Assessment of Poly-ADP-ribose as a potential biomarker in Parkinson's Disease

Master thesis in Pharmacy

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1. Contents

2. Abbreviations

PAR	Poly ADP-ribose
WB	Western Blotting
IHC	Immunohistochemistry
PD	Parkinson's disease
BSA	Bovine Serum Albumin
NR	Nicotinamide riboside
NAD^+	Nicotinamide adenine dinucleotide
PARP1	Poly ADP-ribose polymerase
CSF-	Cerebrospinal fluid
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween

3. Abstract

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder that affects millions of people worldwide. It is characterized by the loss of dopaminergic neurons in the substantia nigra region of the brain. Currently there is no cure, and the absence of a reliable biomarker complicates the diagnosis of PD. Recently, poly-ADP-ribose (PAR), a NAD derived biomolecule involved in DNA repair, stress response and cell death pathways, was suggested to be a possible biomarker for PD. The main aim of this study was to evaluate PAR in cerebrospinal fluid (CSF) as a potential biomarker in PD.

Using a combination of immunodetection methods including ELISA, dot blotting and immunohistochemistry, CSF samples and brain sections from individuals with PD and neurological healthy controls were investigated. The results suggest that PAR levels are not significantly altered in PD. NAD replenishment therapy by supplementation of the NAD precursor nicotinamide riboside did not lead to elevated PAR levels in the CSF of individuals with PD. Finally, expression levels of CD38, an enzyme suspected to mediate age dependent NAD-decrease, were not different in brain sections from individuals with PD and controls.

In conclusion, the results of this study suggest that CSF levels of PAR are not a suitable biomarker for PD. Moreover, NR treatment did not increase PAR levels in the CSF, further supporting the safety of this precursor for further clinical studies. While this outcome did not provide a desired biomarker for PD, it contributes to the understanding of PAR metabolism in PD and helps in the development of future research.

4. Introduction

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder. It is the second most common neurodegenerative disease after Alzheimer's disease affecting about 1-2% of the population above the age of 65, and the incidence of PD increases with age (1).

PD has motor and non-motor symptoms

Clinically, the hallmark of PD lies in its motor symptoms, which present a variety of challenges for individuals living with the condition. The motor symptoms include bradykinesia (the slowness of movement), resting tremor, muscle rigidity and gait abnormalities, and are mostly due to the loss of dopaminergic neurons in substantia nigra. Loss of dopamine results in decreased muscle activation that leads to mobility limitations, especially for tasks requiring fine motor skills or smaller movements (2).

While these motor symptoms are clearly observed and their onset often defines the timepoint of diagnosis, PD patients also experience non-motor symptoms that often exist long before the diagnosis of PD. These can include sensory symptoms such as pain and olfactory disturbance (loss of taste or smell) gastrointestinal symptoms such as constipation, sleeping problems, but also neuropsychiatric symptoms such as depression and anxiety as well as cognitive impairments, that may worsen over the course of the disease (3) (4).

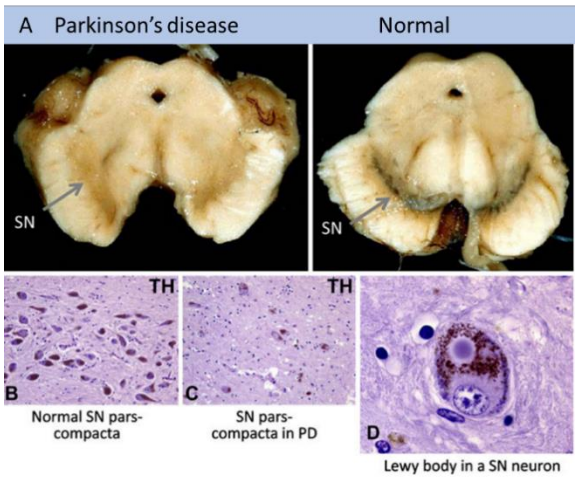


Figure 4.1. Comparison of Parkinson's disease individual to normal individual.

