

# **Cerebrospinal Fluid Biomarkers in Neurodegenerative Movement Disorders**

**Parkinsonian Disorders and  
Huntington's Disease**

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Lui Clara, Julius și Anna fără de care nimic n-ar avea sens

# Cerebrospinal Fluid Biomarkers in Neurodegenerative Movement Disorders

## Parkinsonian Disorders and Huntington's Disease

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### ABSTRACT

**Background:** Parkinson's disease (PD) and atypical parkinsonian disorders (APD) [multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD)] are a large group of idiopathic neurological diseases, together affecting millions of patients worldwide. Huntington's disease (HD) is a rare autosomal dominant neurological disorder with an overall prevalence of about 6/100000. Common to these neurodegenerative movement disorders are combinations of motor, behavioral, psychiatric, cognitive and autonomic symptoms causing much suffering, disability, increased morbidity and mortality. There are no known causal or disease modifying treatments. One of the main obstacles for developing such treatments is the lack of biomarkers for detecting the onset of disease, for discriminating exact diagnosis at an early stage, and for monitoring disease stages and treatment response.

**Aim:** This dissertation explores the biomarker potential of compounds found in the cerebrospinal fluid (CSF) and serum from patients with parkinsonian disorders (PD and APD) and HD.

**Results:** The results of this thesis are gathered into six publications. Increased concentrations of neurofilament light chain (NFL), a marker of axonal degeneration, were confirmed in the CSF of MSA and PSP patients compared with healthy controls and PD patients. This observation was also extended to CBD. CSF NFL levels did not correlate with measures of disease stage and were stable over one year. Thus, NFL may be useful in the

differential diagnosis of parkinsonian disorders but not in measuring disease stage or progression. (Paper I)

In advanced PD patients treated with deep-brain stimulation (DBS) of the subthalamic nucleus, CSF NFL concentrations increased immediately after the surgical procedure, as expected, but decreased thereafter and were normalized at one year and later, thus indicating no accelerated neuronal death due to the DBS treatment itself. (Paper II)

Using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), a panel of four proteins was identified (ubiquitin,  $\beta$ 2-microglobulin, and 2 secretogranin 1 [chromogranin B] fragments), permitting differentiation of APD patients from PD and healthy controls with a sensitivity of 91% and a specificity of 56%. (Paper III)

In males, serum but not CSF urate levels were increased in tauopathies (PSP and CBD) compared with synucleinopathies (PD and MSA). (Paper IV)

Levels of CSF NFL and total tau protein (another marker of neuronal cell damage) were elevated in HD subjects compared to healthy controls. (Papers V and VI)

**Conclusion:** Taken together, the findings presented in this dissertation suggest that CSF NFL is a promising biomarker to differentiate APD from idiopathic PD and healthy controls, and patients with HD from healthy controls. More studies are needed to identify biomarker patterns that could differentiate specific diseases within the APD group, PD patients from healthy controls, and for measuring disease stage and progression.

**Keywords:** Parkinson's Disease, Atypical Parkinsonian Disorders, Multiple System Atrophy, Progressive Supranuclear Palsy, Corticobasal Degeneration, Huntington's Disease, Cerebrospinal Fluid, Neurofilament Light Chain, Tau Protein, Urate, SELDI-TOF Mass Spectrometry

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# SAMMANFATTNING PÅ SVENSKA

Neurodegenerativa motorikstörningar leder till ett för tidigt och sjukligt åldrande av hjärnan. Genom att drabba områden som är inblandade i motoriken leder de till gång-, balans- och finmotoriska svårigheter, ofrivilliga rörelser och koordinationsrubbnings. Påverkan på andra hjärnområden orsakar störningar av beteende och tänkande, psykiatriska symptom så som nedstämdhet, och rubbning av automatiska kroppsfunktioner så som ämnesomsättning, blodtrycksreglering, styrning av vattenkastning och magtarmfunktioner. I denna avhandling berörs två huvudtyper av neurodegenerativa motorikstörningar: (1) parkinsonsjukdomar kännetecknade av rörelsearmod och (2) Huntingtons sjukdom kännetecknad av ofrivilliga rörelser. Parkinsons sjukdom och besläktade sjukdomar kan vara mycket svåra att skilja åt tidigt i förloppet. De drabbar, till följd av den ökade livslängden, ett allt större antal människor över hela jorden. Orsaken till dem är huvudsakligen okänd medan Huntingtons sjukdom är ärftlig. Det saknas idag bot mot dessa sjukdomar som obönhörligt leder till mycket lidande och en för tidig död. En av huvudorsakerna till avsaknaden av effektiva behandlingar är bristen på markörer för att påvisa sjukdomsprocessen, signalera sjukdomsstarten, säkerställa exakt diagnos, mäta sjukdomens svårighetsgrad, dess utveckling och svar på terapi. Att utveckla markörer för dessa sjukdomar är en av läkarvetenskapens främsta utmaningar.

Syftet med de arbeten som beskrivs i denna avhandling är att i ryggvätskan, som sköljer hjärnan och därmed ger information om vad som sker i nervcellerna, undersöka beståndsdelar som skulle kunna tjänstgöra som markörer för neurodegenerativa motorikstörningar.

Halten av neurofilament (NFL), en nervcellsspecifik substans som frisätts vid nervcellsskada, undersöktes i **det första arbetet**. Den visade sig vara normal vid Parkinsons sjukdom och hos friska försökspersoner men förhöjd vid parkinsonlikande sjukdomar inklusive kortikobasal degeneration, där detta inte hade beskrivits förut. De höga halterna kunde oftast påvisas redan vid den första undersökningen och verkade inte öka med tiden. Dessa fynd skulle kunna hjälpa till att tidigt ställa korrekt diagnos, vilket idag kan vara svårt.

Höga NFL ses vid många olika tillstånd som drabbar hjärnan och leder till ökad nervcellsdöd. Det var därför betryggande att, i **det andra arbetet** finna normala NFL halter hos svårt sjuka parkinsonpatienter behandlade med

inopererade hjärnelektroder med kontinuerlig elektrisk ström på. Dessa resultat kan indicera att denna behandling inte orsakade ökad nervcellsdöd.

I det **tredje arbetet** gjordes en överblick över samtliga av de vanligare proteinerna i ryggvätskan hos patienter med parkinsonsjukdomar. Man fann fyra proteiner som tillsammans bildade ett mönster som kunde skilja åt Parkinsons sjukdom från de besläktade, atypiska parkinsontillstånden.

**Det fjärde arbetet** undersökte blod- och ryggvätskehalterna av urat som verkar ha en gynnsam effekt vid både Parkinsons sjukdom och multipel system atrofi. Hos män var urathalterna högre vid tauopatier än vid synucleinopatier, de två huvudgrupperna av parkinsonsjukdomar.

I de **femte och sjätte arbetena** beskrivs höga halter av NFL och även av total tau protein, en annan beståndsdel av nervcellerna, även vid Huntingtons sjukdom.

**Slutsats:** NFL halten i ryggvätskan kan ofta skilja patienter med Parkinsons sjukdom från patienter med besläktade, parkinsonliknande sjukdomar, och även patienter med Huntingtons sjukdom från friska kontrollpersoner. Ingen av de i denna avhandling undersökta substanserna kan idag anses vara en fullvärdig markör för neurodegenerativa motorikstörningar. Sökandet efter tillförlitliga markörer för dessa svåra sjukdomar måste fortsätta.





# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Constantinescu R, Rosengren L, Johnels B, Zetterberg H, Holmberg B. Consecutive analyses of cerebrospinal fluid axonal and glial markers in Parkinson's disease and atypical Parkinsonian disorders. *Parkinsonism Relat Disord.* 2010 Feb;16(2):142-5.
- II. Constantinescu R, Holmberg B, Rosengren L, Corneliusson O, Johnels B, Zetterberg H. Light subunit of neurofilament triplet protein in the cerebrospinal fluid after subthalamic nucleus stimulation for Parkinson's disease. *Acta Neurol Scand.* 2011 Sep;124(3):206-10.
- III. Constantinescu R, Andreasson U, Li S, Podust VN, Mattsson N, Anckarsäter R, Anckarsäter H, Rosengren L, Holmberg B, Blennow K, Wikkelsö C, Rübtschi U, Zetterberg H. Proteomic profiling of cerebrospinal fluid in parkinsonian disorders. *Parkinsonism Relat Disord.* 2010 Sep;16(8):545-9.
- IV. Constantinescu R, Andreasson U, Holmberg B, Zetterberg H. Serum and cerebrospinal fluid urate levels in synucleinopathies versus tauopathies. *Acta Neurol Scand.* 2013 Feb;127(2):e8-12.
- V. Constantinescu R, Romer M, Oakes D, Rosengren L, Kiebertz K. Levels of the light subunit of neurofilament triplet protein in cerebrospinal fluid in Huntington's disease. *Parkinsonism Relat Disord.* 2009 Mar;15(3):245-8.
- VI. Constantinescu R, Romer M, Zetterberg H, Rosengren L, Kiebertz K. Increased levels of total tau protein in the cerebrospinal fluid in Huntington's disease. *Parkinsonism Relat Disord.* 2011 Nov;17(9):714-5.

# CONTENT

ABBREVIATIONS.....	V
DEFINITIONS IN SHORT .....	VI
1 INTRODUCTION .....	1
1.1 Neurodegenerative Movement Disorders .....	1
1.1.1 Parkinsonian Disorders .....	2
1.1.1.1 Parkinson’s Disease .....	3
1.1.1.2 Multiple System Atrophy .....	7
1.1.1.3 Progressive Supranuclear Palsy .....	9
1.1.1.4 Corticobasal Degeneration .....	11
1.1.2 Huntington’s Disease .....	13
1.2 Biomarkers.....	16
1.2.1 Definition .....	16
1.2.2 Why Biomarkers for Neurodegenerative Movement Disorders?..	17
1.2.2.1 The Differential Diagnostic Issue .....	17
1.2.2.2 The Disease Onset, Stage and Progression Issue .....	18
1.2.2.3 The Effects of Therapy Issue .....	20
1.2.2.4 The Patho-Etiological Issue .....	20
1.2.2.5 Clinical Applications of Biomarkers.....	21
1.3 Cerebrospinal Fluid .....	21
1.4 Biochemical Biomarker Candidates for Parkinsonian Disorders .....	23
1.4.1 The “omics” Techniques.....	24
1.4.1.1 Genomics .....	24
1.4.1.2 Transcriptomics.....	25
1.4.1.3 Proteomics.....	25
1.4.1.4 Metabolomics.....	26
1.4.1.5 Conclusion “omics”:	27
1.4.2 Specific Biomarker Candidates in the CSF and Blood .....	27

1.4.2.1	Alpha-Synuclein.....	27
1.4.2.2	Neurofilament Light Chain Protein.....	30
1.4.2.3	Tau Protein.....	30
1.4.2.4	Amyloid- $\beta$ .....	31
1.4.2.5	DJ-1.....	32
1.4.2.6	8-Hydroxydeoxyguanosine (8-OHdG).....	32
1.4.2.7	Urate.....	33
1.4.2.8	Glial Fibrillary Acidic Protein .....	33
1.4.2.9	PGC-1 $\alpha$ .....	34
1.4.3	Combinations of CSF Biomarker Candidates .....	34
1.5	Biochemical Biomarker Candidates for Huntington’s Disease .....	36
2	AIM .....	38
3	PATIENTS AND METHODS .....	39
3.1	Subjects.....	39
3.1.1	Patients.....	39
3.1.1.1	Patients with Parkinsonian Disorders.....	39
3.1.1.2	Patients with Huntington’s Disease .....	41
3.1.1.3	Controls.....	42
3.2	Methods .....	42
3.2.1	Clinical Scales.....	42
3.2.1.1	Hoehn and Yahr Scale.....	42
3.2.1.2	The Unified Huntington’s Disease Rating Scale .....	42
3.2.2	Cerebrospinal Fluid and Serum Sampling .....	42
3.2.3	Laboratory Methods.....	43
3.2.3.1	Sandwich ELISA.....	43
3.2.3.2	Uricase/Peroxidase Enzymatic Method .....	44
3.2.3.3	SELDI-TOF Mass Spectrometry .....	44
3.2.4	Ethics .....	46
3.2.5	Statistics .....	47
3.2.5.1	Generalities .....	47

3.2.5.2	Comparing Means .....	47
3.2.5.3	Correlations .....	47
3.2.5.4	OPLS Discriminant Analysis .....	48
3.2.5.5	Receiver Operating Characteristics.....	48
4	RESULTS.....	49
4.1	Markers for Differential Diagnosis.....	49
4.2	Markers for Disease Stage and Progression .....	57
4.3	Markers for Effects of Therapy .....	57
4.4	Markers with Patho-Etiological Implications? .....	58
5	DISCUSSION AND IMPLICATIONS.....	60
5.1	Markers for Differential Diagnosis.....	60
5.2	Markers for Disease Onset, Stage and Progression .....	61
5.3	Markers for the Effects of Therapy.....	62
5.4	Markers with Patho-Etiological Implications? .....	63
5.5	Limitations.....	66
6	CONCLUSIONS .....	68
	ACKNOWLEDGEMENTS .....	69
	REFERENCES .....	71
	APPENDIX.....	94

# ABBREVIATIONS

A $\beta$	Amyloid- $\beta$
AD	Alzheimer's Disease
APD	Atypical Parkinsonian Disorders
CBD	Corticobasal Degeneration
CSF	Cerebrospinal Fluid
ELISA	Enzyme-Linked Immunosorbent Assay
GFAP	Glial Fibrillary Acidic Protein
HD	Huntington's Disease
MSA	Multiple System Atrophy
NFL	Neurofilament Light Chain
OPLS DS	Orthogonal Projections to Latent Structures Discriminant Analysis
PD	(Idiopathic) Parkinson's Disease
PSP	Progressive Supranuclear Palsy
SELDI-TOF MS	Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

## DEFINITIONS IN SHORT

Parkinsonism	Combination of hypokinesia (slowness of movement) and at least one of two motor symptoms: rest tremor, and rigidity.
Parkinsonian disorders	Neurological disorders characterized by parkinsonism and other movement abnormalities in addition to non-motor symptoms. The most common and well known parkinsonian disorder is Parkinson's disease.
Atypical parkinsonian disorders	Other parkinsonian disorders except Parkinson's disease. Those discussed in this dissertation are multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD).
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [1].

# 1 INTRODUCTION

The ultimate goal of any medical endeavor is to find cures for the diseases afflicting us. Despite the tremendous progress achieved in the field of neuroscience, we are often no better off than our ancestors with respect to having cures for many of the diseases affecting the brain. And as our longevity increases due to improved living conditions and better health care, many neurologic diseases which previously laid beyond the average span of human life, now have time to manifest themselves, causing suffering which most of our ancestors did not live long enough to know. To find a cure for any of these diseases has eluded us mainly because of our inability to grasp their very essence: What are they? What causes them? What are they doing to the brain? Current levels of understanding are restricted to observing their effects and at that time it is already too late; the damage has been done and the effect of available interventions limited. We need something to provide prediction or early stage detection of the disease process, to discriminate between different diseases that have similar clinical presentations, to tell us how the disease is progressing and how it responds to our interventions, and we need to trust it: we need a disease marker or a biomarker.

The goal of this work is to examine compounds in the cerebrospinal fluid and in the blood from patients with parkinsonian disorders and Huntington's disease, and investigate their potential as biomarkers for these diseases.

This thesis consists of three parts: (I) part one is an overview of parkinsonian disorders and of Huntington's disease, presenting basic aspects of these diseases from a clinical perspective; (II) part two brings to attention the topic of biomarkers, highlighting the need for biomarkers for these disorders, and reviewing compounds with biomarker potential; (III) part three presents the research on which this dissertation is based, and its potential implications.

## 1.1 Neurodegenerative Movement Disorders

The neurodegenerative movement disorders are a large group of diseases, encompassing both a common disorder, Parkinson's disease (PD), and a variety of rare disease entities. The common denominator for them all is an insidious, continuous and unrelenting neuronal loss manifested, among other symptoms, through disorders of movement, with either too little movement -

hypokinesia, or too much of it - hyperkinesia. The ultimate cause of these incurable disorders eludes us, and we still do not have any disease modifying treatment, neither one that stops the ongoing neurodegeneration (neuroprotective), or reverses it (neurorestaurative). It is conceivable that any future breakthrough in regard to the etiology or treatment of any of these disorders will find applications for the others too.

The scope of this thesis is restricted to parkinsonian disorders, characterized by hypokinesia, and to one hyperkinetic movement disorders, Huntington's disease.

### **1.1.1 Parkinsonian Disorders**

The parkinsonian disorders have in common, to various degrees, the parkinsonism, defined as the presence of at least two of six movement abnormalities, of which either no. 1 or no. 2 are compulsory: 1) hypokinesia or diminished movement activity (also called bradykinesia, slowness of movement); 2) rest tremor; 3) rigidity (muscular stiffness); 4) loss of postural reflexes; 5) flexed posture; 6) and the freezing phenomenon (when the feet seem temporarily to be glued to the floor) [2]. In addition to motor abnormalities, specific combinations of non-motor symptoms such as autonomic and neuropsychiatric disorders, balance and ocular movement abnormalities, developing at various disease stages, characterize each particular parkinsonian disorder, with major implications in regard to morbidity, treatment and prognosis.

The parkinsonian disorders represent a large group of neurodegenerative diseases affecting a considerable number of patients, most of whom are elderly. PD dominates the group by far, as the most prevalent in the population but also on scientific grounds, as a flagship for neurodegeneration in general, and due to the overwhelming impact which levodopa, its highly efficacious symptomatic treatment, has had on neurology. To the more uncommon atypical parkinsonian disorders (APD) belong multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and dementia with Lewy bodies (DLB). Depending on the nature of the abnormal proteins which aggregate in the nervous tissue in these diseases, they can be subclassified as either synucleinopathies (PD, MSA, DLB) with alpha-synuclein accumulation, or tauopathies (PSP and CBD) with tau protein accumulation. The oftentimes deceptively similar clinical pictures of these diseases can make the differential diagnosis difficult, especially in early stages. Generally, the clinical diagnostic accuracy is lower for APD compared with PD [3]. Due to the global aging of the population, the number of patients affected by these,



for now, incurable disorders will expand in the future, with considerable strains on the society at large, increasing the need for efficacious therapies.

### **1.1.1.1 Parkinson's Disease**

By far the most common parkinsonian disorder, PD was probably already observed in Antiquity, although it got its name first in the 1860's, from the legendary Parisian neurologist Jeanne-Marie Charcot. He called it Parkinson's Disease, an eponym for paralysis agitans as it had been known previously, in honor of Dr. James Parkinson who wrote the first widely read description of the disease in 1817 - "An essay on the shaking palsy". The work initiated by Prof. Arvid Carlsson and his collaborators finally led to the development of the modern treatment of PD [4].

