical parkinsonian disorders, such as early severe autonomic involvement, cerebellar signs or early severe dementia need to be absent for a clinical diagnosis of PD, whereas supporting features include a progressive nature of the disease, a positive effect of dopamine replacement therapy and a unilateral onset [5,6].

Clinico-pathological studies indicate that a clinical diagnosis of PD does not perfectly match the pathological diagnosis [6]. In particular in the early stages, PD can easily be confused with clinically overlapping disorders such as essential tremor, vascular parkinsonism, progressive supranuclear palsy (PSP), multiple system atrophy (MSA) or other atypical parkinsonian syndromes. Clinico-pathological studies have established that the final clinical diagnosis of PD by a general neurologist has an accuracy of 76% [6], which means that only 76% of PD patients are correctly diagnosed by general neurologists during life. Movement
disorder specialists reach a much higher diagnostic accuracy with 98.6% of PD patients ultimately correctly diagnosed [22]. Diagnostic accuracy of the initial clinical diagnosis is probably substantially lower, considering that even movement disorders specialists revise their initial clinical diagnosis in almost one third of patients with parkinsonism within the first five years of the disease [22].

Imaging techniques are used increasingly to improve the certainty of a diagnosis of PD. MRI scanning can help to exclude secondary causes of parkinsonism, such as normal pressure hydrocephalus and extensive subcortical vascular pathology [5]. MRI scanning may further show specific abnormalities associated with atypical degenerative parkinsonian syndromes; a hyperintense putaminal rim or hot cross bun sign in multiple system atrophy (MSA) or midbrain atrophy in progressive supranuclear palsy (PSP) [23]. However, the sensitivity of these findings is low [23]. Other imaging techniques, single photon emission computed tomography (SPECT) or Positron Emission Tomography (PET) can be applied in patients with diagnostic uncertainty to assess the presynaptic functional integrity of dopamine neurons. These functional imaging techniques can facilitate the differential diagnosis between PD and drug-induced, psychogenic and vascular parkinsonism or essential tremor, but do not help to differentiate PD from other neurodegenerative parkinsonian syndromes, such as MSA or PSP [24].

Apart from the overlap in clinical features between the various neurodegenerative disorders, there is neuropathological overlap that also influences clinical diagnostic accuracy. Frequently, different types of pathology associated with specific neurodegenerative diseases, e.g. misfolded alpha-synuclein in Lewy bodies, extracellular amyloid-beta plaques and intracellular aggregates consisting of hyperphosphorylated tau in neurofibrillary tangles, co-occur in the aging brain [25].

A high level of diagnostic accuracy in the early phase of PD is important, in the first place because related disorders with which PD can be confused differ in their rate of clinical progression and response to medication. An accurate and early diagnosis will enable physicians to provide patients and their caregivers with a more precise prognosis and therapeutic options, which is essential for the patients’ future planning. In addition, a higher degree of diagnostic accuracy would improve selection of PD patients for therapeutic clinical trials. The erroneous inclusion of non-PD patients confounds the interpretation of trial results and raises trial costs by requiring larger group sizes to detect treatment effects [26].

BIOMARKERS

Biomarkers are objective measures of a disease state [27]. Biomarkers can be classified into different types, based on their application. Diagnostic markers serve to improve the
diagnostic accuracy of a disease. In addition, this type of biomarker can play a role in
the early detection of a disease. Biomarkers can also be implemented to stratify patients
into subtypes that require different therapeutic approaches and/or have a different rate of
progression of the disease (prognostic biomarkers). For example, PD patients have previously
been segregated into subgroups based on a clinical data-driven approach: early disease
onset, tremor-dominant, non-tremor dominant and rapid disease progression without
dementia [28]. Patients with non-tremor dominant disease were more frequently demented
than patients with early disease onset and had higher pathological grading of cortical Lewy
bodies and more cortical amyloid plaques post mortem. By contrast, the group with early
disease onset had the longest disease duration until death and the greatest delay to the onset
of falls and cognitive decline [29]. Lastly, a progression biomarker reflects the severity of the
underlying disease process, for example the distribution of Lewy body pathology. This type
of biomarker can serve as a marker of disease progression and therefore also be used to
monitor the effect of disease-modifying therapies.

The ideal diagnostic biomarker is sensitive and specific, meaning in the case of PD that
it correctly identifies all patients with PD without erroneously identifying a non-PD
patient as having PD. Independent of the type of biomarker, the ideal biomarker should be
reproducible, easy to measure, inexpensive, closely associated with the disease process and
non-invasive [30].

It is important to determine what biomarkers exactly measure: do they reflect the clinical
phenotype or the disease pathology [30]. A marker that reflects the clinical phenotype,
such as a neurophysiologic measurement of tremor, is sensitive to symptomatic treatment
effects, but not suitable to measure the effects of a disease-modifying treatment. Moreover,
a marker that reflects the clinical phenotype cannot be used in the prodromal phase of
a disease, i.e. before clinical symptoms appear. Therefore, great efforts are being put into
the identification of biomarkers that reflect the underlying pathology of the disease. A
biomarker that reflects pathology should not be influenced by symptomatic treatment, be
detectable in the earliest stages of disease, allow monitoring of disease progression and
provide information about the disease state [30].