A- Brain image of substantial nigra of an individual with Parkinson's disease showing light shade indicating the loss of dopaminergic neuron. While the brain image of substantial nigra of normal individual has darker shades indicating the existence of dopaminergic neurons. **B-C** Tyrosine hydroxylase (TH) staining shows dopaminergic neurons in healthy (B) and PD (C) substantia nigra, indicating neuronal loss in PD. **D**- A dopaminergic neuron with a Lewy body (the intracellular round purple structure surrounded by the brown pigment within the neuron. Its formation is believed to be involved in the neuron's functional impairment. Figure adapted from Mandel et al.2010 (5).

Pathology related to PD

PD is characterized by the loss of dopaminergic neurons in the Substantia nigra pars compacta (SN; Figure 4.1). This loss of dopaminergic neuron is believed to be caused due to various factors. The following are some of the known causes related to the death of dopaminergic neuron.

Protein aggregation contributes to PD

The development of PD is influenced by various factors, and one significant contributor is alpha synuclein. Alpha synuclein is a dynamic protein involved in pre-synaptic transmission, playing a role in regulating the transport of neurotransmitter vesicles for synaptic transmission. It is primarily abundant in neurons (6). Through interactions and combinations, alpha synuclein can adopt various shapes and sizes, forming chains that are soluble and transient. However, in certain situations, these alpha synuclein structures may not dissolve properly. In normal circumstances, chaperones and proteasomes help dissolve and regulate the size and forms of alpha synuclein (1).

However, when aberrant or abnormal forms of alpha synuclein arise, they are not recognized and cleared by the chaperones and proteasomes. As a result, these abnormal alpha synuclein structures start to accumulate, forming aggregates known as fibrils. Over time, these fibrils further aggregate to create Lewy bodies (Figure 4.1D) (7). The enlargement of Lewy bodies disrupts dopamine production, leading to damage and eventual neuronal death. In the case of PD, Lewy bodies tend to form in dopaminergic neurons located in the substantia nigra, which is responsible for various functions of the central nervous system such as movement and working memory (8) (9) but can be observed in other areas of the brain such as the frontal cortex.

Genetic factors associated with PD

Most cases of PD are idiopathic, that is, with unknown cause. However, a minor portion of cases (about 5%) are genetic disorders and can be attributed to genetic mutations that often involve mitochondrial dysfunction. Individuals who have genetic disorders affecting mitochondria may develop PD at a younger age and it is thought that this is due to the impact of mitochondrial dysfunction on the disease (10). For example, the PINK1 gene is involved in regulating mitochondria and the removal of damaged mitochondria. Mutation in the PINK1 gene results in the accumulation of damaged mitochondria and an increase in reactive species (10). This genetic abnormality is believed to disrupt normal mitochondrial function, leading to impaired energy conversion and ATP production, increased oxidative stress and cellular damage, all are associated with the development and progression of PD. However, mitochondrial dysfunction has also been observed in idiopathic PD, and is thought to contribute to the progression of the disease (11).

Moreover, mutations in the SNCA gene coding for alpha-synuclein can lead to increased levels of aggregated alpha-synuclein protein, which is a characteristic feature of PD (10).

Mitochondrial dysfunction and oxidative stress associated with PD

Oxidative stress is the accumulation of reactive oxygen species (ROS), radical molecules that are highly reactive and can lead to lipid and protein oxidation, membrane- and cellular damage. It is also involved with aging, and age is a major risk factor for PD. Radical oxygen species (ROS) cause oxidative stress and can be induced by external factors such as smoking and environmental toxins or internal metabolism. ROS are unstable radical molecules that can easily react in a cell, for example damaging the cell membrane by reacting with membrane lipids. A certain amount of ROS in cells can be beneficial, for example in the immune system.

It triggers the immune system to be alert and fight against foreign invasion. However, extensive ROS production can damage among others mitochondrial functions (12) (13). Aberrant alpha-synuclein has also been shown to induce oxidative stress in cells, which could in turn lead to DNA damage and activate PARP1.