#### **1.1.1.1.1 Epidemiology**

PD is the second most common neurodegenerative disorder after Alzheimer's disease (AD), affecting roughly more than 1,5% of the population aged 60 years or more [5, 6]. Prevalence rates differ from country to country and study to study. In Europe, they could be as high as 12.500 per 100.000 people or as low as 65.6 per 100.000 [7]. In a study from Olmsted County, USA, the incidence was 10.8/100 000 person-years, whereas in Finland, the crude annual incidence was 17.2/100 000 population [8-10]. The different results in different studies probably reflect more methodological differences than differences in the real world. The disease is present on all continents, although it is more prevalent in the industrialized world; it is slightly more common in males than in females, and it increases with age. Due to the global aging of the population, the number of PD patients is increasing. It has been predicted that the number of individuals over age 50 with PD in Western Europe's five most and the world's ten most populous nations was between 4.1 and 4.6 million in 2005 and will double to between 8.7 and 9.3 million by 2030 [11]. While mortality was three times higher than expected in the pre-levodopa era, it has decreased with the advance of symptomatic treatment, and for some PD patients, survival may be the same as in a non-affected population although it is strongly related to levodopa response and disease severity [12].

#### **1.1.1.1.2 Pathology**

The classic definition of PD acknowledges the loss of dopaminergic neurons in substantia nigra pars compacta and the presence of alpha-synuclein-rich intracytoplasmatic Lewy bodies in the remaining neurons [13, 14]. However, it has become increasingly apparent that the degenerative process is spread far beyond the substantia nigra, both chronologically, as it starts

before changes in substantia nigra can be seen, and topographically, as it affects extensive regions not only in the brain but also outside it, in the periphery. According to Braak's theory, widely discussed although controversial, the pathological process starts in the dorsal motor nucleus of the glossopharyngeal and vagal nerves, and in the anterior olfactory nucleus, only thereafter spreading to higher structures in the brain stem and eventually to the cerebral cortex itself [15]. Not only is the central nervous system affected but also the enteric nervous system and the innervation of the heart [16, 17]. The degenerative process afflicts not only the dopaminergic pathways but also other neurotransmitter systems such as serotonin of the raphe nuclei, acetylcholine of the nucleus basalis of Meynert, and noradrenaline in the locus coeruleus [18].

This widespread degeneration explains the large extent of motor and non-motor, levodopa responsive and levodopa non-responsive symptoms in PD and may motivate a redefinition of the disease itself [19].

#### 1.1.1.1.3 Etiology

Despite a plethora of hypotheses, the ultimate cause of PD remains unknown. The vast majority of cases are sporadic but approximately 5-10% are genetic. A combination of both environmental and genetic factors is thought to underlie the pathological processes. In part due to these circumstances, the term itself, PD, does not necessarily mean the same to different researchers and clinicians [20]. Considerable evidence implicates oxidative stress in the degeneration of dopaminergic neurons, through deficiencies in the major antioxidant systems, and not only in the brain, but also in the periphery [21, 22]. Closely linked to oxidative stress is mitochondrial dysfunction [23]. Several hereditary forms of parkinsonism are caused by mutations in genes related to mitochondria, such as PINK1 and PARK2 [24, 25]. Environmental toxins such as rotenone and paraquat, which can disturb mitochondrial function, are positively associated with PD [26]. Alpha-synuclein, a major component of Lewy bodies, inhibits the mitochondrial complex I [27] and may cause impaired protein degradation and accumulation of abnormal proteins by disturbing the two major systems which remove damaged proteins: 1) the ubiquitin-proteasome pathway; and 2) the autophagy-lysosome pathway. The latter is affected as either the migration of proteins to the lysosomes or the degradation of proteins or mitochondria in the lysosomes may be disturbed by accumulation of alpha-synuclein or by genetic abnormalities [28]. Transcription abnormalities caused by alpha-synuclein may disturb metabolic pathways [29]. Abnormal inflammation in the central nervous system has also been suspected to cause

parkinsonism. A different assumption questions the truth in the traditional dogma of neuronal cell body death and proposes axonopathy as the key event in the pathogenesis of PD [30].

The bewildering complexity of the current etiological theories may just confirm that we still do not understand the etiology of PD but it could also imply that treatment must also be complex and oriented towards several potential targets at the same time [31]. The same may apply to biomarkers; it could be preposterous to expect to find a single biomarker covering such a complex disease.

#### 1.1.1.1.4 Clinical Manifestations

Before the diagnostic parkinsonian symptoms are manifest, there is a long prodromal period in which patients may have non-specific symptoms. Thus, depression, constipation, hyposmia, fatigue, stress sensitivity may emerge several years before the movement abnormalities, but cannot by themselves lead to a PD diagnosis without reliable biomarkers.

The movement abnormalities, manifested as parkinsonism, are still the fundament on which the diagnosis of PD rests. Hypokinesia / bradykinesia, and at least one of the other two cardinal symptoms, rigidity and rest tremor, must be present for a PD diagnosis to be made. These symptoms are most often levodopa responsive and emerge in the early phases of PD. Later, three other parkinsonian motor abnormalities usually develop (loss of postural reflexes, flexed posture and the freezing phenomenon) but they are non-dopaminergic and non-levodopa responsive [2]. In most cases, the movement abnormalities dominate the clinical picture for several years and they can be addressed quite well initially, with adequate medication. However, despite treatment and also, to a certain degree due to the treatment itself, as time passes, the movement abnormalities aggravate and in parallel, the treatment response diminishes. After 4-6 years of levodopa treatment, more than 40% of the patients experience motor fluctuations (wearing-off, on-off phenomenon, freezing of gait, off dystonia) or dyskinesias (uncoordinated involuntary movements) [32, 33] and at 15 years follow-up, 94% of PD patients suffer from dyskinesias or wearing off [34].

Non-motor symptoms, more subtle but often present already at the disease onset, may become with the passage of time the principal cause of morbidity and disability. They encompass a large range of symptoms such as neuropsychiatric (affective disorders, impulse-control disturbances, stress intolerance, anxiety, apathy, psychosis, cognitive impairment), dysautonomic (hypotension, incontinence, impotence, constipation),

dermatologic, sensory (pain, paresthesia), balance impairment, dysphagia, speech difficulties and sleep disorders [2]. Non-motor symptoms are often non-dopaminergic and most of the time they do not respond to dopaminergic therapy. Six years after disease onset, three non-motor symptoms were quoted among the five most troublesome symptoms seen from a patient's perspective [35].

The most widely used clinical scale for assessing the impact of PD on motor and non-motor aspects of patient's function and life, is the Unified Parkinson's Disease Rating Scale (UPDRS) developed in the 1980s and revised in 2008 [36, 37].

#### 1.1.1.1.5 Diagnostic Considerations

Due to the lack of reliable and clinically feasible biomarkers, the diagnosis of PD still rest firmly on clinical grounds, as encoded for example in the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria [38] with negative implications in regard to prognostication, treatment and development of a causal therapy [20]. Basically, the diagnosis requires the presence of hypokinesia together with either rest tremor or "lead pipe" rigidity, and the absence of atypical features which would instead often point towards a different diagnosis, such as an atypical, hereditary or secondary parkinsonian disorder. Consistent levodopa unresponsiveness calls the PD diagnosis into question. Supportive features, such as unilateral debut, disease course longer than 10 years, and the presence of rest tremor endorse a PD diagnosis [2]. A confident diagnosis is practically impossible early at disease onset and requires years of follow up until it can be made with a reasonable degree of certainty. In the daily clinical praxis, the available investigations confirm the plausibility of an early clinical PD diagnosis primarily by showing normal results and no pathological findings which would indicate other diagnoses. The gold standard for diagnosis remains neuropathological, requiring the presence of Lewy bodies in substantia nigra and other brain regions, a fact which offers no consolation to the occasional diagnostically difficult cases.

Due to diagnostic difficulties, a reliable diagnosis in movement disorders requires the expertise of movement disorders specialists and that is not always feasible, leading to delays. In a report from a general practitioner setting, of 402 suspicious cases, parkinsonism was confirmed by a movement disorder specialist in 74% and clinically probable PD in 53%, concluding that patients with parkinsonism should be referred to specialists early [39]. The historic diagnostic error rate has been shown to be as high as

24% [38] but it gets considerable lower when strict diagnostic criteria are applied by movement disorder specialists [3]. However, in a study from 2010, high false positive (17.4-26.1%) and negative (6.7-20%) rates were found for the diagnosis of PD, even when movement disorder specialists evaluated tremor dominant parkinsonism and dystonic or other non-parkinsonian tremor [40].

Considering all this, a reliable and clinically accessible biomarker would greatly improve the accuracy of PD diagnosis early in the course of the disease, when it is mostly needed.

#### 1.1.1.1.6 Treatment Highlights

There is a large array of symptomatic pharmacologic and non-pharmacological treatment options for PD, especially addressing the motor abnormalities, but no causal, disease-modifying, neuroprotective or neurorestorative therapeutic alternatives (for a recent review see [41]). A major impediment for the development of an efficacious causal therapy is the lack of biomarkers [42]. Treatment strategies are tailored to the individual patient's needs and background, and to the stage of the disease. The mainstay of PD pharmacotherapy is still levodopa, which is highly efficacious for ameliorating primarily motor symptoms in PD but which, with the passing of years, loses in part its effectiveness and may contribute to the development of motor and non-motor complications. In addition to levodopa a large number of drugs have been developed over time for treating mainly the motor aspects of the disease. Treating advanced patients with motor complications is a challenge. Treatment strategies include combinations of drugs with different pharmacological properties, dose and drug regimen adjustments, advanced drug deliverance systems such as duodopa and apomorphine infusions, and neurosurgery with deep brain stimulation of nucleus subthalamicus or globus pallidus. Not to be forgotten, vigorous physical activity may be associated with a lower risk for developing PD [43] and it has been shown to improve not only motor but also cognitive parameters in animal models and in PD patients [44].

Despite available treatments, PD has a large impact on patients' quality of life, morbidity and mortality, and new, efficient therapies must be developed.

### 1.1.1.2 Multiple System Atrophy

When Graham and Oppenheimer proposed the term "multiple system atrophy" (MSA) as "a temporary practical convenience" to combine the

entities of striatonigral degeneration, olivopontocerebellar ataxia, and Shy-Drager syndrome, they probably did not think it would still be used more than 40 years later [45].

#### 1.1.1.2.1 Epidemiology

Compared to PD, MSA is a rare disease, with an average annual incidence rate of 3/100,000 person-years for the age group of 50 to 99 years [46] and a prevalence varying between 1.9 and 4.9/100,000 people [47]. Prognosis is much worse compared with PD, with a survival of less than 9 years from diagnosis [48]. However, more benign forms of MSA with late onset of dysautonomia and long survival have been reported [49].

#### 1.1.1.2.2 Etiology

The cause of MSA, a synucleinopathy, is not known. As for PD, mitochondrial dysfunction and oxidative stress, genetic predisposition, microglial activation, pesticides and other environmental toxins have been suggested as putative causes [50-52]. Alpha-synuclein accumulates in the oligodendrocytes but its source is not known, neither why it leads to neuronal death. Presumably, disturbances in the neurotrophic support offered by oligodendroglia to neurons result in their degeneration [53].

#### 1.1.1.2.3 Pathology

The neuropathological changes in MSA are widespread, engaging neuronal loss and gliosis in the basal ganglia, cerebellum, pons, inferior olivary nuclei, locus coeruleus, nucleus of Onuf, and spinal cord. In contrast to PSP, which often is a differential diagnosis, the midbrain is spared, and the lack of cortical atrophy differentiates it from CBD [52]. On MRI, the “hot cross bun sign” can sometimes be seen, caused by atrophy in the pontocerebellar tracts. The pathognomonic histopathological feature is the presence of glial cytoplasmic inclusions, containing alpha-synuclein, mostly in oligodendrocytes [54].

#### 1.1.1.2.4 Clinical Manifestations

While the clinical presentation in PD can be dominated throughout many years by extrapyramidal symptoms only, in MSA a mixture of extrapyramidal, pyramidal, cerebellar and autonomic symptoms emerges from the early disease stages. Dysautonomia with orthostatism, syncope and falls, cold hands and feet, urinary incontinence, impotence, and constipation is what most often distinguishes MSA from the other APD. In MSA with predominant parkinsonism (MSA-P), the parkinsonian features are predominant; in MSA with predominantly cerebellar symptoms (MSA-C) the cerebellar symptoms, with ataxia, ataxic gait and speech, [55, 56].

Disease progress is accelerated compared with PD and with the passage of time, the clinical picture may become dramatic, with prominent, levodopa non-responsive parkinsonism, spasticity, dysautonomia, dysarthria, dysphagia, neuropsychiatric abnormalities and respiratory difficulties due to laryngeal dysfunction and immobility. In the late disease stages, patients may become wheelchair-bound and bed-ridden, unable to talk and feed themselves.

#### 1.1.1.2.5 Diagnostic Considerations

In addition to parkinsonism, atypical features pointing towards MSA include the early presence of autonomic dysfunction, poor levodopa responsiveness, a tendency of the head to be drawn forward (antecollis), prominent dyskinesias in the face, pyramidal and cerebellar signs. Given the wide range of symptoms, there is more need of diagnostic stringency in the management of MSA. The latest diagnostic criteria encompass clinical symptoms and signs, and also neuroimaging findings. Definite diagnosis requires neuropathological confirmation [57].

#### 1.1.1.2.6 Treatment Highlights

Treatment in MSA is purely symptomatic. The parkinsonian symptoms may respond to dopaminergic therapy, primarily levodopa, but the response is generally poor and transient. The dysautonomic symptoms may be partially addressed both pharmacologically and non-pharmacologically [58]. Minocycline, rifampicine, rasagilline, transplantation of striatal cells, and neurotrophic factors are but a few of the proposed therapeutic approaches in the future [59].

### **1.1.1.3 Progressive Supranuclear Palsy**

The disease entity we call PSP today was once known as the Richardson-Steale-Olszewski syndrome, after the three doctors who described it.

#### 1.1.1.3.1 Epidemiology

PSP is the second most common parkinsonian disorder after PD, with a prevalence of 6.4 per 100,000 [60] and an annual incidence rate of 5.3 per 100,000 person-years in people above age 50. The incidence increases steeply with age and is higher in men [46]. Survival, as with MSA, is much lower than in PD, and is estimated to average 6 years. Reduced survival is predicted by the presence of early falls, early dysarthria and severe dysphagia requiring an early insertion of a percutaneous gastrostomy [61].

#### 1.1.1.3.2 Etiology

PSP is a four-repeats (4-R) tauopathy (4 repeats in the tau microtubule-binding domain), and so are also CBD and frontotemporal dementia. The chromosomal region where the tau gene is located consists of two haplotypes, H1 and H2. A majority of PSP patients have the H1H1 haplotype. Far less have the H1H2 haplotype while the H2H2 haplotype is extremely rare. Interestingly, patients who developed a PSP like phenotype in the tropics due to alimentary neurotoxins, had the H1H1 haplotype [62].

As for the other parkinsonian disorders, the ultimate cause of PSP is not known. Again, a combination of environment and genetics may start the pathological process resulting in tau protein accumulation, oxidative stress and neurodegeneration. Inflammation is also involved; using PET, microglia cell activation could be found in the same regions where the PSP pathology is usually located [63].

#### 1.1.1.3.3 Pathology

In PSP, hyperphosphorylated tau isoforms with four repeats (4-R) protein accumulates in the basal ganglia, the brainstem medulla, and, more restricted, in the cerebral cortex, mostly in the motor areas. Tau accumulation occurs in neurons as neurofibrillary tangles and causes in astrocytes a characteristic appearance - tufted astrocytes.

Midbrain atrophy is the most striking feature in PSP and can give rise to specific MRI findings, called the “hummingbird sign” or the “standing imperial-penguin sign”. Although the basic neuropathological abnormalities are the same, the different clinical variants of PSP correspond to specific pathoanatomical distribution patterns: more cortical pathology presents more like a corticobasal syndrome, frontotemporal dementia or aphasia, while more engagement of substantia nigra, striatum and nucleus subthalamicus results in a more parkinsonian presentation [64]. Compared with CBD, where pathological changes occur mostly in forebrain and are cortical, PSP tau pathology predominates in hindbrain structures and is subcortical.

#### 1.1.1.3.4 Clinical Manifestations

Lately, four clinical PSP presentations have been recognized although not all patients fit into these categories: (1) the Richardson’s syndrome (PSP-RS) is closest to the classical description and includes parkinsonism, eye movement abnormalities with gaze palsy, early falls due to impaired balance, and neuropsychiatric deterioration with frontal lobe dysfunction; (2) pure akinesia with gait freezing stands out through prominent gait difficulties, freezing of gait, hypophonia, and micrographia; (3) speech apraxia may lead to progressive non-fluent aphasia; (4) PSP pathology and



corticobasal symptomatology may be combined in the PSP-corticobasal syndrome variant. The parkinsonian features in PD and PSP are slightly different: rigidity and bradykinesia are more axial in PSP as compared with appendicular in PD; the gait, turning and seating are “en bloc”; the levodopa response is poor or waning and dyskinesias are rare; there is a tendency for retrocollis and of dystonia in musculus frontalis resulting in a surprised facial expression. The gaze abnormalities with saccade slowness, vertical gaze palsy, eyelid apraxia with blepharospasm may lead to patient being unable to read, to see the food on the plate or to see downwards while walking. The neuropsychiatric abnormalities may result in a picture resembling subcortical dementia with psychomotor slowing, apathy and dysinhibition which can lead to an increased fall risk [65]. Pyramidal signs are less common than in MSA and in CBD, but may be present [62].

#### 1.1.1.3.5 Diagnostic Considerations

As for the other parkinsonian disorders, the diagnosis of PSP is based on the clinical picture and a definite diagnosis demands neuropathological confirmation. Early in the course of the disease, when parkinsonism is the dominant feature and neither eye movement abnormalities nor falls have become established, it may be very difficult to differentiate PSP from PD. Warning signs for PSP are impaired balance with early falls, eye movement abnormalities, early cognitive impairment with apathy and frontal signs, and poor levodopa response [66].

#### 1.1.1.3.6 Treatment Highlights

The levodopa response is modest. Management is focused on the most disabling symptoms and includes physiotherapy, occupational therapy, counseling on swallowing, and symptomatic pharmacotherapy for ameliorating neuropsychiatric symptoms such as anxiety, depression, impulsivity, and sleep disturbances. Cholinesterase inhibitors are usually not efficacious for the cognitive symptoms. As for the other parkinsonian disorders, future trials may engage drugs that aim to improve energy turnover, decrease inflammation, promote secretion of neurotrophic factors or inactivate free-radicals.

### 1.1.1.4 Corticobasal Degeneration

#### 1.1.1.4.1 Epidemiology

CBD is the most rare of the APD, with a prevalence of approximately 6/100.000 and an incidence rate of only 0.02/100.000/year [67, 68]. Survival is similar to other APD, lying around 7 years after disease onset [69].