PD BIOMARKERS

Examples of PD biomarkers studied so far include imaging markers such as striatal
dopamine transporter (DAT) binding measured by DAT-SPECT imaging [31], echogenicity
of the substantia nigra using transcranial ultrasound techniques [32], clinical measurements
including tests of the sense of smell [30,33], genetic tests [34] and blood constituents.
Although each of these biomarkers meet some of the requirements of the ideal biomarker,
none of the studied biomarkers has satisfied them all.
DAT-SPECT imaging, for example, is expensive, invasive (injection of a radiopharmacon) and cannot discriminate PD patients from patients suffering from other diseases with a presynaptic dopaminergic deficit, such as PSP and MSA. Substantia nigra echogenicity requires experienced investigators and can be difficult to measure when bone window quality is low [35]. Furthermore, hyperechogenicity of the substantia nigra is not only observed in PD, but also in other disease entities, such as amyotrophic lateral sclerosis [36]. Yet, hyperechogenicity of the substantia nigra may serve as a pre-motor marker. It is observed in 10% of asymptomatic individuals and appears to predispose to the development of PD [37,38].

Tests of the sense of smell can also be used to identify patients in the pre-motor disease stage [39]. However, disease specificity is low, as hyposmia can be observed in other diseases, such as Alzheimer’s disease (AD) [40]. Genetic tests can be considered biomarkers as well. However, they measure an inherited trait, for example a risk for developing a disease, rather than the disease itself [41]. Genetic tests for monogenetic forms of PD, such as LRRK2 [42] or point mutations in the alpha-synuclein gene [43] may only be useful as biomarkers in a minority of individuals that have a high probability of a hereditary form of PD [34]. Risk genes for PD, for example variants at the MAPT locus [44], are probably not valuable for individual risk assessment [41], as carrying a mutation does not unequivocally predict development of PD during life. Finally, several blood constituents have been studied as PD biomarker candidates. Urate, an acid with anti-oxidant properties, is a well-studied example and a promising blood biomarker candidate. Higher levels of urate are associated with a lower risk for developing PD [45] and slower rates of clinical disease progression [46]. Yet, disease specificity appears to be low, as urate levels have been linked to other diseases as well, such as AD and multiple sclerosis [47,48].

**CSF PROTEINS**

Proteins in the cerebrospinal fluid (CSF) have the potential to meet many major biomarker requirements. The CSF is in close contact with the extracellular fluid surrounding brain cells and could therefore reflect the pathological brain processes occurring in PD [49]. Furthermore, CSF is relatively easily accessible by lumbar puncture and – when performed with an atraumatic spinal needle – with low rates of adverse events and with good acceptability in patients [50].

Proteins in CSF originate from three main sources [51]: (1) metabolic processes of neurons within the central nervous system (CNS), (2) diffusion from circulating blood into CSF [52] and (3) secretion by choroid epithelial cells [53]. Approximately eighty percent of CSF proteins are blood-derived, such as albumin and several immunoglobulins [54]. The fraction of CNS-derived proteins is smaller, but has a larger biomarker potential, because CNS-derived proteins are more directly linked to specific neuropathological changes in the
brain. For example in AD, three brain-derived proteins have substantial diagnostic value [55]. CSF concentrations of amyloid-β-42, total and phospho-tau are strongly associated with the future development of AD in patients with mild cognitive impairment [55]. In PD, several CSF biomarker studies have been performed so far and yielded a number of promising candidates. For an overview of the CSF proteins that have been studied in PD, we refer to chapter 2. In this chapter, we also describe important pre-analytical factors that have to be taken into account in CSF biomarker studies.

**METHODS TO IDENTIFY CSF BIOMARKERS IN PD**

CSF biomarkers can be identified using two fundamentally different approaches that each have their specific merits.

The **targeted approach** depends on existing background knowledge of pathogenetic mechanisms to identify proteins that might be sensitive and specific biomarkers. Targeted mass spectrometry is an example of a method used in these hypothesis-driven CSF biomarker studies. In targeted mass spectrometry, peptide ions derived from predetermined proteins of interest are selected and quantified [56]. Also, antibody-based methods such as western blotting and enzyme-linked immunosorbent assays (ELISA) are examples of methods used in hypothesis-driven CSF biomarker studies. In western blotting, gel electrophoresis is performed to separate proteins, after which proteins are transferred to a membrane and stained with protein-specific antibodies. In sandwich ELISA, proteins are quantified in a liquid sample. Protein antigens bind both to antibodies attached to the surface of a plate and to protein specific antibodies coupled to an enzyme. After addition of the enzyme's substrate a signal can be measured that is quantifiable.