Mitochondrial dysfunction and oxidative stress are strongly linked. When mitochondria are dysfunctional, this can result in oxidative stress, and equally, oxidative stress can also contribute to mitochondrial dysfunction. There are various factors that can cause mitochondrial dysfunction. Genetic mutations, exposure to environmental toxins and mutation in mitochondrial respiratory complex 1 can all disrupt mitochondrial function (14) (15). Mitochondria are also a natural source of ROS, for example as byproducts of the respiratory chain, an electron transfer chain in the inner mitochondrial membrane that is the primary source of ATP production under oxidative conditions. However, in the case of mitochondrial dysfunction, there can be excessive production of ROS. This imbalance of ROS levels leads to oxidative stress. The reactive nature of ROS allows them to damage essential elements within the mitochondria, including mitochondrial DNA, lipids of the mitochondrial membranes and proteins. Such damage impairs the normal function of mitochondria, leading to mitochondrial dysfunction (14). Thus, oxidative stress poses a significant threat to cellular components and cell survival when uncontrolled.

It is important to understand that the exact causes and mechanisms behind the loss of dopaminergic neurons in the substantia nigra are still unknown but are suspected to be a complex interaction between genetic and environmental factors, and that mitochondrial dysfunction, oxidative damage and protein aggregation play a central, but not yet fully understood role. Therefore, research is needed to fully understand the causes and develop targeted treatments for PD.

Current treatments for PD

Currently, there is no known cure for PD or any treatment that can slow down its progression. However, there are various therapeutic approaches available that aim to improve symptoms and enhance the quality of life for individuals living with PD. One of the primary treatments for the motor symptoms of PD is levodopa. Levodopa functions by replenishing the dopamine levels in nerve cells. To minimize side effects such as vomiting and low blood pressure, levodopa is often administered in combination with carbidopa (16). Carbidopa, a medication classified as decarboxylase inhibitor, acts by inhibiting the breakdown of levodopa prior to its arrival in the brain. This mechanism is beneficial in preventing the reduction of levodopa before

it reaches its intended target. Levodopa itself can cause gastrointestinal side effects. By reducing the breakdown of levodopa before it reaches the brain, Carbidopa also helps prevent or minimize unwanted gastrointestinal side effects (17). However, a significant challenge with levodopa treatment is the occasional occurrence of diminished effectiveness over time (16).

In addition to levodopa, enzyme inhibitors such as inhibitors for MAO-B are used to slow down the breakdown of dopamine in the brain. Monoamine oxidase (MAO-B) is an enzyme responsible for the degradation of dopamine in the brain. By inhibiting the enzymes that metabolize dopamine, these medications can help prolong its effects and enhance symptom control (18).

Non-motor symptoms associated with PD, such as depression, panic and anxiety are often addressed through medications like Selective Serotonin Reuptake Inhibitors (SSRIs). These medications help to regulate serotonin levels in the brain and improve mood-related symptoms. For individuals with Parkinson's who experience long-term complications, deep brain stimulation surgery can be considered as an option (16).

Non-drug treatments, such as speech therapy and exercise, also play a significant role in improving the quality of life for individuals with PD. Speech therapy helps individuals with PD maintain clear and loud speech, addressing any speech related difficulties that may arise. Exercise on the other hand, aids in activating and relaxing muscles, promoting mobility and overall physical well-being (16).

These treatments contribute to a better quality of life for people with PD. However, it is important to understand that these treatments do not offer a cure or the ability to halt the progression of the disease itself.

Diagnosis of PD

The diagnosis of PD currently depends on a combination of clinical symptoms, response to medication (such as levodopa) and imaging data. Pathological confirmation of PD can only be achieved through the presence of Lewy bodies at autopsy. Importantly, there is a complete lack of a valid biomarker for the diagnosis of PD (19).

Clinical symptoms play a crucial role in the diagnosis of PD. These symptoms involve the cardinal motor features mentioned above, including tremors, bradykinesia, rigidity or stiffness in the limbs or body, and postural instability. Non- motor symptoms such as pain, constipation and loss of sense can also be present. A positive response to levodopa treatment is also important in the diagnosis of PD, as it supports the hypothesis that the symptoms are caused by dopamine deficiency in the substantia nigra (19).