#### 1.1.1.4.2 Etiology

The etiology of CBD is unknown, but is somehow related to the accumulation of 4-R hyperphosphorylated tau protein, as in PSP. It is not known what causes the hyperphosphorylation of tau protein, but microglia may be involved and genetic factors could play a role [69].

#### 1.1.1.4.3 Pathology

The presence of cortical symptoms is matched by engagement of corresponding cortical areas, with atrophy of superior fronto-parietal regions and relative sparing of temporal and occipital regions. In addition, substantia nigra, striatum, thalamus, parts of the brainstem and cerebellum are affected. Involved areas present neuronal loss and ballooned neurons. Tau protein accumulates as neuropil threads in both white and gray matter and as astrocytic plaques in astrocytes [70].

#### 1.1.1.4.4 Clinical Manifestations

As different protein-tau-related pathologies (CBD but also AD, PSP, frontotemporal lobar degeneration) can manifest with similar clinical presentations, it is more correct to talk about the corticobasal syndrome when discussing the clinical picture, and not specifically about the diagnosis CBD. The corollary is also true: CBD pathology can lead to different clinical presentations, with different profiles of motor, cognitive or behavioral symptoms, including the classical corticobasal syndrome, but also Richardson's syndrome, forms of frontotemporal dementia and of primary progressive aphasia. Therefore, not having any in vivo biomarkers, the only reliable diagnosis of CBD is still neuropathological [69, 70].

The corticobasal syndrome is dominated by a mixture of motor, sensory, cognitive and behavioral symptoms and a persistent symptom asymmetry for several years. The movement disorder component is parkinsonism, with no or poor levodopa response, and atypical motor features such as myoclonus, dystonia which may be severe and lead to fixed contractures, and loss of voluntary control over the most affected limb ("alien limb syndrome"). Balance is impaired but falls occur later compared with PSP, and first when symptoms become bilateral. Oculomotor abnormalities can occur with gaze apraxia, increased saccade initiation latency, but normal saccade velocity both in the vertical and the horizontal planes, in contrast to PSP [62]. Cortical dysfunction leads to dyspraxia, neglect, cortical sensory deficits, visuospatial impairment and speech abnormalities which may progress to non-fluent aphasia. Neuropsychiatric symptoms such as depression irritability and frontal behavioral dysfunction are common.

#### 1.1.1.4.5 Diagnostic Considerations

Due to the poor relationship between the underlying neuropathology and the clinical presentation, as previously discussed, there has been a shift in the way CBD diagnosis is conceptualized. Although the diagnosis has been found to be relevant, it is no longer considered reliable ante mortem but only after neuropathological examination [69, 71, 72]. The most common diagnostic mimicker is PSP, but atypical presentations of AD, Creutzfeldt-Jakob disease, MSA, DLB, frontotemporal dementia and sometimes even PD may have to be considered. Although, when initially described, CBD and PSP were considered distinct clinical and pathological entities, there is now a growing opinion for grouping them together, as opposite poles on a disease spectrum, due to overlapping clinical, pathological, genetic and biochemical features. However, for the time being, there seem to be enough differences between them to motivate for maintaining both diagnoses [73].

#### 1.1.1.4.6 Treatment Highlights

There is no causal or disease modifying therapy and the only available therapeutic interventions are symptomatic and supportive.

## 1.1.2 Huntington's Disease

Probably observed already in the Renaissance by Paracelsus, who reported on patients with involuntary, dance-like movements or “chorea” (χορεία, khoreia = dance in Greek), HD was first described in 1872 by Dr. George Huntington whose name it bears. Because it is caused by a single, fully penetrant gene mutation which is possible to detect in still asymptomatic gene carriers, HD represents a model for neurodegenerative diseases including the parkinsonian disorders and can be used as a paradigm for studying them [74].

### 1.1.2.1.1 Epidemiology

A recent metaanalysis of several epidemiological studies found in Western populations an overall HD incidence of 0.38 per 100,000 people per year and an overall prevalence of 5.70 per 100,000 people in the Western world. Both incidence and prevalence were significantly lower in Asian populations [75]. In contrast to parkinsonian disorders, due to heritability, there are on average five people at risk for being carriers for every manifest patient with HD [76], which has profound existential, practical and sometimes legal implications at the individual and family levels. Although it is possible for adults to undergo genetic tests for the HD mutation, the vast majority abstain from testing, as possibly an act of self-preservation and for maintaining hope [77].

#### 1.1.2.1.2 Etiology

HD is an autosomal dominant disease caused by a mutation in the gene coding for huntingtin, on chromosome 4. The gene is vital for survival as mutant mice homozygous for expressing low huntingtin levels die already during embryogenesis [78]. Although huntingtin is widely expressed in the whole body, its exact function in the cell is not known. The mechanism by which mutant huntingtin leads to neurodegeneration is not sufficiently understood either [79]. Mutant huntingtin accumulates and disrupts many functions in the cell such as energy production, transcription and posttranslational processes, protein clearance and production of neurotrophic factors.

The HD mutation consists of an expanded repetition of the cytosine-adenine-guanine (CAG) trinucleotide coding for an expanded polyglutamine section in the huntingtin protein [80]. The average length of CAG tract in healthy people is 16 to 20 repeats. Expansions over 36 repeats lead to manifest HD, if the gene carrier lives long enough, but the disease penetrance may be reduced in expansions between 36 and 39 repeats; expansions between 27 and 35 do not generally lead to disease in the carrier but are unstable and may expand when transmitted to the next generation [81, 82]. About 8% of HD cases have a negative family history and are caused by new mutations in the huntingtin gene [83].

#### 1.1.2.1.3 Pathology

Striatal atrophy and white matter loss start long before the onset of symptoms [84, 85] and, at death, the brain of a typical HD patient has lost about a third of its mass due to profound atrophy of nucleus caudatus, putamen, and to a lesser degree the cerebral cortex, hippocampus, thalamus, cerebellum, and corpus callosum [86]. In addition, as huntingtin is expressed ubiquitously throughout the body, the pathologic process affects not only the central nervous system but also the periphery. This may explain, at least in part, other symptoms associated with more advanced disease stages such as muscular and testicular atrophy, cardiac failure, osteoporosis and weight loss [87]. Mutant huntingtin aggregates into intracellular deposits. It is not known whether these aggregates are being built as a reaction toward the pathological process or if they cause this process themselves.

#### 1.1.2.1.4 Clinical Manifestations

Several studies have shown that subtle but non-symptomatic abnormalities are present long before the emergence of diagnostic motor symptoms. In asymptomatic gene carriers, Stout et al. found slight abnormalities in

recognition of facial emotions, 15 years prior to diagnosis; at nine years before diagnosis almost all given cognitive tests showed abnormalities [88]. Asymptomatic carriers could be differentiated from healthy controls by subtle motor abnormalities [89]. Sometimes, irritability, apathy, depression, suicidal behavior may predate the diagnostic symptoms, but are not recognized at the time of their emergence, due to their non-specific character [85]. Despite this, the typical HD mutation carrier can enjoy a normal life, enriched by personal, familial and professional achievements until about the age of 40 years, when the first manifest motor HD symptoms emerge, often as involuntary movements (chorea). The next 15-20 years are marked by increasing motor, cognitive and psychiatric symptoms resulting in severe disability and premature death [76]. Other motor symptoms include bradykinesia, dystonia, dyscoordination, oculomotor abnormalities, ideomotor apraxia and motor impersistence. Later in the disease, balance impairment leads to frequent falls. In advanced disease stages, chorea may diminish while dystonia increases, sometimes leading to fixed postures and severe gait disabilities. Patients may become unintelligible due to profound dysarthria. With time, severe dysphagia may require the insertion of a PEG. Behavioral abnormalities manifest as personality changes, irritability, cognitive inflexibility, apathy and attention deficit. Twenty percent of the patients present with severe psychiatric symptoms such as depression, suicidality, anxiety or psychosis. Cognition deteriorates and leads to dementia. As in advanced parkinsonian disorders, the non-motor symptoms often become the greatest burden for the patient and the caregivers in later disease stages [90].

The most widely used clinical scale for assessing the impact of HD on motor and non-motor aspects of patient's function and life, is the Unified Huntington's Disease Rating Scale (UHDRS) developed in the 1990s [91].

#### 1.1.2.1.5 Diagnostic Considerations

The diagnosis HD requires the emergence of typical motor symptoms together with a positive genetic test and/or a history of HD in the family. As in the case of PD, these criteria imply that the disease process has already been present for several years at the time for diagnosis. The real time of onset is not possible to ascertain. Age at onset is inversely correlated with the CAG repeats number and an algorithm has been created which, starting with the age of the gene carrier and the CAG repeats number computes the probability of developing manifest HD at different subsequent ages [92]. While the size of the CAG expansion contributes about 70% to the age at onset, environmental and other genetic factors contribute with the rest [93].

The CAG repeat size impacts also on the course of the disease and on the degree of striatal neurodegeneration: the larger the expansion, the greater the clinical and the neuropathological deterioration [94, 95]. The range of disease onset stretches from childhood to octogenarians. When disease onset is during childhood or adolescence, HD tends to present more often as an akinetic–rigid variant, called Westphal, rather than with choreatic movement abnormalities.

#### 1.1.2.1.6 Treatment Highlights

Treatment is entirely symptomatic as there is no causal or disease modifying therapy. However, optimal treatment has a positive impact on quality of life, morbidity and mortality. Neuroleptics and tetrabenazine are used for suppressing chorea. Antidepressants, anxiolytics or antipsychotics may be needed for treating psychiatric symptoms. Of special importance due to the nature of the disease is genetic counseling. There is a great need for non-medical services including rehabilitation, supportive psychotherapy, physio- and occupational therapies, access to dieticians, social workers, and psychologists. The best way to provide an optimal management of this complex and relatively rare disorder is through dedicated HD medical centers.

## 1.2 Biomarkers

### 1.2.1 Definition

The word “biomarker” is being used widely but not always correctly. The term was defined in 2001 by the Biomarkers Definitions Working Group: “Biological marker (biomarker): A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [1]. Surrogate endpoints are a subgroup of biomarkers. They are substitutes for clinical endpoints which is what we really are interested in, reflecting how the patient is doing in reality. The requirements for a biomarker to serve as a surrogate endpoint are very strict and, at the present time, we do not have any surrogate endpoints in neurodegenerative movement disorders. However, any reliable biomarker, even if not strong enough to be a surrogate endpoint, would be tremendously valuable. In order for a parameter to be considered a biomarker for a certain disease, it must also fulfill several requirements: 1) Validity: there must be a correlation between the biomarker and the disease which it stands for; a treatment must affect the disease and not only the biomarker itself; 2) Performance: how good is the biomarker? How well does it differentiate

between affected and non-affected? The biomarker assessment must be reliable and reproducible, both in the same patient at different points in time, and at different centers. It must be feasible in a clinical context and that implies safety, tolerability, simplicity and low cost; 3) Generalizability: the performance in different patient subsets, based e.g. on age, gender, disease stage, medication, must be known [96, 97]. It is easy to use the word “biomarker”, but the implications of this word are profound, and despite all effort, we cannot say, for the time being, that we really have a biomarker for neurodegenerative movement disorders. What we do have in neurological sciences are: 1) biomarkers for certain disease-related processes, such as neurofilament light chain as a biomarker of axonal degeneration, particularly damage to large-calibre, myelinated axons; and 2) different forms of protein inclusions, such as the 42 amino acid isoform of amyloid  $\beta$  ( $A\beta_{42}$ ) as a biomarker of Alzheimer-related senile plaque pathology.

## **1.2.2 Why Biomarkers for Neurodegenerative Movement Disorders?**

The ultimate reason for needing a biomarker is the fact that we still do not have any disease-modifying treatment in movement disorders. The lack of biomarkers is considered to be one of the greatest limitations for developing such a treatment [42]. Over years, there has been no shortage of therapeutic hypotheses or compounds to be tested; the list with failed compounds is very long. The real problem has been the lack of a reliable way to assess the underlying disease process and whether an intervention could influence it and alter the course of the disease [98-100].

It has been assessed that it takes five years of follow-up and 600 subjects participating in a randomized placebo-controlled trial in order to detect a 20% slowing of functional decline. A biomarker could dramatically reduce the resources needed [101].

Considering the very nature of neurodegenerative movement disorders and the limits it puts on the process of developing disease-modifying therapies, biomarkers could be useful for solving many limiting issues.

### **1.2.2.1 The Differential Diagnostic Issue**

Differential diagnosis can be difficult during early phases of parkinsonian disorders. What might look as PD in the beginning, could turn out to be PSP, MSA or even CBD. What was initially considered to be a synucleinopathy, may end as a tauopathy. Considering the substantial differences between these disorders, mixing together patients with different

diagnoses may lead to negative or inconclusive results in any therapeutic trial, even when the therapy itself is efficient for one of the diagnoses. A biomarker pointing early towards the right diagnosis would increase the probability of success.

Even though there are a number of HD-like disorders without the HD mutation, the differential diagnostic issues in HD are generally easily settled by doing genetic testing [102]. Difficulties are of a different nature in HD: how to determine the time of disease onset in an asymptomatic HD mutation carrier?

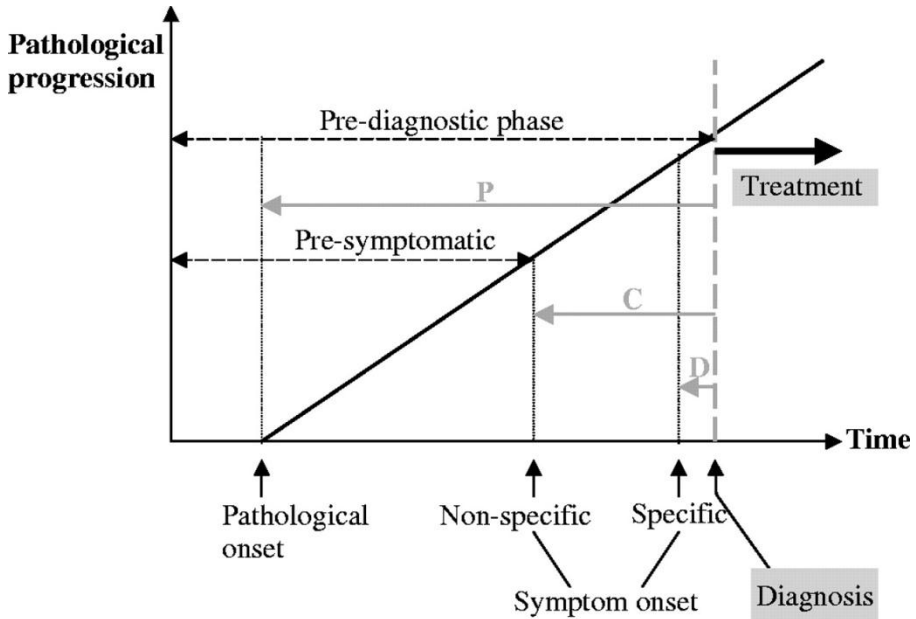
A diagnostic biomarker would decrease the cost, time and effort necessary to secure a diagnosis. Currently, that is best achieved through an assessment done by a movement disorders specialist. A biomarker would simplify the diagnostic process.

Even when there is no doubt regarding diagnosis, an ideal biomarker could help stratify patients in subgroups which may show different responses to a given therapy. That would make possible a distinction between respondent and non-respondent diagnostic subgroups, preventing the dismissal of a therapy when it does not benefit the diagnostic group as a whole. Such a distinction would also permit, within a given diagnostic group, to differentiate and individualize treatment according to expected benefits or risks, and expected disease progression and complications [96]. For example, young PD patients with an increased risk for developing dyskinesias once levodopa therapy is instituted, might need a different treatment approach compared with patients with late disease onset and a low risk for dyskinesia but high for dementia.

### **1.2.2.2 The Disease Onset, Stage and Progression Issue**

To date it is impossible, in both parkinsonian disorders and in HD, to determine the exact date of disease onset, marking the point in time when the pathological process started. Once started, the disease is asymptomatic for some time, followed by non-specific, non-diagnostic symptoms. The current “early” diagnosis based on the emergence of specific motor symptoms probably describes relatively advanced disease (Fig. 1).





**Figure 1. Schematic presentation of the disease course for parkinsonian disorders and Huntington's disease. D=earlier diagnosis may be possible with improved clinical skills and if patients present earlier with typical symptoms; C=earlier diagnosis may be possible if we develop clinical tests/biomarkers that can positively identify the earlier symptoms that are currently regarded as non-specific; P=biomarkers could theoretically detect the pathological onset even before symptoms develop. Adapted from Michell AW, Lewis SJ, Foltynie T, Barker RA. Biomarkers and Parkinson's disease. *Brain*. 2004 Aug;127(Pt 8):1693-705. Epub 2004 Jun 23 (permission for reuse granted).**

Thus, in PD, it has been calculated that up to 50-70% of substantia nigra neurons are lost before symptomatic motor abnormalities develop [103] and the premotor period could be between 5 and 20 years long [96]. In one positron emission tomography study in PD, a mean preclinical period of 5.6 +/- 3.2 years was calculated [104]. Results from the Honolulu-Asia Aging Study do also place the onset of non-motor symptoms, such as bowel movement abnormalities, ten years or more before the emergence of diagnostic motor symptoms [105].

The same applies to HD. Advanced imaging techniques have shown pathological changes in presymptomatic HD gene carriers in form of striatal atrophy and white matter loss, as early as 15 years prior to diagnosis [84, 85], and abnormalities can be detected years prior to diagnosis in asymptomatic gene carriers if they are assessed [89].

The fact that the disease onset predates with years the time when enough symptoms emerge for a diagnosis to be made, implies that even efficacious therapies may show themselves powerless if given when neurodegeneration

has gone that far [19]. An ideal biomarker could detect the disease in presymptomatic individuals or early in the disease course allowing an efficacious disease-modifying therapy to act and “cure” or at least delay the progression of disease.

For now, there is also no way of measuring disease stage and progression. The tools we have been using are clinical scales of which UPDRS [36] is the most widespread for PD and UHDRS for HD [91]. However, these scales are no biomarkers and they are subject to both investigator and patient bias and cannot be considered truly objective; they are not reliable as their score can vary from hour to hour due to medication, placebo, food intake or a myriad of other causes; in parkinsonism, they measure a combination of dopaminergic and nondopaminergic effects and not the disease process itself, nor the direct effects of treatment over this process. Biomarkers are needed to identify the onset of disease, monitor its stage and measure its progression,

### **1.2.2.3 The Effects of Therapy Issue**

At the present time we do not have a way of assessing whether and to which degree a therapeutic intervention has an impact on the disease process: we cannot measure the effects of a therapy. The clinical scales which we use today are subject to error, as discussed before. In addition, as it was shown in the ELLDOPA study, clinical measures such as UPDRS, and a more objective assessment, radiotracer imaging, moved in different directions after the therapeutic intervention, levodopa treatment, leading to confusion in regard to interpretation [106]. A further problem is that radiotracer imaging, which, currently, is the best we have achieved in regard to a PD biomarker, does only assess the integrity of the dopaminergic pathways in the striatum and, maybe, although it is controversial, the impact of therapy on these dopaminergic pathways [107]. However, PD and also the APD, are not only disorders of the dopaminergic system, but of several other neurotransmitter systems, which these radiotracers do not visualize.