The **untargeted discovery approach** of proteomics is primarily unbiased and involves the analysis of the expression, structure and interaction of proteins, usually by means of mass spectrometry instrumentation [57]. This technique enables the discovery of proteins and molecular mechanisms that are not *a priori* expected to be linked to a specific disease [58]. Very relevant to biomarker discovery is quantitative proteomics that can be used to measure differences in expression profiles between protein mixtures [57]. However, protein mixtures in CSF are complex and have extremely wide dynamic ranges, which reduces the methods’ sensitivity [59]. To reduce complexity, multiple separation techniques such as gel-electrophoresis and liquid chromatography can be applied. Furthermore, protein fractions can be selected for analysis or, alternatively, high-abundant proteins can be removed from the mixture prior to their digestion to peptides [59,60] and ionisation by either electrospray or soft laser desorption. Quantitative proteomics is a powerful tool in biomarker discovery. However, it is costly and requires subsequent validation steps [61] that can be either technical – aiming to confirm the methods’ accuracy using other methods – or biological,
in order to evaluate the biological significance of the findings. Of note, multiple sandwich ELISAs may also be used for the untargeted discovery approach.

**AIMS, RESEARCH QUESTIONS AND OUTLINE OF THIS THESIS**

The main objective of the studies described in this thesis was to identify candidate biomarkers in CSF for PD. Our biomarker search was based on the assumption that the most promising biomarker candidates are those that not only have changed expression levels in CSF of PD patients compared to healthy controls, but also relate to PD pathogenesis.

Therefore, we used a dual approach to the identification of candidate biomarkers. We combined a targeted approach, in which literature-based candidates were selected and evaluated, with the untargeted discovery approach of proteomics on affected brain tissue and CSF. The latter approach would enable us to discover entirely novel biomarker candidates, and hence also potentially improve our understanding of PD pathogenesis.

We addressed the following main research questions:

1. Which candidate CSF biomarkers for PD are known from literature?
2. Are selected proteins, chosen based on literature findings, successful in differentiating PD patients from neurologically healthy controls and is their expression level associated with disease duration and/or severity?
3. Which proteins are differentially expressed in post-mortem locus ceruleus tissue of PD patients compared to controls, and are these proteins related to previously suggested or potentially new processes in PD pathogenesis?
4. Which proteins are differentially expressed in CSF of drug-naïve PD patients compared to neurologically healthy controls and are these proteins potential PD biomarker candidates?

To answer the first research question, we summarize the literature on PD pathogenesis and describe a literature search for the most promising CSF biomarkers in chapter 2.

The second research question is addressed in chapter 3, in which the results of three studies on CSF levels of selected candidate proteins, based on literature findings, are described. Alpha-synuclein is a promising biomarker candidate, as this protein is the primary component of Lewy bodies and Lewy neurites that characterize PD neuropathologically [8] and is thought to play a central role in PD pathogenesis [62]. Chapter 3.1 describes the analysis of total alpha-synuclein levels in CSF of PD patients and neurologically healthy controls, to determine whether CSF alpha-synuclein levels differ between PD patients and controls, and whether CSF alpha-synuclein levels relate to disease duration and/or severity. The aggregation of misfolded alpha-synuclein in PD brains suggests that an impaired
degradation of proteins may be an important factor in PD pathogenesis [63]. One of the systems implicated in alpha-synuclein degradation is the endolysosomal pathway [63-65]. For that reason, we describe the analysis of endolysosomal enzyme activities in CSF of PD patients and controls in chapter 3.2. A potential pathogenetic role for clusterin in PD is suggested by the finding of an association between PD and a specific single-nucleotide polymorphisms of the clusterin encoding gene [66]. Clusterin has frequently been linked to AD pathogenesis [67]. Therefore, we performed a comparative study of CSF and plasma levels of clusterin and CSF levels of the AD related proteins amyloid-β-42, total and phospho-tau in PD patients and controls, as described in chapter 3.3.

The third and fourth research question are addressed in chapter 4 that comprises two discovery studies, one performed on brain tissue and the other on CSF, both aimed at identifying fully novel biomarker candidates. In chapter 4.1, we describe the exploration of the proteome of the locus ceruleus, one of the brain regions affected in the earliest pathological stages of PD. We compared the locus ceruleus proteome of PD patients with that of controls in order to improve our understanding of PD pathogenesis and to discover novel proteins related to PD pathogenesis that may ultimately serve as candidate biomarkers in CSF. The results of the second discovery study, a comparative proteomic analysis of CSF obtained from drug-naïve PD patients and controls, are presented in chapter 4.2. The deregulated CSF proteins identified in this cohort were verified in a second, independent cohort of PD patients and controls to select the most promising candidate biomarkers.

In the final chapter, chapter 5, a summary of this thesis is given, followed by a general discussion of the main findings and recommendations for future research.

REFERENCES


64. Fratantoni SA, Piersma SR, Jimenez CR. Comparison of the performance of two affinity depletion spin filters for quantitative proteomics of CSF: Evaluation of sensitivity and