Brain imaging techniques like DaT scan can provide supportive evidence for the diagnosis of PD. However, neuroimaging alone is insufficient to establish a definitive diagnosis due to the absence of distinct PD-specific characteristics or signals. Moreover, PD shares similar symptoms and signs with other neurodegenerative diseases like multiple system atrophy (MSA) and other so called parkinsonisms, making it difficult to differentiate between them (20).

The lack of reliable biomarker for PD results in significant challenges. Biomarkers could not only assist in diagnosing PD, but also in differentiating PD from other neurodegenerative disorders that are similar to PD. The discovery of a biomarker would assist in identifying the population at risk and enable early interventions that could help to delay the onset of the disease and protect dopaminergic and other neurons.

Recently, a study suggested that poly ADP ribose (PAR) levels in the cerebrospinal fluid (CSF) may serve as a representative biomarker of the brain. CSF, which surrounds the brain and spinal cord, is commonly used as a representative for brain samples since direct biopsy of the brain in living individuals is not possible. The presence and measurement of PAR in the CSF could potentially provide valuable insights into the activity and process occurring within the brain (21).

Poly-ADP-ribose – an important biopolymer with many functions

Poly-ADP-ribose (PAR) is a biopolymer with a heterogeneous structure that can be long and branched. PAR is synthesized from nicotinamide adenine dinucleotide (NAD^+), a vital coenzyme in all cells, by cleavage of the nicotinamide ring, and transfer of the remaining ADP-ribose to the polymer (Figure 4.2). One PAR molecule can consist of up to 200 ADP-ribose units. Its formation is catalyzed by enzymes called poly-ADP-ribose polymerases (PARPs) and it is usually transferred as posttranslational modification onto an acceptor protein (often the PARPs themselves) (22) (23).

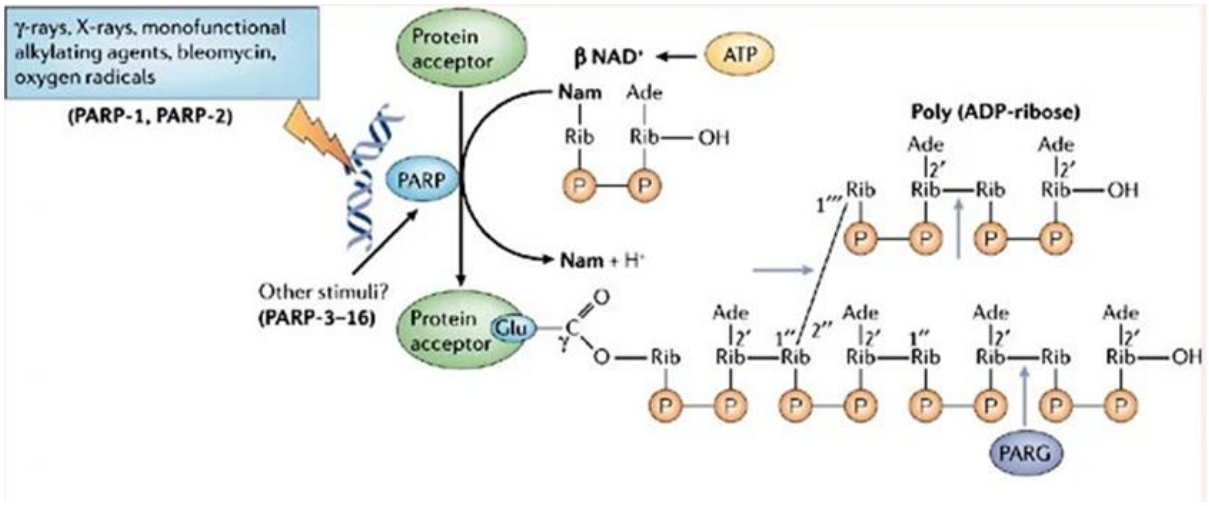


Figure 4.2. The synthesis of poly-ADP-ribose.
When DNA is damaged, PARP is triggered to synthesize poly-ADP-ribose (PAR) using NAD^+ as substrate. The polymers are usually transferred onto an acceptor protein, often the PARP itself. The PAR molecules are very heterogenous and can be build in different shapes varying in length, level of branching and number of ADP-ribose units. Figure adapted from Schreiber et al. 2012 (24)