In conclusion, biomarkers that can identify and monitor the biochemical effect of drugs would greatly benefit the search for disease-modifying therapies as well as be usefully employed as surrogate markers in clinical trials.

### **1.2.2.4 The Patho–Etiological Issue**

The ultimate cause of parkinsonian disorders and of HD remains unknown, despite an abundance of theories. In HD, the mutation is known and can be

detected, but we still do not understand how that mutation causes neurodegeneration. A biomarker reflecting the etiology of the disease might offer insights into the pathological mechanism itself, thereby opening the way for a potentially successful intervention.

### **1.2.2.5 Clinical Applications of Biomarkers**

In conclusion, putative biomarkers could assist in the development of disease modifying therapies and in the management of neurodegenerative movement disorders, if there was a disease modifying therapy by:

1. Indicating promising therapeutic approaches derived from a patho-etiological understanding of the disease;
2. Translating results of drug tests in animals to human populations;
3. Enriching study populations by identifying patients at risk for a disease;
4. Determining disease onset at an early stage;
5. Stratifying populations according to estimated disease progression, anticipated complications, expected therapy benefits and potential risks;
6. Measuring the effects of a therapy on the disease process and on disease progress;
7. Determining when a therapeutic intervention can be discontinued;
8. Simplifying the drug regulatory process.

In regard to parkinsonian disorders in general and PD in particular, and considering the heterogeneity of clinical presentations at onset, the variability in clinical progression, the multitude of genetic variants and of possible etiologies, it is conceivable that several biomarkers will need to be developed, covering biochemical, imaging, pathological and clinical aspects of the diseases [96].

## **1.3 Cerebrospinal Fluid**

The first lumbar puncture (LP) was done in London 1889 and cerebrospinal fluid (CSF) studies have a long tradition in neurology, both in research and in clinical practice [108]. We know mainly from AD research that CSF studies in patients with neurodegenerative disorders are feasible with a low rate of post LP headache or other complications [109] and CSF analysis for assessing tau protein and beta-amyloid belongs now to the standard of care

in the management of dementias. Brain-derived proteins do not usually appear in the blood due to the blood-brain-barrier. In contrast, CSF, in the other side of the blood-brain-barrier, is very close to the pathologic processes in the brain and to the source of brain-derived proteins and brain metabolites [110]. While 80% of all proteins in the CSF derive from blood, only 20% are brain-derived [111]. The protein concentration in blood is much higher than in the CSF, due to the brain blood barrier which isolates the CSF space. However, CSF is dynamic. Proteins that diffuse in the CSF from plasma have a concentration gradient with a 2.5 times higher lumbar concentration than cranial. Proteins secreted in the CSF from the brain have about the same concentration in the CSF space, but some, including tau protein, may actually have a lower concentration distally, in the lumbar region. There are also diurnal variations, as the secretion of proteins into the CSF is higher at night. In addition, the protein concentration decreases between the first ml CSF tapped at the LP and the later portion which is the preferred one as it more accurately reflects the environment in the brain. All this makes imperative the standardization of the CSF sampling protocol [112].

It has been suggested that CSF itself mediates humoral signaling which is distinct from synaptic neurotransmission. In one study, spherical nanometric-scale structures were identified in the CSF containing synaptic vesicles [113]. Cell-line studies have shown that CSF from PD patients affects dopaminergic cells differently than CSF from healthy controls, implying that there are differences in their composition [114]. Due to all this, CSF has been widely investigated in neurodegenerative movement disorders and it might be considered to offer the most promising insights in the disease processes [115].

There have been concerns regarding CSF sample handling and its impact on the acuity of CSF data as post-translational modifications, protein loss and degradation can be caused by non-optimal procedures including sampling, freezing, thawing and storage. Therefore it is important to have standard operating procedures in place [116]. A consensus protocol for the standardization of CSF collection and handling has been published in 2009 and is being followed by many European centers [117]. In regard to analysis, for increasing the reliability of results, a study should ideally include a training subgroup and a validation subgroup, the latter preferably run by a different research group [118, 119]. Recently, an update on recommendations to standardize preanalytical confounding factors in AD and PD cerebrospinal fluid biomarkers has been published [120].

## 1.4 Biochemical Biomarker Candidates for Parkinsonian Disorders

There are different types of potential biomarkers for neurodegenerative disorders: biochemical analysis of blood, CSF, urine or brain tissues, genetics, and multiple imaging modalities. In addition, several clinical markers are used to measure different aspects of the diseases and to track their progression: motor analysis, assessments of olfaction, autonomic functions, cognition, sleep, speech and swallowing, neuropsychological and psychiatric investigations [96]. This overview is only concerned with biochemical markers, mostly in the CSF but to a lesser degree also in the blood.

In a review from 2008 of all then current publications regarding CSF biomarkers in PD, MSA, PSP, CBD, and DLB, no less than 67 tested compounds were identified, most of them in PD. However, several limitations were found in most of the studies: sensitivity and specificity were low; there was a lack of reproducibility of results by independent cohorts; and the analysis methods in use were still in their infancy [118]. Thus, there is no scarcity of investigations on CSF compounds with biomarker potential in parkinsonian disorders. What we barely have are mature CSF biomarker candidates and what we still lack is a real biomarker.

Due to the prominence of the dopaminergic abnormalities in these disorders, the first compounds to be tested were dopamine and other monoamines and their metabolites. As these results were prone to be influenced by a multitude of other factors, the quest went further to compounds which were already known and tested in other diseases such as tau protein, beta amyloid and neurofilament light chain (NFL). With advancing knowledge and technical capabilities, the search turned further towards specific targets following theoretical considerations in regard to patho-etiology, such as alpha-synuclein, oxidative stress or inflammatory markers. Later on, the newer and far-reaching possibilities offered by the “omics” techniques led to broad searches surveying large, non-discriminate entities like the genome or the proteome. The overview presented here has no claim on being exhaustive; instead it focuses on a number of compounds perceived to be more mature and/or promising for the future.

According to the aims of the investigation and the technique utilized, there are two main approaches to assess body fluids and body/brain tissues for biomarkers:

- 1) Targeted search investigates one or several a priori defined compounds in patients and in healthy controls and looks for differences, patterns and associations.
- 2) Untargeted search investigates broadly a large amount of components in a sample and compares patients with healthy controls. Nowadays, this is achieved by the “omics” techniques.

### **1.4.1 The “omics” Techniques**

The relatively new “omics” techniques present both an enormous potential, through their capacity of screening wide and complementary areas of different biological materials, and a significant challenge, through the huge amounts of data that are generated and need interpretation. In biologic materials, transcriptomics, proteomics and metabolomics evaluate the transient, momentaneous or “state” characteristics of a sample while genomics mirror its permanent or “trait” characteristics.

#### **1.4.1.1 Genomics**

Genomic studies survey and compare genomes in patients and controls, looking for associations between gene alleles, genetic risk factors, and disease. The more restricted candidate gene approach investigates specific genes in the context of a certain disease, such as mutations in the alpha-synuclein gene causing a rare form of autosomal dominant PD. The genome-wide association studies, a more recent technique, investigate the whole genome. Comprehensive, genome-wide association studies and metaanalyses have found more than 16 PARK loci associated with PD and 11 genes for PARK loci, and new insights are gained every year [121, 122]. Five of the identified genes induce a roughly typical PD presentation (alpha-synuclein, parkin, PTEN induced putative kinase 1, DJ-1, and leucine-rich repeat kinase 2) while mutations of ATP13A2 (PARK9) cause Kufor-Rakeb disease, characterized by both parkinsonism and many atypical features [123]. A genetic biomarker is unchangeable and indicates a trait, a propensity to develop a disease. However, it does not indicate whether the disease has started or how advanced it is; it does not provide information about a state. Due to environmental factors, age or reduced penetrance, the trait may or may not induce a state of disease during the lifetime of the bearer. The huntingtin mutation and the leucine-rich repeat kinase 2 (LRRK2) mutation are examples of genetic traits for HD and an autosomal dominant form of PD respectively. Performing genome-wide association studies, Simon-Sanchez et al. found a strong association in PD with the

alpha-synuclein gene (SNCA) and, surprisingly for a synucleinopathy, also with the MAPT locus, related to tau protein [124].

An emerging research field is epigenetics which may bridge the gap between the apparently unchanging genome and the ever changing environment. There is evidence from both human but mostly from in vitro and animal models that DNA methylation, histone modifications, and small RNA-mediated mechanisms, could modify the expression of PD-related genes such as the alpha-synuclein gene, DJ-1, LRRK2, and parkin-gene, contributing to the development of the disease [123, 125].

### **1.4.1.2 Transcriptomics**

Transcriptomics investigates mRNA levels of expressed genes coding for proteins. Several studies have examined cells from substantia nigra in patients, controls and animal models. Differences were found between controls and patients but the results in regard to particular genes were not similar between studies. However, looking at categories of genes according to their function, findings became more consistent across studies and a pattern could be discerned showing that genes involved in oxidative stress, mitochondrial function, protein degradation, dopaminergic transmission, and axonal guiding were expressed differently in PD and PD models [126, 127].

### **1.4.1.3 Proteomics**

Proteomics characterizes the protein content - the proteome- of samples coming from tissues, cell components or body fluids. By comparing the proteomes of patients and controls, differences may be found, with possible patho-etiological or diagnostic implications. The technology is based on three components: 1) separation of proteins; 2) analyzing protein patterns through mass spectrometry; and 3) quantifying and identifying interesting proteins through advanced data processing [127]. Using this technique, a comprehensive characterization of the proteome in substantia nigra was made by Kitsou et al. [128]. Many of the proteins known to be involved in PD such as DJ-1 and UCHL-1 were identified. Using proteomics, Abdi et al. characterized the proteome of the CSF and over 1500 proteins were identified and grouped according to their functions, such as cell cycle, signal transduction, cellular transport. In addition, a large number of proteins unique to AD, PD and DLB were identified [129]. Seventy two of them were uniquely altered in PD compared with healthy controls. Apolipoprotein H (Apo H) and ceruloplasmin appeared to be able to segregate PD from healthy controls and from non-PD (AD, DLB). Using the same material,

Zhang et al. validated a multianalyte CSF profile, identifying a panel of eight CSF proteins that were highly effective at recognizing PD [130].

**Subcellular proteomics** investigates the proteome at the subcellular level, in compartments of the cell. Such a compartment is neuromelanin, a granular pigment associated with lysosomes and present in catecholaminergic neurons. It interacts with compounds in the cytoplasm such as iron, lipids, pesticides, neurotoxins and it sequesters them, thus having a cytoprotective function. However, if it malfunctions, it could turn out to become cytotoxic and be involved in neurodegeneration. The proteins associated with neuromelanin were investigated using proteomics [131]. Several were associated with mitochondrial function and chaperons. Interestingly, antibodies against neuromelanin have been found in serum from PD patients [132]. The same technique was used for analyzing Lewy bodies. Several proteins thought to be involved in the pathogenesis of PD were found, associated to alpha-synuclein, such as chaperons, proteins involved in oxidative stress and proteosomal degradation [133]. Analyzing mitochondrial fractions, 119 proteins were found to differ in PD compared with controls, among them mortalin, involved in mitochondrial function and oxidative stress reactions. Low levels of mortalin were found in substantia nigra from PD patients compared with controls [134].

Ideally, proteomics should identify promising proteins which could function as candidate biomarkers and be further investigated. A shortcoming of the proteomics technique is that it is often biased towards identification of abundant proteins. In the CSF, 70% of the proteins are represented by albumin and immunoglobulins and a way to enhance the discovery of proteins present in small amounts is to exclude the abundant proteins from the sample through fractionation. Blood contamination with its high protein content can dramatically alter the proteomic pattern in the CSF and it has been suggested to exclude from proteomic analyses CSF containing more than 10 erythrocytes per microliter [127].

#### **1.4.1.4 Metabolomics**

Metabolomics investigates end products of metabolic pathways. These are molecules with low molecular weights required for the maintenance, growth and normal function of a cell [135]. Adequate sample collection and preparation prior to analysis is very important for accurate results. Metabolomic studies conducted by Bogdanov et al. have confirmed the inverse association between blood urate levels and the risk for PD. In addition, they found higher levels of glutathione and 8-hydroxydeoxyguanosine in PD compared with controls. These compounds are markers of oxidative processes and support the oxidative stress



hypothesis in PD [136]. Using metabolomics, the same group could differentiate controls from idiopathic PD patients, patients with idiopathic PD from those with hereditary PD caused by a specific LRRK2 mutation, and also symptomatic LRRK2 mutation carriers from asymptomatic carriers [137].

#### **1.4.1.5 Conclusion “omics”:**

Ideally, findings from the four “omics” techniques applied on different materials (e.g. substantia nigra cells or the CSF) should be consistent. Thus, if genomics shows an altered gene in neuronal nuclei, then the mRNA (transcriptomics) should reflect that in the cytoplasm, and further, after translation, in proteins and through them metabolic products detected in the cell or in the CSF by proteomics and ultimately by metabolomics. Findings in the CSF should be replicated in substantia nigra cells. Unfortunately, this congruence of findings is not often to be seen. That may be due to the limitations of the techniques or experimental incongruences, along with the use of different techniques and the inherent complexities of living organisms [127]. Better equivalence is achieved when findings from different techniques are categorized within pathways such as oxidation, synaptic transmission, mitochondrial function, or protein degradation. Of these, the oxidative stress pathway is the most robust with similar results from both cellular and CSF analysis, from genomics, transcriptomics, proteomics and metabolomics. Thus, oxidative stress appears to be the final common pathway in the neurodegenerative process in PD [127]. Better integration of these techniques should lead to a deeper understanding of the pathophysiology of PD as well as other neurodegenerative disorders, and open venues for developing new treatment strategies.

### **1.4.2 Specific Biomarker Candidates in the CSF and Blood**

#### **1.4.2.1 Alpha-Synuclein**

##### Background

Alpha-synuclein is the main component of intracytoplasmatic Lewy bodies and of Lewy neurites in neuronal processes. These structures are found in PD and in DLB in the remaining dopaminergic neurons in substantia nigra, and also in non-dopaminergic cortical and non-cortical neurons [18, 138]. In MSA, alpha-synuclein is a component of the characteristic glial intracytoplasmatic inclusions.

Mutations affecting the gene coding for alpha-synuclein cause rare hereditary forms of PD, such as in PARK1 (missense) and PARK4 (duplication, triplication) [139] but are also important for sporadic forms of PD [140]. In addition, in both PD and MSA, genome wide association studies showed a strong association between disease risk and distinct single nucleotide polymorphisms (SNPs) in the  $\alpha$ -synuclein encoding gene [124]. There seems to be a dose-effect of alpha-synuclein as increased levels of synuclein caused by duplications and triplications of the gene cause PD [124, 141].

#### Alpha-Synuclein's Role in the Pathogenesis of Synucleinopathies

Although it is widely expressed in the brain, the precise function of alpha-synuclein is not known. It might play an important role in neurotransmission by regulating synaptic vesicle size and recycling. Mutant alpha-synuclein builds fibrils, aggregates, resists degradation and ultimately interferes with vital cell functions such as transcription, the ubiquitin-proteasome system, lysosomes and mitochondria, disrupting protein metabolism and energy production. Oxidation, pesticides, and mitochondrial dysfunction can damage alpha-synuclein and initiate its metamorphosis to toxic forms [142]. It has been proposed that alpha-synuclein pathology and subsequent neurodegeneration could represent a common event for different forms of PD, with different etiologies. A recent theory proposes pathologic "seeding" throughout the nervous system of abnormal alpha-synuclein which, after finding its way in the body, might, through a prion-like induction, spread from cell to cell, causing the neurodegenerative process in PD [143, 144]. Due to alpha-synuclein's prominence in the pathogenesis of these disorders, PD, MSA and DLB are considered to be synucleinopathies.

#### Previous Findings in Parkinsonism

CSF alpha-synuclein levels in PD have been investigated using different techniques in over 10 studies. A majority of them showed decreased levels in PD [145-148] but not all [149, 150].

Four studies have investigated CSF alpha-synuclein levels in MSA. Three of them found decreased levels in MSA compared with controls but not with PD patients [148, 151, 152]. In one of them levels were similar in MSA, PD and controls [153]. In one study, PD and MSA could be differentiated by the CSF Flt3 ligand, not by alpha-synuclein [152].

In one study, CSF alpha-synuclein levels in PSP and CBD were not significantly different compared with controls. However, levels in PSP but not in CBD were higher than in PD [151].

Alpha-synuclein levels have also been investigated in plasma in PD and MSA but with conflicting results. Both higher [154] and similar [155] levels compared with controls have been found and there was no correlation with PD severity. A major difficulty in measuring both alpha synuclein and DJ-1 in plasma is the risk for contamination with erythrocytes or platelets as more than 95% of these compounds reside in erythrocytes and about 4% in platelets. However, even after controlling for that, there were no statistically significant differences between PD patients and controls in regard to these compounds although there was a trend for lower levels in PD. It does not seem that plasma alpha-synuclein can be used as a biomarker for PD for the time being [156].

Oligomeric forms of alpha-synuclein protein in plasma were higher in PD than in controls, in one study [157]. However, in another study, phosphorylated alpha-synuclein, but not total alpha-synuclein, nor oligomers of alpha-synuclein, was higher in PD than in controls [158]. Interestingly, antibodies directed against monomeric alpha-synuclein were found in plasma in PD patients, with higher response in earlier disease phases [159]. Studies in animal models suggest that immunomodulatory interventions such as vaccination with alpha-synuclein [160] or administration of alpha-synuclein antibodies [161] may have a positive impact on the intraneuronal accumulation of alpha-synuclein, presumably reflected by reduced neuropathological and behavioral deficits. Intravenous immunoglobulin reduced alpha-synuclein oligomer neurotoxicity in human neuroblastoma cells [162]. These results may motivate further research aiming to find whether immunomodulation might be a novel therapeutic approach in PD.

Alpha-synuclein was found not only in the brain and the blood but in other peripheral locations too. It was present in the colonic mucosa years before the emergence of PD symptoms and the question was raised whether it can be a biomarker for premotor PD stages [163, 164]. In saliva, alpha-synuclein was lower in PD patients than in controls and it inversely correlated with the UPDRS score [165].

CSF alpha-synuclein levels increase non-specifically in Creutzfeldt-Jakob's disease, presumably due to massive neuronal death [146]. The same phenomenon but on a smaller scale occurs in AD, with increased CSF alpha-synuclein levels [151].

Although alpha-synuclein is a strong biomarker candidate due to its important role in the pathogenesis of synucleinopathies and to several

promising results, it cannot, for the time being, be considered a mature biomarker. However, in a group of parkinsonian patients, low CSF alpha-synuclein levels could help with their stratification, due to its high positive predictive value for synucleinopathies. An additional marker (e.g. non-motor prodromal symptoms) would strengthen the stratification process and help to select a group of patients who may benefit from future synuclein-reducing therapies [148]. Longitudinal studies and studies in early disease stages are needed in order to better understand the value of alpha-synuclein as a potential biomarker in parkinsonism.

### **1.4.2.2 Neurofilament Light Chain Protein**

#### Background

Neurofilaments (NF) are major neuronal structural elements, composing the intermediate filaments present in nerve fibers. They are mainly involved in maintaining the axonal caliber and the neuronal shape and size [166] and are thereby critical for the morphological integrity of neurons and for the conduction of nerve impulses along the axons [167]. The NF are composed of three subunits of different molecular weights: light chain NF (NFL), medium chain NF (NFM) and heavy chain NF (NFH). The NFL forms the backbone to which NFH and NFM chains copolymerise to form neurofilaments. Increased levels of CSF NF primarily reflect axonal degeneration of large myelinated axons, such as those present in the pyramidal tracts. NFL is a mainly non-phosphorylated protein, whereas NFH is substantially phosphorylated (pNFH), and can be measured in that form. CSF NFL has been shown to be increased in a variety of acute and chronic neurological diseases [168-170] (for review, see [171]).

#### Previous Findings in Parkinsonism

NFL has been investigated in parkinsonian disorders in a relatively large number of studies [172-175]. A review from 2009 concluded that NFL could differentiate between PD and controls on one side and MSA and PSP on the other side, although with overlap. NFL could not discriminate between MSA-P and MSA-C, nor between MSA and PSP [176]. Several studies have been conducted since then with similar findings. Hall et al. found increased NFL in MSA, PSP and CBD and that NFL was the best discriminator between PD and APD [151].

### **1.4.2.3 Tau Protein**

#### Background

Tau protein is important for the function of axonal microtubules and thereby for the structural integrity of the neuron and for axonal transport. In

hyperphosphorylated form it has reduced binding affinity for microtubules and leads to their malfunction. At the same time, it adopts an abnormal configuration favoring aggregation and inclusion formation [69]. Tau protein is the main structural element of neurofibrils in Alzheimer's disease (AD) but it has also been found in neurofibrillary tangles in PSP, in neuronal cytoplasmic inclusions and in ballooned neurons in CBD and PSP [177]. Increased tau levels indicate cortical axonal degeneration.

#### Previous Findings in Parkinsonism

CSF tau protein levels in parkinsonism have been investigated in many studies in the past, with inconclusive results. In PD, most studies found normal values, but both higher and lower values were reported. In atypical parkinsonism, tau levels tended to be higher in MSA than in PD, but not in PSP. The results for CBD are mixed, with both higher and lower levels than in controls being reported (for review of older literature, see [176]).

Recently, in a large study on patients with dementia, total tau and phosphorylated tau levels were not significantly different in PSP and CBD compared with controls (patients with subjective memory complaints) [178]. In four recent large studies, tau protein was investigated along with other CSF compounds (see "Combinations of CSF compounds").

### **1.4.2.4 Amyloid- $\beta$**

#### Background

A $\beta$ 42, derived from the proteolytic processing of a larger protein, amyloid precursor protein, is a major component of neuritic plaques in AD. Due to its sequestration in plaques, the characteristic pattern in AD is low CSF A $\beta$ 42 levels. Low CSF concentrations have also been found in Creutzfeldt-Jakob's disease, in DLB, in frontotemporal and vascular dementias, and in PD with dementia.

#### Previous Findings in Parkinsonism

Previous studies in parkinsonism were inconclusive, with both normal and decreased levels in the same disorder, and did not allow any conclusions ([151], for review, see [176]). However, in vitro studies have shown that A $\beta$ 42 promotes accumulation of alpha-synuclein making it interesting in a PD context [179].

More recent studies have found a correlation between A $\beta$ 42 and cognitive dysfunction in PD, with significantly lower CSF A $\beta$ 42 and higher total tau protein levels in PD with dementia (PDD) compared with PD [180]. In addition, this pattern also distinguished AD from PD, DLB and MSA, although CSF A $\beta$ 42 was lower in DLB compared with controls and PD

[130, 151, 152]. In a study from Norway, non-demented PD patients with memory impairment had lower A $\beta$ 42 than those without memory impairment [181]. Significant associations were found between cognitive performance and CSF levels of A $\beta$ 42 and A $\beta$ 42/total-tau in non-demented PD patients in one more study [182]. Interestingly, in a rare occurrence, the ratio fractalkine /A $\beta$ 42 correlated with PD severity assessed by UPDRS-III [152].

### **1.4.2.5 DJ-1**

#### Background

DJ-1 is a gene product associated with PD in both familial and sporadic forms. Its exact function is not known but it seems to play an important role in oxidative processes where it probably acts as a protease, chaperon or antioxidant [183]. Loss of DJ-1 function leads to neurodegeneration.

#### Previous Findings in Parkinson's Disease

Previous studies have found both higher [184] and lower [147] CSF DJ-1 levels in sporadic PD compared with non-PD controls. DJ-1 will be investigated in the ongoing Parkinson Progression Markers Initiative study aiming to identify markers for disease progression [185].

### **1.4.2.6 8-Hydroxydeoxyguanosine (8-OHdG)**

#### Background

8-hydroxydeoxyguanosine (8-OHdG) is produced when reactive oxygen radicals react with guanine residues in DNA. When the oxidized DNA is repaired, 8-OHdG is excreted in the blood and eventually in urine, where it can be measured. As such, it has emerged as a marker of oxidation and mitochondrial dysfunction, not only in neurodegenerative disorders but also in cancer research.

#### Previous Findings in Parkinson's Disease

Sato et al. found that the mean urinary 8-OHdG increased with the disease stage in PD patients [186] and another group found an association between hallucinosis in PD and urinary 8-OHdG levels [187].

The CSF 8-OHdG levels were increased in non-demented PD compared with controls [188]. 8-OHdG is one of the parameters selected for assessment in the FS-ZONE study, investigating the effect of pioglitazone, a potential antioxidant, in early PD ([http://www.ninds.nih.gov/disorders/clinical\\_trials/NCT01280123.htm](http://www.ninds.nih.gov/disorders/clinical_trials/NCT01280123.htm)).

### 1.4.2.7 Urate

#### Background

In humans, uric acid is the major product of the catabolism of the purine nucleosides adenosine and guanosine. Purines are derived from dietary intake as well as from endogenous metabolic processes (synthesis and cell turnover). The enzyme uricase which breaks down urate is absent in humans and apes, due to mutations which occurred millions of years ago [189]. As a result, together with extensive reabsorption of filtered urate (> 90%), humans have high serum urate levels (about 5 mg/dL in men), close to the maximum solubility. Levels above the saturation limit (7 mg/dL) can result in hyperuricemia which may be a cause of disease in humans. However, higher urate levels may account for the greater longevity of humans, e.g. due to lower cancer rates compared with shorted-lived mammals. During the evolution, urate has replaced ascorbate as the most potent antioxidant in humans.

#### Previous Findings in Parkinson's Disease

There is a substantial amount of evidence showing a relationship between urate and PD. Higher serum urate levels and higher dietary urate intake are associated with lower risk for developing PD, and with slower disease progression, better cognitive performance, and reduced loss of striatal [123I]  $\beta$ -CIT uptake in those already having PD [190-193]. In a recent study, the ratio between the immediate precursor of urate, xanthine and homovanillic acid, the major catabolite of dopamine, was different in PD patients compared with controls and correlated with disease severity as measured by the sum of UPRDRS Activities of Daily Living and Motor Exam ratings, suggesting that this quotient provides both a state and trait biomarker of PD [194]. The odds for having parkinsonism but without signs of dopaminergic deficit on iodine-123-labeled 2- $\beta$ -carboxymethoxy-3- $\beta$ -(4-iodophenyl) tropane ([123I]b-CIT) scan were higher in subjects with higher urate levels [195].

The title of a recent article reflects the encouraging data centered on urate and its future perspectives: "Urate: a novel biomarker of Parkinson's disease risk, diagnosis and prognosis" [196].

### 1.4.2.8 Glial Fibrillary Acidic Protein

#### Background

GFAP is a protein expressed mainly in the fibrillary astrocytes. High CSF levels have been seen in the context of acute central nervous system injury leading to disintegration of astroglial cells, such as brain infarction [197],

traumatic brain injury [198], and in chronic brain disorders with astrogliosis such as hydrocephalus [199] and dementia [200].

#### Previous Findings in Parkinsonism

Previous studies have shown normal levels in parkinsonian disorders [173, 201].

### 1.4.2.9 PGC-1 $\alpha$

#### Background

Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ) is a key transcriptional co-regulator involved in mitochondrial respiration, oxidative stress defense and adaptive thermogenesis [202].

#### Previous Findings in Huntington's Disease and in Parkinson's Disease

Reduced mRNA levels of PGC-1 $\alpha$  leading to mitochondrial dysfunction and neurodegeneration were found in HD models [203], opening up for new therapeutic targets by artificially increasing PGC-1 $\alpha$  levels [204]. The same phenomenon seems to occur in PD [205, 206] and PGC-1 $\alpha$  dysregulation may cause malfunction of neuronal energy production and ultimately neuronal damage and death [207]. PGC-1 $\alpha$  is under investigation in new PD studies such as Pioglitazone in Early PD (FS-ZONE)

(<http://clinicaltrials.gov/ct2/show/NCT01280123>).

## 1.4.3 Combinations of CSF Biomarker Candidates

**Hong et al.** investigated PD patients, healthy controls and AD patients, and found that both DJ-1 and alpha-synuclein were decreased in PD compared with the other groups. Alpha-synuclein discriminated PD from controls with a sensitivity of 92% and a specificity of 58%. For DJ-1 the sensitivity was 90% and the specificity 70%. There was no association with disease severity. Combining alpha-synuclein with DJ-1 did not enhance the performance of the test model. They emphasized that blood contamination must be an exclusion criterion for sample analysis as it influenced the results; likewise, age must be taken into consideration as both DJ-1 and alpha-synuclein increased with age [147].

**Mollenhauer et al.** investigated a large number of patients with both synucleinopathies (PD, MSA, DLB) and tauopathies (PSP, AD) plus neurological controls, first in a training set and afterwards in a validation set. They found that a CSF alpha-synuclein concentration of 1.6 pg/ $\mu$ L discriminated PD from non-synucleinopathies with a 70% sensitivity and a



53% specificity. At this cutoff, the positive predictive value for any synucleinopathy was 91%. In the training set, a combination of alpha-synuclein, tau protein and age discriminated between and neurological controls and AD with an area under the curve (AUC) of 0.908. In the validation cohort the AUC was 0.702 for discriminating between synucleinopathies and a mixture of PSP, normal pressure hydrocephalus and neurological controls. Age, not diagnosis, was the strongest factor affecting total tau protein levels. Only mean alpha-synuclein levels and not total tau, or A $\beta$ 42 levels differentiated PD and MSA from neurological controls [148].

**Hall et al.** assessed patients with synucleinopathies (PD, MSA, DLB and PDD), tauopathies (PSP, CBD, AD) and healthy controls using a panel of compounds: alpha-synuclein, total tau protein, hyperphosphorylated tau, A $\beta$ 42, and NFL. Alpha-synuclein levels were decreased in synucleinopathies compared to controls, PSP and AD, and increased in AD compared to controls. NFL levels were substantially increased in APD. A receiver operating characteristics (ROC) analysis showed that NFL alone had the same ability to discriminate between PD and APD as the entire panel of five proteins, with an AUC of 0.93. Total tau protein was decreased in PD compared with controls, but increased in MSA and CBD compared with PD. No significant change was seen in PSP. A $\beta$ 42 did not differ significantly between controls and PD, MSA, PSP and CBD [151].

**Shi et al.** examined patients with PD, MSA, AD and healthy controls. The fractalkine/A $\beta$ 42 ratio correlated positively with PD severity (in cross-sectional studies) and with PD progression (in longitudinal studies). No other marker had shown this association before. Fractalkine is important for the proper function of microglia. In addition, the Flt3 ligand, a cytokine which acts as a neurotrophic and anti-apoptotic factor in CNS, could alone differentiate between PD and MSA with a sensitivity of 99% and a specificity of 95%. A $\beta$ 42 levels were lower in PD and MSA than in controls but higher than in AD. They could not differentiate between PD and MSA. Total tau levels were also lower in PD and MSA than in controls and AD. A combination of alpha-synuclein and phosphorylated tau/total tau could differentiate PD from MSA with a sensitivity of 90% and a specificity of 71% but only when samples with blood contamination were excluded. Alpha-synuclein was decreased in both PD and especially in MSA compared with controls, presumably reflecting aggregation or metabolic abnormalities [152].

**Bech et al.** investigated a group of patients with parkinsonian disorders (PD, MSA, PSP, CBD, DLB, PDD). They could confirm previous results concerning NFL. Thus, a ROC analysis of NFL showed a sensitivity of 86% and a specificity of 81% with a cut-off value of 284.7 ng/L for differentiating PD from atypical parkinsonism. A $\beta$ 42 was low in DLB. Neither phosphorylated-tau nor total tau differed between the diagnostic groups [208].

## 1.5 Biochemical Biomarker Candidates for Huntington's Disease

Compared with PD, there are fewer reports concerning potential CSF biomarkers in HD. In one study, CSF F2-isoprostanes, markers of oxidative stress, were elevated in HD [209]. CSF N(epsilon)-(gamma-L-Glutamyl)-L-lysine (GGEL), a marker of transglutaminase, a protein involved in mutant huntingtin's deleterious effects, was increased in HD [210]. Levels of transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2(1)), a cytokine involved in neuroprotection, were decreased in the blood from asymptomatic HD gene mutation carriers [211]. Using two-dimensional electrophoresis and mass spectrometry, Huang et al. analyzed the CSF proteome in six pairs of HD patients and controls. They found that the haptoglobin level, and the ratios of CSF prothrombin/albumin and Apo A-IV/albumin, were significantly elevated in HD. In addition, the ratio of CSF prothrombin/albumin significantly correlated with the disease severity assessed by UHDRS [212]. Using proteomics, Fang et al. created a comprehensive profile of the human HD CSF proteome. They compared it with genomics data and could conclude that 81% of the quantitative changes in brain-specific proteins corresponded to known transcriptional changes. There was a general decrease of brain-specific proteins in HD patients compared with controls and the proteins affected reflect known pathological phenomena in HD such as neurodegeneration, microgliosis and astrogliosis. They identified a number of proteins which differed significantly between HD patients and controls and they propose them as candidate biomarkers in HD [213].

Plasma proteomics revealed that levels of several proteins engaged in the regulation of inflammatory activities increased with increasing disease stages [214]. Also in plasma, 24S-hydroxycholesterol (24OHC) which is a brain-generated cholesterol metabolite associated with neurodegeneration, was found to be decreased in symptomatic HD patients compared with controls and with presymptomatic HD mutation carriers. It correlated with the decrease in caudate volume observed in gene-mutation carriers from pre-

manifest to HD stage 1 but with not with disease progression [215]. A study comprising both asymptomatic gene-carriers and symptomatic HD patients showed a widespread immune activation in HD, which could be detected in plasma. Increased plasma IL-6 levels could be measured in premanifest subjects with a mean of 16 years until predicted clinical onset [216]. In another study, the characteristic weight loss seen even in premanifest HD gene-mutation carriers, despite high caloric intake, correlated with low levels of the branched chain amino acids (BCAA), valine, leucine and isoleucine in plasma. The authors suggested that BCAA levels could be used as a biomarker, indicative of disease onset and early progression [217].

In a clinical trial, HD subjects were found to have increased serum 8-OHdG, a marker of oxidation and mitochondrial dysfunction, with lower levels following treatment with creatine [218]. In presymptomatic carriers of the mutant huntingtin gene, plasma 8-OHdG levels correlated with the proximity to HD diagnosis. In addition, the rate of annual increase in 8-OHdG correlated with the rate of disease progression [219].

## 2 AIM

Overall aim: To investigate compounds found in the CSF and serum from patients with parkinsonian disorders and HD and assess their biomarker potential in respect to: (1) differential diagnosis; (2) disease marker; (3) association with clinical measures; (4) disease progression; (5) effects of therapy; and (6) pathoethiological considerations.

Specific aims:

1. To investigate CSF NFL and CSF GFAP in healthy controls and in patients with parkinsonian disorders (PD, MSA, PSP and CBD) in order to evaluate their potential as an aid in the differential diagnosis and for measuring disease stage and progression (paper I).
2. To investigate the CSF NFL response over time in advanced PD patients treated with deep brain stimulation of nucleus subthalamicus in order to assess its potential for measuring long-term adverse events and therapy effects (paper II).
3. To investigate whether a proteomic profile can assist in the diagnostic differentiation of healthy controls and parkinsonian patients with PD, MSA, PSP and CBD (paper III).
4. To investigate CSF and serum urate in healthy controls and in parkinsonian patients and look for differences which may have patho-etiological implications (paper IV).
5. To investigate CSF NFL in healthy controls and in patients with HD and evaluate its potential as a disease marker and the association with clinical measures (paper V).
6. To investigate CSF total tau protein levels in healthy controls and in patients with HD and evaluate its potential as a disease marker and the association with clinical measures (paper VI).

## 3 PATIENTS AND METHODS

### 3.1 Subjects

Table 1 offers a summary of all subjects pertinent to this thesis.

**Table 1** Number of subjects in papers I-VI

<i>Diagnosis</i>	<i>C</i>	<i>PD</i>	<i>MSA</i>	<i>PSP</i>	<i>CBD</i>	<i>HD</i>
Paper I	59	10	21	14	5 <sup>1</sup> +6 <sup>2</sup>	-
Paper II	-	8	-	-	-	-
Paper III	24	56	42	39	9	-
Paper IV	18	6	13	18	6	-
Paper V	35	-	-	-	-	35
Paper VI	35	-	-	-	-	35

C = healthy controls; CBD = corticobasal degeneration; HD = Huntington's disease; MSA = multiple system atrophy; PD = Parkinson's disease; PSP = progressive supranuclear palsy; <sup>1</sup>CBD subjects with two LPs; <sup>2</sup>only one LP.

#### 3.1.1 Patients

##### 3.1.1.1 Patients with Parkinsonian Disorders

Subjects were recruited consecutively among patients with parkinsonian disorders treated by the movement disorders team at the Department of Neurology, Sahlgrenska University Hospital, between 1997 and 2009. Some of the patients were examined in all four studies, but many not, as they did not comply with the specific inclusion and exclusion criteria, or there was no more CSF or serum from them. As time passed, new patients were recruited.

###### 3.1.1.1.1 General inclusion criteria

All patients had established diagnoses of: definite PD, probable MSA, probable or definite PSP, and CBD.