PAR is an important molecule involved in a variety of cellular functions. For example, PAR formation is one of the initial sensors to signal DNA damage repair due to reactive oxygen species (ROS) UVB light exposure or ionizing radiation (23). When DNA is damaged, PAR plays a crucial role in initiating the DNA repair process. PARP1, a nuclear enzyme and main producer of PAR (75-95% of PAR formation is mediated by PARP1), binds to the damaged DNA site, and automodifies itself with PAR. This then acts as a signaling molecule, attracting DNA repair proteins like chromatin remodelers and scaffolding proteins to the damaged site, facilitating DNA repair (25). PARylation, the addition of PAR chains to target proteins, is involved in various DNA repair pathways such as base excision repair (BER) and single-strand break repair (SSBR) (22). PAR formation happens very quickly in a matter of minutes after DNA damage and is equally fast removed by PAR glycohydrolases (PARGs) (22).

PAR signaling is also involved in gene expression, protein translation and signaling cellular responses to viral infection and stress such as inflammation and immune response (26).

Moreover, PAR has been shown to be involved in the regulation of apoptosis. PAR molecules are commonly generated in the nucleus of cells such as during DNA repair. But when DNA damage becomes too extensive, PAR may be released to the cytoplasm and signals cellular stress to the mitochondria. This in turn leads to mitochondrial depolarization and the release of apoptosis-inducing factor (AIF) from mitochondrial membranes. It then binds to macrophage migration inhibitory factor (MIF) and together translocate to the nucleus, where MIF cleaves genomic DNA into large fragments, thus initiating cell death. This mechanism is called

parthanatos (after “PAR” and “Thanatos” the Greek personification of death) and activated after severe DNA damage for example after exposure to oxidative stress or inflammation (Figure 4.3) (23) (22) (27) (28).

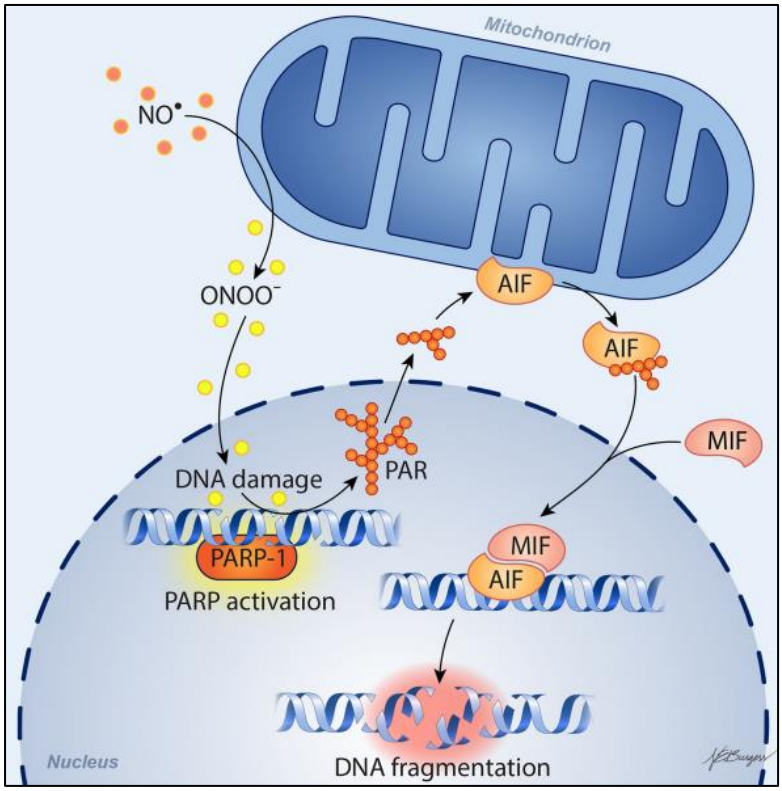


Figure 4.3 – PAR-mediated cell death pathway:“parthanatos”.
DNA damage triggers the synthesis of PAR. In the presence of excessive PAR levels, AIF is induced and transferred into the cytoplasm, reaching the mitochondria. This signals to release apoptosis inducing factor (AIF), which can bind to macrophage migration inhibitory factor MIF, be transported to the nucleus, and lead to DNA degradation, initiating cell death. Figure adapted from Koehler et al. 2021 (29).