The diagnosis of definite PD according to the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria requires: 1) the presence of parkinsonism; 2) absence of exclusion criteria, most of which point toward an atypical parkinsonian syndrome or secondary parkinsonism; and 3) three or more supportive prospective positive criteria for PD, such as unilateral onset, rest tremor, progression over time, persistent asymmetry, excellent response to levodopa sustained over at least five years, duration ten years or more, and levodopa induced dyskinesia [38].

The diagnosis of probable MSA, including MSA-P and MSA-C, according to Gilman's criteria from 1999 requires: 1) autonomic failure or urinary dysfunction; 2) poorly levodopa responsive parkinsonism or cerebellar dysfunction [55].

The diagnosis of probable PSP according to National Institute of Neurological Disorders and Stroke and Society for Progressive Supranuclear Palsy, Inc. clinical criteria requires: 1) gradually progressive disorder; 2) onset at age 40 or later; 3) vertical supranuclear gaze palsy; 4) prominent instability with falls in the first year of disease onset; 5) absence of exclusion criteria pointing towards other parkinsonian syndromes, dementias, Whipple's disease or other primary disease cause; 6) supportive criteria such as retrocollis, poor levodopa response, early dysphagia and dysarthria, early onset of cognitive impairment of frontal type [220]. Neuropathological examination of one patient confirmed the PSP diagnosis which in that case was definite.

The diagnosis of CBD according to Lang et al. requires: rigidity plus one cortical sign (apraxia, cortical sensory loss, or alien limb) OR asymmetric rigidity, dystonia and focal reflex myoclonus. Exclusionary criteria rule out other parkinsonian syndrome or other primary diseases [221].

#### 3.1.1.1.2 General Exclusion Criteria

1. Total follow up time from symptom onset less than 3 years.
2. Any kind of dementia at the time of LP.
3. CSF contaminated with blood (CSF erythrocytes > 500/ $\mu$ L in studies I, II and IV and CSF erythrocytes > 5/ $\mu$ L in study III).
4. STN DBS with the exception of subjects in study II in which it was an inclusion criterion.

#### 3.1.1.1.3 Particular Inclusion or Exclusion Criteria

##### Paper I

In the first study, only patients who had undergone two consecutive LP-s with one year interval were included. An exception was done for CBD patients, due to their scarcity; those with just one LP were analyzed separately in a subsection of the study. Some of the PD patients had initially been referred to the Movement Disorders Unit due to perceived levodopa unresponsiveness or other atypical features. However, all of them could sooner or later receive a definite PD diagnosis

## Paper II

In this study were included only PD patients who had normal CSF NFL at baseline, were operated with STN DBS, and underwent at least four consecutive LP-s according to the study protocol. This is the only study in which CSF was collected in PD patients after STN DBS.

## Paper IV

Only PD patients who had not taken any dopaminergic drugs for one month prior to the LP were included. There is a slight mistake in the published paper where it is written that the patients did not want to be treated. In reality, there were several reasons for the lack of dopaminergic treatment: some patients did not accept treatment, some were disappointed with the results and had stopped taking medications, and some had not been offered treatment yet.

### **3.1.1.2 Patients with Huntington's Disease**

The subjects investigated in studies V and VI were enrolled in a drug trial, the INTRO-HD study, conducted by the Huntington Study Group in 1995. This study was a multicenter, double-blind, randomized, placebo controlled clinical trial, examining the tolerability of OPC-14117, a free-radical scavenger, in adult HD subjects. Subjects were assessed at baseline and then randomized to three different dosages of OPC-14117 or matching placebo. As part of the study procedures, LPs were done at baseline and on the final dosing day at week 12.

The two HD studies investigated the same group of 35 subjects. They had: 1) characteristic clinical features of HD; 2) a confirmatory family history of the disorder or a confirmatory genetic test; and 3) no standard exclusionary criteria. Subjects were in stage I-III (I = normal; V = severely impaired, total care facility only), implying that they could stay in their own home although they had some disability; they were ambulatory and able to give consent for participation in the study.

### **3.1.1.3 Controls**

CSF and serum from four different subsets of Swedish healthy controls were used in studies I, III and IV - VI, for comparison with patients. All controls underwent a normal medical examination and had no history of neurological or psychiatric disease. None of them were related to the patients.

## **3.2 Methods**

### **3.2.1 Clinical Scales**

#### **3.2.1.1 Hoehn and Yahr Scale**

The oldest scale for assessment of parkinsonian disorders, the H&Y scale offers a simple measure of the disease stage, in five steps. In addition to its high acceptance and utilization, the scale has been shown to correlate with neuroimaging findings and to some standardized scales of motor impairment, disability and quality of life. Its major disadvantages are its non-linearity, the mixture of impairment and disability, the scarcity of motor parameters beyond gait and balance, and the absence of non-motor parameters [222, 223].

#### **3.2.1.2 The Unified Huntington's Disease Rating Scale**

The Unified Huntington's Disease Rating Scale (UHDRS) is a comprehensive instrument for assessing in HD: a) motor function (normal 0 points, to grossly abnormal 142 points); b) behavior (higher scores - more disability); c) function in daily activities (higher scores - less disability); and d) independence (0 to 100% independent) [91].

##### **3.2.1.2.1 Total Functional Capacity Scale**

A subscale of UHDRS, the Total Functional Capacity (TFC) scale, assesses the overall function of the patient in respect to basic domains of daily life such as occupation, finances, domestic chores, and care level. The range is between 13 points (normal) and 0 points (unable to do anything, full time skilled nursing) [91].

### **3.2.2 Cerebrospinal Fluid and Serum Sampling**

#### **3.2.2.1. Papers I-IV**



Generally, all patients with atypical parkinsonian syndromes and a large part of those suspected of having PD undergo LP and blood tests as part of the routine initial investigations at our Movement Disorder Unit. CSF and serum are collected both for immediate analyses and for storing in a Biobank. The samples used in these studies came both from this biobank and from specific sampling dedicated to these studies.

The CSF was collected in the morning in polypropylene tubes via LP, performed at our Clinic in the lateral recumbent position. Serum was collected in vacutainer tubes by venipuncture. CSF was gently mixed to avoid gradient effects. The first 10 ml portions of serum and CSF were taken for immediate routine analysis, including CSF erythrocytes. The second 10 ml portions were taken separately, aliquoted in 2-ml tubes and stored in a biobank. All samples were kept frozen at  $-80^{\circ}\text{C}$  until the time for analysis. All analyses pertaining to this study were conducted from the second portion of serum and CSF. CSF and serum from controls are handled in the same way as described for patients.

### **3.2.2.1. Papers V and VI**

As part of the INTRO-HD study procedures, LPs were performed at baseline and on the final dosing day of week 12, after eight hours of bed rest. Seventeen milliliters of CSF were collected at each LP. The samples were stored at  $-70^{\circ}\text{C}$  at Cornell University, Neurology Department, New York, NY, USA. In 2005, they were send frozen to Gothenburg, Sweden, for analysis and were thawed only once, at the time of NFL and tau analyzes.

## **3.2.3 Laboratory Methods**

All samples were coded, and the analyst was unaware of any patient data. All analyses were performed by certified laboratory personnel at the Neurochemical Laboratory, University of Gothenburg, Sweden. The laboratory is accredited by the Swedish Board for Accreditation and Conformity Assessment.

### **3.2.3.1 Sandwich ELISA**

#### **3.2.3.1.1 Neurofilament Light Chain (NFL) Assay (Papers I, II)**

CSF NFL levels were measured using an in-house developed sandwich Enzyme Linked Immunosorbent Assay (ELISA). CSF samples and reference NFL were incubated for two hours, at room temperature, together

with microtitre plates coated with hen anti-NFL IgG. Rabbit polyclonal anti-NFL IgG was thereafter used as secondary antibody. For detection, peroxidase-conjugated donkey anti-rabbit IgG was used. The detection limit of the assay was 250 ng/L for these studies [168].

#### 3.2.3.1.2 Glial Fibrillary Acidic Protein (GFAP) Assay (Paper I)

The GFAP assay was identical with the NFL assay except that the plates were coated with anti-GFAP IgG and the detection antibodies were also anti-GFAP. The detection limit of the assay was 32 ng/L.

#### 3.2.3.1.3 Total Tau Protein (Paper VI)

CSF total tau (T-tau) concentration was determined at the University of Göteborg, Sweden, using a sandwich ELISA (Innotest hTAU-Ag, Innogenetics, Gent, Belgium) specifically constructed to measure all tau isoforms irrespectively of phosphorylation status as previously described [224]. The detection limit of the T-tau ELISA was 75 ng/L and the coefficient of variation was below 10%.

### 3.2.3.2 Uricase/Peroxidase Enzymatic Method

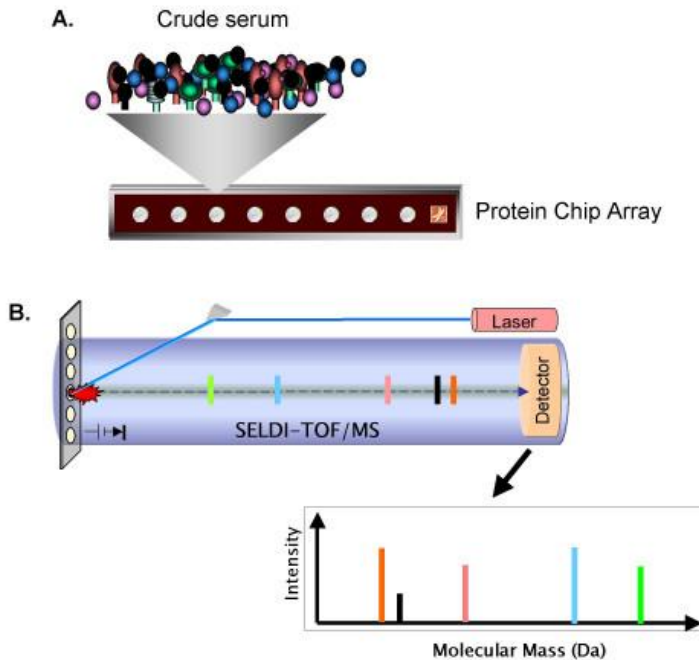
#### 3.2.3.2.1 Urate Assay (Paper IV)

Urate concentrations were measured by the uricase/oxidase enzymatic method on a Modular system (Roche Diagnostics GmbH, Basel, Switzerland). This method implies oxidation of urate by uricase with production of hydrogen peroxide which is then coupled to a reagent, in the presence of peroxidase. The resultant compound can be quantified.

### 3.2.3.3 SELDI-TOF Mass Spectrometry

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF / MS) (Fig. 2), first described by Hutchens and Yip [225], and further developed by CIPHERgen Biosystems [226], is an easy-to-use system with high output [227]. It requires low sample volumes and is best for low molecular weight proteins. The process consists of four steps: 1) Each CSF sample is added to a specific array surface for selective adsorption, followed by washing, thus removing non-specific proteins and salts. This simplifies the complexity of the protein content in the sample. In our study (paper III) three different array surfaces were used: cation-exchange, anion-exchange and metal-ion binding; 2) Adsorbed proteins are eluted from the array surfaces by a laser and are ionized and accelerated. Sinapinic acid was used as the energy-absorbing molecule; 3) Depending on the mass-to-charge ratio ( $m/z$ ), each protein or protein fragment (peptide)

travels with a certain velocity, is separated from other proteins with other  $m/z$ -s, and, after a certain time of flight, is captured by a detector. The detector records an ion current of the separated analytes and creates a mass spectrum where the ion current is plotted against  $m/z$ ; 4) The last step is protein identification. In our study, proteins from interesting peaks in the mass spectra were purified from CSF by liquid chromatography sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by tandem mass spectrometry [228].



**Figure 2. Analysis of serum proteins using ProteinChip arrays and SELDI-TOF/MS. A.** Crude serum sample is placed (and processed) on a ProteinChip array which contains chemically (cationic, anionic, hydrophobic, hydrophilic, etc.) or biologically treated surfaces for specific interaction with proteins of interest. Proteins, thus, bind to chemical or biological "docking sites" on the ProteinChip surface. Non-binding proteins, salts, and other contaminants are washed away, eliminating sample "noise". **B.** Retained proteins are "eluted" from the ProteinChip array by Surface-Enhanced Laser Desorption/Ionization (SELDI). Ionized proteins are detected and their mass accurately determined by Time-of-Flight Mass Spectrometry (TOF/MS). From

Abramovitz M, Leyland-Jones B. A systems approach to clinical oncology: focus on breast cancer. *Proteome Sci.* 2006 Apr 4;4:5.<sup>1</sup>

## 3.2.4 Ethics

### 3.2.4.1. Papers I - IV

At our Clinic, all patients who undergo LP and blood tests must give their informed consent to these procedures and to the storage of samples in a biobank for present and future analyses. The Biobank for parkinsonian patients complies with necessary Swedish regulations concerning biobank activities. The medical ethical committee at the University of Gothenburg approved the creation of this biobank and the use of data generated from it for research and publications, as long as full patient confidentiality and anonymity is respected.

For studies I and II: All subjects consented to participate in these studies which implied two or more LPs. In study no. I were also included patients who had undergone two LPs with one year interval as part of their regular medical investigations. The studies were approved by the medical ethical committee at the University of Gothenburg, Sweden.

### 3.2.4.2. Papers V and VI

HD subjects: The study protocol and consent form were reviewed and approved by the University of Rochester Research Subject Review Board. In addition, the Human Subjects Review Boards at each site-institution (where patients were actually enrolled and underwent study procedures) provided complete ethical review and approval of the protocol and consent process.

### 3.2.4.3. Healthy controls:

The healthy controls gave their informed consent for the LP and for donating their CSF for research purposes. The sampling of CSF from healthy controls was approved by the Ethical Committee at the University of Gothenburg.

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## 3.2.5 Statistics

### 3.2.5.1 Generalities

Analyses were performed using commercially available statistical software: SYSTAT 11.0; SYSTAT GmbH, Erkrath, Germany (paper I); Prism 5, Graphpad Software Inc, La Jolla, CA, USA (paper II and III); and SPSS software, SPSS Science, Chicago, IL, USA (paper IV).

P-values  $<0.05$  were considered statistically significant in papers I- II and IV-VI. A P value  $\leq 0.01$  was considered significant in paper III.

For the statistical analysis, all NFL values lower than the detection limit of the assay (250 ng/L) were entered as 250 ng/L. Data analysis was performed by correction for age.

### 3.2.5.2 Comparing Means

#### 3.2.5.2.1 Papers I, III and IV

The distribution of the variables was significantly skewed. Therefore, single marker statistics were calculated using the non-parametric Mann-Whitney U test for 2-group analyses on the comparisons that were significant using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

#### 3.2.5.2.2 Paper II

In paper II, the CSF NFL values originating from the same group of patients were compared at different time points using Wilcoxon signed test (the nonparametric analogue of the paired t-test), as the distribution of the variables was significantly skewed.

#### 3.2.5.2.3 Papers V and VI

Both paired and unpaired t-tests were used to compare CSF NFL and total tau protein levels in HD patients and controls, with identical results. Chi-square tests were used for comparing categorical variables.

### 3.2.5.3 Correlations

#### 3.2.5.3.1 Papers I-IV

Spearman correlation coefficient (non-Gaussian populations) was used for estimating the relationships between levels of CSF and serum compounds and demographic and disease related variables.

#### 3.2.5.3.2 Papers V and VI

The relationships between CSF NFL levels and demographic and disease related variables were examined with Pearson correlations (Gaussian populations), both adjusting for age at collection and without. Variables were prespecified as of primary interest or as of secondary interest. Scatter plots of CSF NFL levels versus each variable were created along with the Pearson correlation.

### 3.2.5.4 OPLS Discriminant Analysis

#### Paper III

In paper III, after calculating single marker statistics, the combined data from all the four identified markers was further analyzed using the orthogonal projections to latent structures discriminant analysis (OPLS-DA) algorithm [229] implemented in the software SIMCA-P+ (v12, Umetrics, Umeå, Sweden). Prior to the analysis the data was randomly divided in equally sized training and prediction sets. The training sets were used to construct the model, which was then tested for robustness using the prediction set.

### 3.2.5.5 Receiver Operating Characteristics

#### Paper III

The discriminating quality of the OPLS Discriminant Analysis model described above (3.2.5.4) was judged by the area under the curve (AUC) of the receiver operating characteristic (ROC) plot. The ROC plots sensitivity against specificity and the AUC represents the probability that a randomly chosen positive instance (i.e. a test value from an APD patient) is ranked higher than a randomly chosen negative instance (i.e. a test value from an PD patient). Thus, the higher the AUC, the better the test. ROC curves permit comparisons between different types of biomarkers as they do not depend on the absolute scale or raw data. The Youden's index, with values ranging between -1 and 1, is the difference between the true positive rate and the false positive rate. Maximizing this index allows to find the optimal cut-off point from the ROC curve.

## 4 RESULTS

### 4.1 Markers for Differential Diagnosis

#### **4.1.1. CSF NFL levels were increased in atypical parkinsonian disorders including CBD (paper I)**

The CSF NFL levels were found to be similar in PD patients and in controls but significantly higher in the atypical parkinsonian disease group, including CBD<sup>2</sup>, at both the first and the second LP, one year later (Table 2 and Fig. 3A and B). The overlap between CSF NFL levels in PD/controls and MSA/PSP/CBD was minimal.

At the group level, NFL levels could differentiate PD and controls on one side from MSA, PSP and CBD on the other side (age-adjusted  $P < 0.001$ ) but they could not differentiate between PD and controls, between MSA, PSP and CBD, nor between MSA-P and MSA-C.

#### **4.1.2. CSF GFAP levels were similar in controls and in parkinsonian disorders (paper I)**

The CSF GFAP levels were normal in all parkinsonian disorders at both the first and the second LP, one year later. There were no associations with any demographic or clinical parameters. Thus, the only way CSF GFAP levels might be useful in the context of parkinsonism is to warn for some other diagnosis, if increased.

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<sup>2</sup> When Paper I was published, it was, to the best of our knowledge, the first report on CSF NFL levels in CBD.