PAR is generated from NAD^+, a vital molecule in all living cells, and thus, together with other NAD-degrading signaling reactions (mediated for example by the protein deacetylase family of sirtuins), PAR formation contributes to the degradation of the cellular NAD pool (30). Since NAD is a vital cofactor in all living cells and essential for cell survival, NAD needs to be constantly replenished. NAD synthesis has many entry points, such as nicotinamide and nicotinic acid. NAD synthesis from nicotinamide, the byproduct of PAR formation and other NAD dependent signaling reactions, is the main route in mammalian cells. However, other precursors such as nicotinamide riboside (NR) can also be used and have been intensively studied in recent years both in preclinical and clinical studies. This is due to the beneficial effects of increased NAD levels, which include improved viability, neuroprotective effects, and

improved life and health-span in several animal models. Clinical studies using NR as supplement have focuses among others on metabolic syndromes such as obesity and type 2 diabetes. More recently, NR supplementation has also been studied in a first clinical trial for Parkinson`s disease with promising results (31) and is currently under further investigation in a larger trial (NOPARK; ClinicalTrials.gov Identifier: NCT03568968).

PAR-mediated cell death (parthanatos) is also involved in neuronal death, and is believed to be responsible, at least in part, for progressive neuronal loss in various neurodegenerative diseases including Alzheimer`s disease and PD (32). Here, this can for example be triggered by excessive activation of glutamate receptors and activation of nitric oxide synthase. Its product, nitric oxide (NO) in term can react with superoxide radicals (O^{2-}), lead to DNA damage and induce PARP1 activation. This may lead to massive PAR formation and induction of cell death (32).

Moreover, with regard to PD, PAR has recently been shown to interact with aggregates of a-synuclein and to mediate neurotoxicity (21). These aggregates are believed to be central for the degeneration of dopaminergic- and other neurons in the pathology of PD. The study by Kam et al (21) showed that, when PARP1 was present in cells and could generate PAR, the neurotoxic effect of a-synuclein preformed fibrils (PFFs) was much stronger than in cells lacking PARP1 and consequently PAR formation. Moreover, mixing PFFs with PAR before incubation with cells or injection into mice had a much stronger neurotoxic effect than PFFs alone (21). Thus, PAR metabolism seems to be involved in the pathology of PD, and possibly mediation of neurotoxicity and neuronal loss in the substantia nigra and other areas of the PD brain.

As mentioned above, PD is diagnosed based only on clinical features, and usually first when motor-symptoms appear and are recognized, and there are no suitable biomarkers for PD available to date. However, Kam et al (21) also reported that PAR was elevated in the cerebrospinal fluid of individuals with PD compared to controls. This further supported the importance of PAR in the pathology of PD and possible mediation of a-synuclein neurotoxicity. Moreover, if these observations could be confirmed, this could indicate that PAR in CSF could potentially serve as diagnostic biomarker for PD, possibly even before onset of the disease. This possibility was very intriguing.

Therefore, this study was set up to investigate whether PAR would be a practical biomarker in PD, using CSF samples from individuals with PD and neurological healthy controls from clinical studies carried out at Haukeland university hospital and the NeuroSysMed center for

clinical treatment research. Moreover, it was sought to investigate whether NAD precursor supplementation, which had been shown to increase cerebral NAD, the substrate of PARPs, would alter PAR levels in the CSF. Finally, it was sought to establish whether expression levels of PAR related proteins were altered in PD which could have an impact on PAR levels in the brain and CSF.

5. Aims of the study

Poly-ADP-ribose (PAR) is an important molecule involved in various cellular processes and it has been implicated in the neurodegenerative process of PD.