**Table 2**      **Paper I: CSF NFL and CSF GFAP levels at the time for the first LP**

<i>Diagnoses</i>	<i>Controls</i>	<i>PD</i>	<i>MSA</i>	<i>PSP</i>	<i>All CBD</i>
Number of subjects	59	10	21	14	11
Median (range) CSF-NFL at first LP (ng/L)	250 (250-710)	250 (250-347)	1207 (250-6030)	956.5 (228-1570)	870 (492-1811)
Median (range) CSF-GFAP at first LP (ng/L)	483 (111-1363)	499 (300-920)	558 (160-999)	760 (334-1580)	840 (400-1614)

**CBD = corticobasal degeneration; CSF = cerebrospinal fluid; GFAP = glial fibrillary acidic protein; LP = lumbar puncture; MSA = multiple system atrophy; NFL= neurofilament light chain; PD = Parkinson’s disease; PSP = progressive supranuclear palsy.**



Fig. 3A

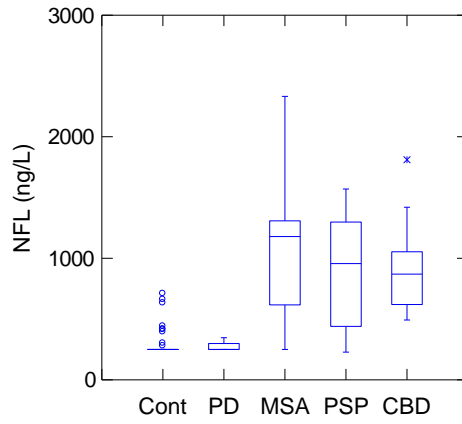
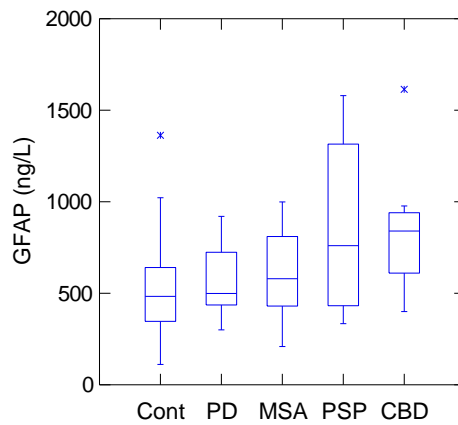


Fig. 3B



**Figure 3. (A) CSF NFL levels at the time for the first lumbar puncture (LP1) in controls and in parkinsonian disorders; (B) CSF GFAP levels at the time for the first lumbar puncture (LP1) in controls and in parkinsonian disorders.**

CBD = corticobasal degeneration; CSF = cerebrospinal fluid; GFAP = glial fibrillary acidic protein; LP = lumbar puncture; MSA = multiple system atrophy; NFL= neurofilament light chain; PD = Parkinson's disease; PSP = progressive supranuclear palsy

#### **4.1.3. A proteomic profile could distinguish PD from atypical parkinsonian disorders patients (paper III)**

Using the SELDI-TOF MS technique, a proteomic profile was identified, consisting of four proteins (ubiquitin,  $\beta$ 2-microglobulin, and 2 secretogranin 1 [chromogranin B] fragments) which helped differentiate PD patients from patients with APD (Fig. 4). However, there were no significant differences in the levels of any protein/peptide when comparing PD patients and controls. Approximately 2000 peptide/protein peaks (mass/charge ratios) were detected in the samples.

**Figure 4**

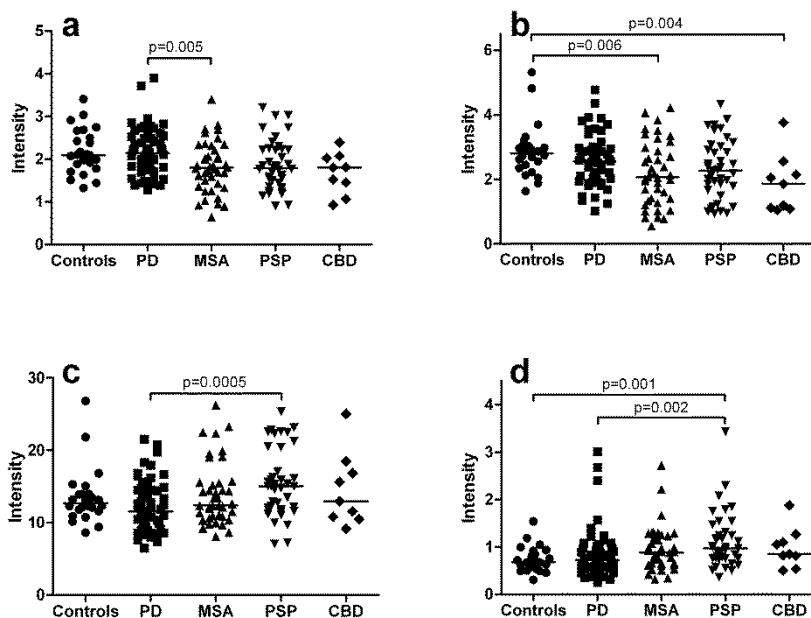


Figure 4. Scatter plots of SELDI-TOF MS intensities for (a) secretogranin 1 (chromogranin B) fragment I, (b) secretogranin 1 (chromogranin B) fragment II, (c)  $\beta$ 2-microglobulin, and (d) ubiquitin. The black bars represent the medians in each group. Mann-Whitney U test significant levels are displayed for the comparisons that were significant after Kruskal-Wallis test using Dunn's post hoc test.

CBD = corticobasal degeneration; MSA = multiple system atrophy; PD = Parkinson's disease; PSP = progressive supranuclear palsy

As none of the four identified proteins could by itself differentiate between PD and APD, they were grouped together and a multivariate discriminant analysis was performed (Fig. 5). Combining information from the four identified peaks made possible a good discrimination between PD and APD in the multivariate analysis, with an AUC of 0.80. For comparison, the AUC for the individual proteins used in the multivariate analysis were in the range of 0.62 to 0.67 (Fig. 5C). As shown in Fig. 5B, all four proteins contributed equally to the separation of PD from APD. The similarity between the training and prediction sets within each group was a strong indicator for a stable model (Fig. 5A).

Figure 5

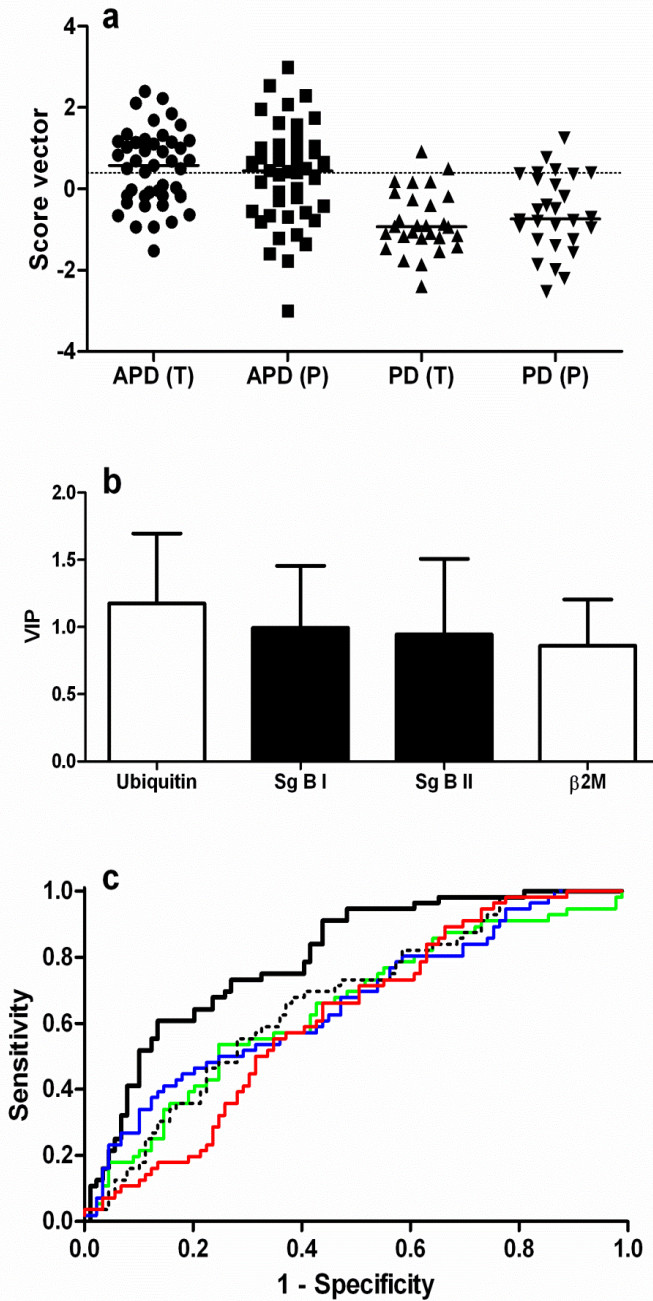


Figure 5. Multivariate discriminant analysis comparing Parkinson's disease (PD) and atypical parkinsonian disorders (APD). (a) The projection to the score vector, resulting from orthogonal projections to latent structures discriminant analysis (OPLS-DA), for training (T) and prediction (P) sets. Solid bars represent the medians in each group and the dotted line the maximum of Youden's index (sensitivity 91% and specificity 56%) when training and prediction set were combined. (b) The variable importance in the projection (VIP). The VIP is a relative measure of the analytes' contributions to the separation. Black or white bars correspond to an increase or decrease, respectively, of the analyte in PD compared to APD. The error bars represent a 95% confidence interval. (c) Receiver operating characteristic (ROC) curves for the OPLS-DA (black solid line) and the data shown in Fig. 4: secretogranin 1 (chromogranin B) (Sg B I) fragment I (black dotted line), secretogranin 1 (chromogranin B) (Sg B II) fragment II (red line),  $\beta$ 2-microglobulin ( $\beta$ 2M) (blue line), and ubiquitin (green line).

#### **4.1.4. CSF NFL levels were higher in HD patients (paper V)**

The CSF NFL levels were significantly higher in all HD subjects [944.6 (432.4) ng/l] [mean (SD)] as compared with controls [154.5 (51.6) ng/l] ( $p < 0.0001$ ) (Fig. 6).

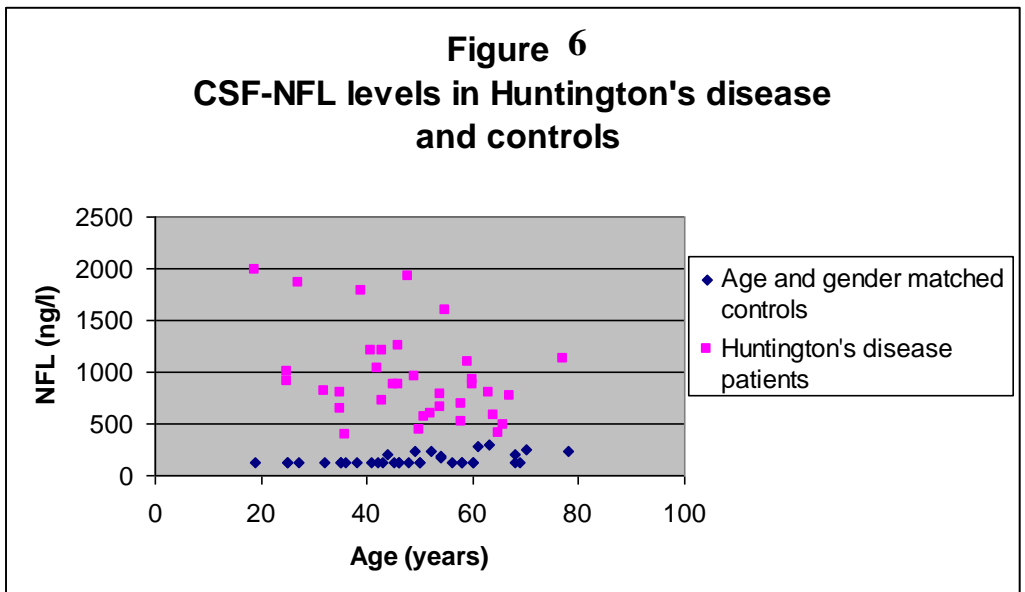


Figure 6. Significantly higher levels of the light subunit of neurofilament triplet protein (NFL) were found in the cerebrospinal fluid (CSF) collected from patients with Huntington's disease, compared with age and gender matched controls ( $p < 0.0001$ ).

#### **4.1.5. CSF Total tau levels were higher in HD patients (paper VI)**

The mean CSF total tau protein level in the HD group ( $322 \pm 143$  ng/L) was significantly higher than that in the control group ( $188 \pm 118$  ng/L) ( $p < 0.001$ ) (Fig. 7).

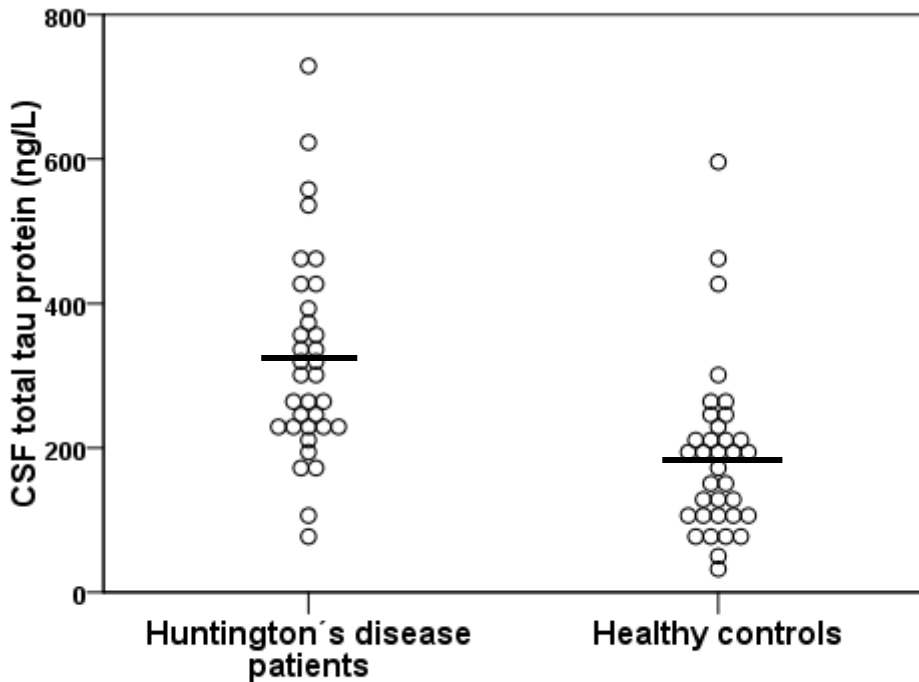


Figure 7. Total tau protein levels in the cerebrospinal fluid were measured in 35 patients with Huntington's disease, and in 35 healthy controls, using ELISA. Total tau protein levels were significantly higher in the Huntington's disease group (unpaired t-test) ( $p < 0.001$ ). Means are marked by horizontal lines.

## 4.2 Markers for Disease Stage and Progression

### **4.2.1. CSF NFL levels do not measure disease stage or progression in parkinsonian disorders (paper I)**

In both PD and atypical parkinsonian disorder, there was no change in CSF NFL levels measured twice with one year interval. However, in the CBD group, there was a tendency towards higher CSF NFL levels one year after the first LP, with borderline significance ( $p = 0.047$ ). On the other hand, the number of CBD patients at the second LP to was much too low to permit any conclusions.

There was no relationship between CSF NFL levels or the change in CSF NFL levels over one year, and measures of disease severity (H&Y stage, disease duration, age at onset) at the time for the first LP. In addition, there was no relationship between measures of disease worsening (changes in H&Y score) and changes in CSF NFL levels over one year, although data available for these calculations were scarce.

### **4.2.2. CSF NFL levels were correlated to the Total Functional Capacity score in HD (paper V)**

In HD patients, the Pearson partial correlations between CSF NFL levels (adjusted for age at CSF draw) and UHDRS-TFC were significant ( $\rho = -0.334$ ,  $p = 0.05$ ). No significant correlations were found with other demographic parameters or clinical assessment scores.

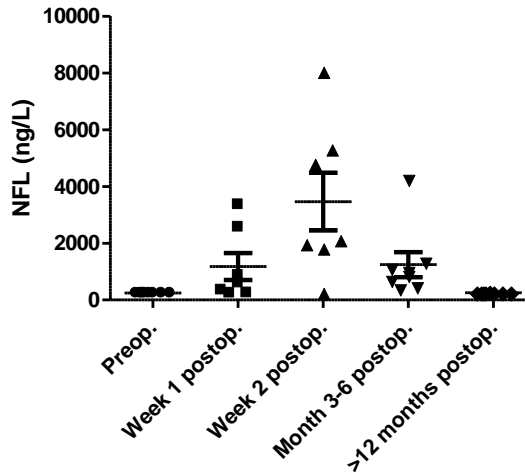
### **4.2.3. CSF Total tau levels were not correlated to clinical scores in HD (paper VI)**

There were no statistically significant correlations between the CSF total tau protein levels and demographic parameters or any of the clinical scores, in the HD population.

## 4.3 Markers for Effects of Therapy

### **4.3.1. CSF NFL levels were not affected, in the long term, by active STN DBS treatment for advanced PD (paper II)**

CSF NFL levels in patients with PD, normal prior to STN-DBS, increased sharply during the first two weeks postoperatively, as expected, due to the operative trauma; they remained elevated for several months but returned to normal at 12 months follow-up and later (Fig. 8).



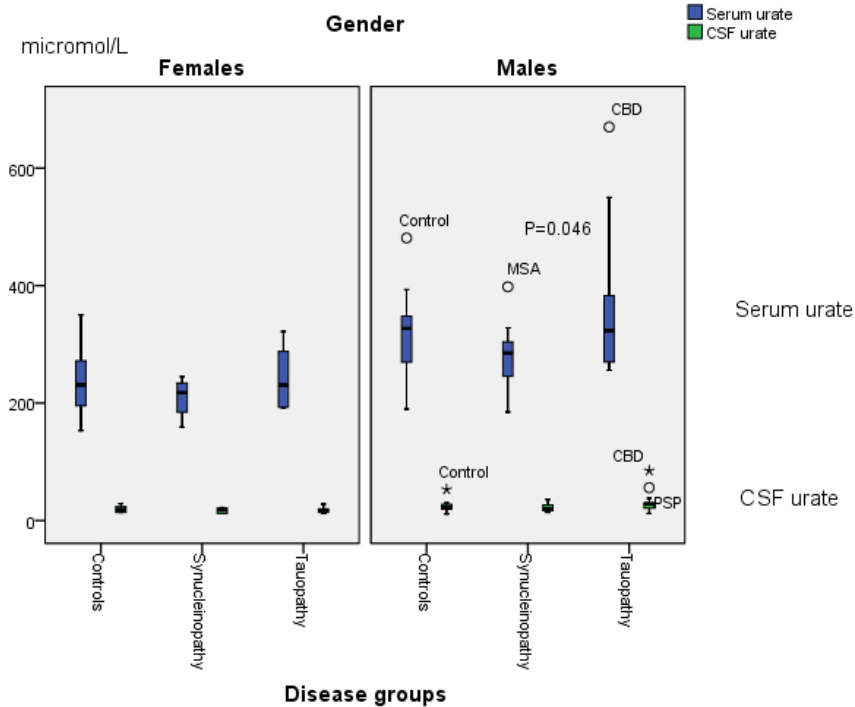
**Figure 8.** Cerebrospinal fluid levels of neurofilament light chain (NFL) increased rapidly during the first and second weeks after surgery for deep brain stimulation of the subthalamic nucleus ( $P = 0.031$  for both comparisons vs the preoperative values); they remained elevated at 3–6 months ( $P = 0.0078$  vs preoperative values), but returned to normal >12 months postoperatively.