The primary aim of this study was to establish whether PAR in cerebrospinal fluid is a suitable biomarker for Parkinson disease. To this end, a reliable and accurate method for detecting and quantifying PAR levels in CSF needed to be established, and the level of PAR in CSF of individuals with PD and neurological healthy controls compared.

A second aim of this study was to investigate whether NAD replenishment, a therapeutic approach currently under investigation for PD treatment, has an effect on the PAR levels in CSF.

Thirdly, this study aimed to investigate the expression levels of PAR-related proteins in brain sections obtained from PD patients and controls. The levels of these proteins in specific regions of the brain sections obtained from PD patients and controls were investigated to gain insights into the molecular mechanisms underlying the disease and find a potential cause for different PAR levels in PD and control samples.

6. Methods

SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to separate proteins and other macromolecules by size, while Western blotting is a method to transfer proteins from an SDS gel onto a nitrocellulose or PVDF membrane to allow for subsequent immunodetection by specific antibodies. For SDS gel electrophoresis, BIORAD precast gels (Any kD gradient gels, cat no: 456903) were used. Samples were loaded along with 3 µl protein ladder (BIORAD, cat no: 1610374) and the gel was run with 25 mA for approximately 60min until the blue running front had left the gel. Protein transfer onto nitrocellulose membrane was achieved using Trans- Blot Turbo prepacked (BIORAD, cat no: 1704156) and the samples were transferred for 30 min at 1 A. Next, the membrane was blocked with 5 % dry milk in TBS-T for 45 min or EveryBlot Buffer (BIORAD) for 5 min. Then, the membrane was incubated with primary mouse anti-PAR antibody (1:10 000) diluted in blocking solution overnight at 4 ˚C. The next day, the membrane was washed 4x 5 min with TBS-T. After that, the membrane was incubated for 1 hour with secondary HRP-conjugated rabbit anti mouse- antibody (1:1000) in blocking solution. Next, the membrane was washed 3x 5 min with TBS-T and 1x with TBS for 5 min. Detection was carried out using the (Clarity Western ECL Substrate, BIORAD, cat no: 170-5061). The membrane was incubated for 5 min with substrate solution. Signal detection was carried out in a ChemiDoc^{TM} XRS+ instrument using the Image Lab Software.

Immunodetection by Dot Blotting

Dot blot is technique for immunodetection of proteins or other macromolecules, in this case poly-ADP-ribose (PAR). Similar to Western blotting (WB), the protein or molecule of interest is immobilized on a membrane (PVDF or nitrocellulose) and detected by a specific antibody. In contrast to WB, the sample solution containing the molecule of interest is pipetted directly ("dotted") onto the membrane.

Here, 2 µl of CSF, either undiluted or diluted 1:2 and 1:4 in deionized water containing 1 % BSA were dotted onto the membrane. PAR standard was also diluted in deionized water with 1 % BSA and 2 µl of each dilution were dotted. Next, the membrane was blocked with EveryBlot Blocking Buffer (Bio-Rad) for 5 min. After that, the mouse anti-PAR primary antibody was diluted 1:10 000 in EveryBlot Blocking Buffer (Bio-Rad), added to the membrane and incubated overnight. The next day, the membrane was washed 3 times with TBS-T for 5 min. Secondary –polyclonal HRP conjugated rabbit anti mouse antibody was diluted 1:1000 in EveryBlot Blocking Buffer, added to the membrane and incubated for 1 hour.

Again, the membrane was washed 3 times with TBS-T and once with TBS for 5 min. Subsequently, the blot was developed using Clarity Western ECL substrate by mixing Solution A and B in a ratio 1:1 and incubating the membrane with the solution for 5 min. Images were taken on a ChemiDoc™ XRS+ instrument (BIORAD) using the Image Lab Software. Signal quantification was then carried out using the Image Lab software.