## 4.4 Markers with Patho–Etiological Implications?

### 4.4.1. Serum urate levels were lower in synucleinopathies compared with tauopathies (paper IV)

In males, serum urate levels were lower in synucleinopathies [median 237 (range 159-398)  $\mu\text{mol/L}$ ] than in tauopathies [median 287 (range 192-670)  $\mu\text{mol/L}$ ], although with a broad overlap (Fig. 9). In this study the synucleinopathies were represented by PD and MSA and the tauopathies by PSP and CBD.





**Figure 9.** Serum and cerebrospinal fluid urate levels in controls, synucleinopathies (PD and MSA), and tauopathies (PSP and CBD), grouped by gender. In males, serum urate levels were significantly lower in synucleinopathes compared with tauopathies ( $p = 0.046$ ). CBD = corticobasal degeneration; CSF = cerebrospinal fluid; MSA = multiple system atrophy; PD = Parkinson's disease; PSP = progressive supranuclear palsy.

No significant differences were seen when comparing serum and CSF urate levels in healthy controls, PD, MSA, PSP and CBD patients. In females, there were no significant differences between controls and the different diagnostic categories, or among the different diagnostic categories in regard to serum or CSF urate levels.

## 5 DISCUSSION AND IMPLICATIONS

### 5.1 Markers for Differential Diagnosis

Reports on CSF NFL levels in parkinsonian disorders have been emerging regularly ever since the first publications in the 1990-s and the observations of increased levels in APD have been consistent over time. The results presented in paper I replicated previous findings showing that CSF NFL can differentiate PD and controls on one side from APD on the other side. In addition, they also extended this finding to CBD, for which, at the time for publication, there were no reports on CSF NFL.

Recently, in a new study in patients with parkinsonian disorders (PD, MSA, PSP, CBD, DLB, PDD), utilizing the ROC analysis, Bech et al. could show that a CSF NFL cut-off value of 284.7 ng/L could differentiate PD from atypical parkinsonism with a sensitivity of 86% and a specificity 81%. Neither phosphorylated-tau nor total tau differed between the diagnostic groups [208]. Even better results were published in 2012 by Hall et al. from a study which also included some of the patients presented here. Multivariate and ROC analysis revealed that CSF NFL levels alone could differentiate PD from APD, with an area under the curve of 0.93 [151].

The usefulness of CSF NFL measurements is greatly diminished by its inability to differentiate between different APD, by its low specificity and low negative predictive value. However, these shortcomings may have some advantages. As NFL does not seem to be directly linked to the disease process and is more an indicator of neuronal damage, it could be regarded as a global marker of accelerated neuronal death irrespective of etiology. As such, it may capture a larger part of the disease than a more specific but at the same time restricted etiological biomarker. A global biomarker may be more helpful for developing treatment strategies in complex diseases such as the neurodegenerative movement disorders which may have multiple causes and disease mechanisms, not amenable to simple analysis. The neurodegenerative process in PD may be of a too low intensity to be detected as an increase in CSF NFL levels. On the other hand it stretches over longer periods of time compared with the more aggressive APD where neurodegeneration seems to be faster, reflected in a shorter survival, and more massive as shown by a heavier symptom burden and mirrored by an increase in CSF NFL levels.

In paper III, a proteomic profile was found, which could, in a similar way as CSF NFL, differentiate PD from atypical parkinsonism, but without any sharper differential diagnostic abilities. While of no immediate diagnostic value, these findings may have some implications from a patho-etiological point of view, as discussed in 5.4. Our results confirmed that SELDI-TOF MS preferentially detected highly expressed proteins, while low abundant neuronal proteins such as tau and neurofilaments remained below the detection limit.

Considering these facts, the question may arise whether information from targeted assays such as CSF NFL analysis, complemented by data coming from a broad proteomic study, would result in a composite test with higher performance than each one of them in isolation.

## 5.2 Markers for Disease Onset, Stage and Progression

Being able to determine the time for disease onset is imperative both for developing disease modifying therapies and even more so in the long-awaited and much-hoped-for eventuality that they would be available one day. This applies to both parkinsonian disorders and to HD. The question of disease onset has not been addressed in the studies presented here, as they only included patients with established diagnoses. However, once it is shown that CSF NFL was increased in atypical parkinsonism and in HD, and that a proteomic profile could reveal APD, the question arises whether this knowledge could be applied to asymptomatic individuals in whom the disease process has already started but is still not manifest. In HD, it is possible to identify asymptomatic gene mutation carriers due to family history and the possibility of performing genetic tests. The same applies to certain genetic forms of PD, such as LRKK2, even if it is not widespread yet. However, for the majority of parkinsonian patients, that is not feasible and the population to be tested for biochemical markers would have to be enriched using other methods, similar to those proposed in PD. There prodromal symptoms, such as hyposmia [230], rapid eye movement related behavioral disorder (RBD) [231], constipation [105] or depression [110, 232] could be used to identify a population at risk for PD.

Given the inherent heterogeneity of neurodegenerative movement disorders, their multifactorial etiology and their capricious clinical expression, there is a need for a way to measure disease stage and the rate of disease progression. The studies presented here tried to address these questions but

the results turned out negative or inconclusive, with one exception: in HD, CSF NFL was found to be correlated with the total functional capacity score. If replicated, the implications of this finding might be investigated, as the total functional capacity score is a robust parameter directly reflecting the overall function of the HD patient in the daily living.

In a recent study, our finding that CSF NFL did not change over one year in MSA patients, was replicated. In a longitudinal study in MSA patients, NFL and NFH levels did not change over a one-year period [233]. However, one year is a very short time and the question should be investigated in longer longitudinal studies. Such studies could presumably also show whether the CSF NFL levels are in any way associated with long time prognosis.

Considering that reliable and sensitive biomarkers for disease onset and rate of disease progression are crucial for the development of disease modifying therapies in HD, especially for the asymptomatic gene-mutation carrier population, several observational studies such as TRACK-HD and its continuation, Track-On HD, and The Neurobiological Predictors of Huntington's Disease (PREDICT-HD) study were established. They investigate presymptomatic HD gene carriers and early HD patients for determining the natural history of the disease and for identifying and characterizing disease markers [85, 234], (<http://hdresearch.ucl.ac.uk/current-studies/trackon-hd/>). In PD, the importance of developing biomarkers for these purposes is recognized in the observational studies conducted over the years and still ongoing, such as Parkinson's Associated Risk Study (PARS) (<http://www.parsinfosource.com/>) or the Parkinson's Progression Markers Initiative (PPMI) (<http://www.ppmi-info.org/>).

## 5.3 Markers for Effects of Therapy

In paper II we report that CSF NFL levels increased postoperatively after STN DBS for advanced PD but normalized one year later and stayed normal thereafter. These finding might suggest that: (1) active STN DBS does not by itself lead to a neuronal death of such magnitude that it can be detected by an increased CSF NFL, once the postoperative trauma has resolved; and 2) the rate of neurodegeneration, as measured by CSF NFL, does not seem to accelerate, after STN DBS for advanced PD. To be able to ascertain that a therapy is not in itself deleterious for the disease being treated remains always a key point, and even more as new therapeutic approaches to PD are envisioned, that employ potentially harmful techniques (e.g. intracranial catheters for injection of neurotrophic factors, cell transplants, genetic

modifications using viral vectors). Thus, in the future there may arise the need to detect adverse events using a sensitive albeit non-specific, marker for brain damage. In this context, CSF NFL with its high sensitivity for detecting more aggressive neuronal death than it occurs in PD, even if enfeebled by a low diagnostic specificity, might be of use.

A question which none of the studies presented here has approached is whether the CSF NFL levels, increased in atypical parkinsonism and in HD, could be influenced by a therapy, and what that would imply. That approach was used in a neuroprotective placebo controlled study, treating MSA patients with recombinant human growth hormone. The results failed to show any significant change in CSF NFL levels following therapy. There was a change in CSF neurofilament stoichiometry, in the ratio between neurofilament heavy chain and neurofilament light chain, but its implications were not clear and the finding must be reproduced [233]. Much work remains to be done until CSF NFL can be used as a therapeutical endpoint in parkinsonism.

## 5.4 Markers with Patho-Etiological Implications?

From our results we can in no way conclude that the compounds we have studied are important from a patho-etiological point of view, and to discuss from that perspective may seem speculative. Nevertheless, contemplating the multitude of hypotheses attempting to explain the patho-etiology of neurodegenerative movement disorders, one may concede that there is still room for speculation in this regard.

In paper III, a proteomic profile was identified, consisting of four proteins (ubiquitin,  $\beta$ 2-microglobulin, and 2 secretogranin 1 [chromogranin B] fragments) which help differentiate PD patients from patients with APD.

**5.4.1. Ubiquitin** ( $m/z$  8590) levels were increased in APD compared with PD. The implications of these facts are not clear but ubiquitin is an abundant and highly conserved protein, important in a PD context. As it normally labels proteins for proteasomal degradation, ubiquitin malfunction may cause impaired protein degradation and accumulation of abnormal proteins with deleterious effects on the neurons. Defective ubiquitin-proteasome pathways are encountered in both sporadic PD and in hereditary PD caused by mutations in alpha-synuclein gene (PARK 1), in the ubiquitin C-terminal

hydrolase L1 (UCHL1) gene (PARK 5), or in the parkin enzymes gene (PARK 2) [235, 236].

**5.4.2.  $\beta$ 2-microglobulin** ( $m/z$  11730) reflects immune activation and the lymphoid cell turnover in the central nervous system. Our finding of increased levels of  $\beta$ 2-microglobulin in APD, but normal in PD, may reflect the more aggressive neurodegeneration occurring in these disorders, presumably leading to a stronger inflammatory response. Abnormal inflammation in the central nervous system, with activated microglia and massive astrogliosis with increased levels of proinflammatory cytokines (tumor necrosis factor -TNF- $\alpha$ , interleukins), has been found in the CSF in PD; these proinflammatory compounds may promote apoptosis and neuronal death [207, 237] and have been suspected to contribute to the development of PD [238] and PSP [239]. Supporting this theory, it has been shown that use of nonsteroidal anti-inflammatory drugs (NSAIDs), particularly ibuprofen, was associated with a lower risk for PD [240, 241]. It is not known whether the glial activation is secondary to neuronal death induced by other factors, or if it is the primary cause to neuronal death [242].

#### **5.4.3. Secretogranin I (Chromogranin B) Fragments**

The implications of the increased secretogranin I (chromogranin B) fragments ( $m/z$  6250 and 7260) are more difficult to understand from the perspective of parkinsonism. The chromogranins are widely distributed in neuroendocrine and nervous system tissues. In neurons, they are processed to active peptides with numerous intracellular and extracellular functions [243, 244]. Chromogranin B, which is neuron-specific, stimulates neurite outgrowth [245] and decreased levels in atypical parkinsonism compared with PD may indicate heavier synaptic or neuronal loss. In a recent CSF proteomics paper, the  $m/z$  6250 peak identified by us was found to be significantly decreased in MSA compared to PD and in PD compared to controls, allowing for a differentiation between these disorders [246].

**5.4.4. Urate** levels in serum but not in the CSF appeared to be lower in synucleinopathies than in tauopathies, in men only, according to the results presented in paper IV. Accumulating evidence implies urate in the pathobiology of PD. Similar findings have been made in MSA, where higher serum urate was associated with a lower rate of disease progression [247]. In DLB, serum urate levels were lower than in controls [248]. Urate does not seem to be of importance exclusively in synucleinopathies. Thus, in HD, the total functional capacity score showed less worsening over time with

increasing serum urate levels at baseline [249]. Comparable findings have been done in other neurodegenerative disorders, such as ALS [250], AD [251] and other dementias [252]. In the light of all this, it is hard to interpret our finding of decreased urate in synucleinopathies compared with tauopathies. Are the fundamental patho-etiological processes different? Are oxidative mechanisms more relevant in the etiology of synucleinopathies? Our findings do not offer any answer to that. It is not known whether urate itself exercises a protective effect on PD or if it is merely a marker of some other neuroprotective process. In a retrospective analysis of DATATOP, only subjects having low urate levels at baseline seemed to profit from treatment with tocopherol raising the possibility that urate could be used for stratifying a population in regard to neuroprotective therapy [196]. Does it also mean that these patients were less protected against oxidative stress, which would explain why they benefited from tocopherol, an antioxidant?

Although high urate levels seem to have a positive impact on neurodegenerative disorders, increased urate levels are associated with higher risks for urolithiasis, cardiovascular disorders, metabolic syndrome and mortality. These both intriguing and encouraging facts motivated the initiation of a phase 2 placebo-controlled double-blind randomized trial, the Safety and Ability to Elevate Urate in Early Parkinson Disease (SURE-PD) trial, in individuals with early PD [253]. Based on the results from this study, a decision will be taken whether to continue with a phase 3 trial of urate in PD.

Our finding that gender had an important role in the context of urate replicates results from several other studies. Thus, Chen et al. found an inverse association between PD and plasma urate levels but in men only [254]. A study enrolling only women found no such relationship [255]. At the same time, several studies have found, on average, lower plasma urate levels in women than in men, both in healthy controls and in PD patients. For example, in controls, mean plasma urate levels were 4.9 mg/dL in women [255], compared with 6.1 mg/dL in men [256]. However, there is evidence that women, presumably due to the protective influence of oestrogens, have lower risk of PD, are older at disease onset, and may have a more favourable phenotype compared with men [257].

In our study, there was a trend for lower serum urate levels in PD compared with controls, but the difference was not large enough to achieve statistical significance. Presumably, one reason for that was the low number of PD patients included in this study due to the exclusionary criterion of ongoing dopaminergic treatment at the time for LP. An alternative explanation could be that these PD patients, who were not treated at the time for LP, represented a subgroup of PD patients with a milder disease form, requiring

treatment at later disease stages than the average PD patients. In such a subgroup, serum urate levels might be higher than in the average PD populations in which most studies have found lower levels compared with controls.

#### **5.4.5. Blood contamination**

When analyzing CSF, it is imperative to avoid blood contamination. Most proteins secreted from the brain in the CSF are of low concentration and the protein profile of the CSF very much resembles that of blood. Proteins such as alpha-synuclein are also present in the blood, in erythrocytes and thrombocytes. Even minor contamination with blood can have a major impact on the CSF protein profile. Therefore it has been advised that samples should not contain more than 10 erythrocytes per microliter CSF according to one American group [127] or 500 erythrocytes per microliter CSF according to an European recommendation [117]. In our studies we discarded CSF samples containing more than 500 erythrocytes/ $\mu$ L CSF except in study III where the limit was 5 erythrocytes/ $\mu$ L.

## **5.5 Limitations**

Diagnostic criteria were strictly applied across all studies which is both a weakness and a strength of this work. Strict criteria lead to lower number of subjects, to less generalizability, but to secure diagnoses in the subjects enrolled. Even though LPs in many cases predate the analyses with several years, there were few instances when LP was done at early disease stages. No LP was done unless enough symptoms supported a strong suspicion of a parkinsonian disorder. And biomarkers are mostly needed for early disease stages when symptoms and diagnoses are not evident. This contradiction could be resolved by collecting clinical data and biological samples longitudinally, over longer periods of time from a broader group of patients and doing retrospective analyses once diagnoses are apparent.

A further limitation is the lack of neuropathological confirmation of the clinical diagnoses underlying these studies, with a few exceptions. As discussed in the Introduction, the diagnostic criteria for APD have changed to a certain extent during the last years, in some instances superseding the criteria used in these studies. However, as the diagnostic criteria were strictly enforced, there is a high probability that all patients would retain the original diagnoses even applying the new diagnostic criteria.



In addition to the low number of subjects, another limitation in the study on urate levels in parkinsonian disorders was the lack of information concerning known confounding factors, such as diet, alcohol consumption, use of diuretics, and body mass index.

## 6 CONCLUSIONS

The CSF NFL was found to be increased in all APD, including for the first time CBD. CSF NFL may thus be useful in differentiating healthy controls and PD patients from APD patients. However, it cannot be used for monitoring disease progression, as it did not change significantly over one year, and it cannot discriminate between different atypical parkinsonian disorders. Normal CSF NFL levels one year or more following implantation of deep brain electrodes for advanced PD, confirm neuropathological reports finding no signs of accelerated neuronal death attributable to this invasive treatment strategy.

With the help of SELDI-TOF MS, a panel of four CSF proteins was identified. Together, they might be useful in the differential diagnosis of parkinsonian disorders, as they were able to differentiate between healthy controls and PD patients on one hand, and APD patients on the other hand.

In males, serum urate levels were lower in patients with synucleinopathies compared with tauopathies, presumably indicating differences in the patho-etiological mechanisms underlying these diseases.

Finally, CSF NFL and total tau protein, both markers of neuronal damage, were increased in HD compared with healthy controls, opening up the possibility to investigate whether they could be useful for more exactly determining the time of disease onset, for monitoring the disease process and response to treatment.

In order to develop efficient therapies for neurodegenerative movement disorders, reliable biomarkers are seriously needed. In the present work, engaging healthy controls, patients with parkinsonian disorders and with HD, the biomarker potential of several compounds of the CSF and serum was explored. Although none of them has been proven so far to be a biomarker, according to the current definition, the results are encouraging and motivate further research.

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## APPENDIX

### LIST OF PAPERS

- I. Constantinescu R, Rosengren L, Johnels B, Zetterberg H, Holmberg B. Consecutive analyses of cerebrospinal fluid axonal and glial markers in Parkinson's disease and atypical Parkinsonian disorders. *Parkinsonism Relat Disord.* 2010 Feb;16(2):142-5.
- II. Constantinescu R, Holmberg B, Rosengren L, Corneliusson O, Johnels B, Zetterberg H. Light subunit of neurofilament triplet protein in the cerebrospinal fluid after subthalamic nucleus stimulation for Parkinson's disease. *Acta Neurol Scand.* 2011 Sep;124(3):206-10.
- III. Constantinescu R, Andreasson U, Li S, Podust VN, Mattsson N, Anckarsäter R, Anckarsäter H, Rosengren L, Holmberg B, Blennow K, Wikkelsö C, Rüetschi U, Zetterberg H. Proteomic profiling of cerebrospinal fluid in parkinsonian disorders. *Parkinsonism Relat Disord.* 2010 Sep;16(8):545-9.
- IV. Constantinescu R, Andreasson U, Holmberg B, Zetterberg H. Serum and cerebrospinal fluid urate levels in synucleinopathies versus tauopathies. *Acta Neurol Scand.* 2013 Feb;127(2):e8-12.
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- VI. Constantinescu R, Romer M, Zetterberg H, Rosengren L, Kiebertz K. Increased levels of total tau protein in the cerebrospinal fluid in Huntington's disease. *Parkinsonism Relat Disord.* 2011 Nov;17(9):714-5.