Enzyme-linked immunosorbent assay (ELISA) for poly-ADP-ribose detection

Enzyme-linked immunosorbent assay (ELISA) is a method that uses antibodies to immobilize, detect and quantify proteins and other biomolecules, in this case poly-ADP-ribose (PAR). A sandwich ELISA in a 96well format from Cell Biolabs (Cat no: XDN-5114) was used in this study. The assay was carried out according to the manufacturer's recommendations with slight adjustments. Briefly, wells were prepared with 100 µl of coating antibody solution 1:500 diluted in 1x PBS, which was added to each well and incubated overnight at 4˚C. The next day, coating antibody solution was removed, and the wells were blocked with 200 µl Assay Diluent for 2 hours at room temperature. Next, the wells were washed 3 times with 200 µl 1x PBS. Then, 100 µl of PAR standard solutions and unknown samples were added to the wells and incubated for 1 hour. After washing 3 times with 200 µL washing buffer, 100 µl of 1:1000 diluted detection (primary) antibody in Assay Diluent were added to the wells and incubated for 1 hour at room temperature. The wells were washed 3 times with a 200 µL washing buffer. 100 µl of 1:1000 diluted secondary antibody in Assay diluent were added to the wells and incubated for 1 hour. The wells were then washed again 3 times with 200 µL washing buffer, and substrate solution was added and incubated for 15-30min, depending on the reaction time. When a nice gradient of blue color on the standard samples was visible, stop solution was added to stop the reaction. The absorbance at 450nm was measured using Infinite F50 microplate reader and Magellan™ software.

Immunohistochemistry (IHC) in human brain tissue.

Immunohistochemistry (IHC) is a technique that uses specific antibodies to detect antigens of interest, most often proteins, in tissue sections. This is commonly used to investigate expression levels of antigen localization and in determining an antigen of interest's distribution.

Here, formalin-fixed paraffin-embedded (FFPE), 3µm thick sections of Frontal Cortex and Substantia nigra of individuals with PD and healthy controls were used. Sections were provided by the Department of Pathology, Haukeland University hospital.

In order to de-paraffinize the sections and make antigens more accessible, the sections were treated in a PT Link instrument (Pre-treatment Module for Tissue Specimens, Agilent Dako). The machine cooked the sections at 98ºC, and the total time consumed by the machine from preheating to cooking took around 1 hour and 30 minutes. The brain sections were then rinsed with TBS-T and then gently marked with a circle around the brain section on the slides using a tissue membrane pen (A-PAP-pen). Next, the sections were blocked using Peroxidazed 1 for 10 min to inhibit endogenous peroxidase activity and then washed in TBST for 5min.

As primary antibodies, mouse anti CD38 (Santa Cruz Biotechnology) and mouse anti PARP1 (Santa Cruz Biotechnology) were tested and titrated to identify optimal staining conditions. 200 µl of primary antibody solution diluted in Da Vinci Green Diluent were added to each sample and incubated at for 1 hour at room temperature. Then, sections were washed in TBST for 5 min. After that, the sections were first incubated in MACH 4 Mouse Probe (Biocare medical, UP534L) for 15 min and later washed with TBST for 5 min. Next, the sections were incubated in MACH 4 HRP-polymer (Biocare medical) reagent for 30 min and then washed in TBST. After that, DAB solution (Biocare medical) was added to the sections and incubated for precisely 4 min. After that, the sections were washed in cool tap water for 30 sec. Tasha's Haematoxylin was then added for 3 minutes, before washing the sections in cool running tap water. Finally, the sections were stepwise dehydrated in graded alcohol and xylene for dehydration.

After drying overnight, sections were investigated, and images were taken using a Leica DM3000 LED microscope with Leica LAS X software.

7. Material

Table 7.1 Poly ADP ribose standard and antibodies used in immunodetection

Target/Standard	Species	Dilution	Supplier	Catalog number
PAR standard	_____	1:20= 5nM	Enzo life sciences	ALX-202-043-C001
poly-ADP-ribose (PAR)	mouse	1:10 000	Enzo life sciences	ALX-804-220-R100
rabbit Anti-Mouse Immunoglobin HRP	Rabbit	1:1 000	Agilent Dako	Ref- P0260

Table 7.2. Antibodies used in IHC.

Target	Species	Dilution	Supplier	Catalog number
Poly-ADP-ribose) polymerase-1 (PARP1)	Mouse (monoclonal)	1:50-1:20000	Santa Cruz Biotechnology	sc-8007
CD38	Mouse (monoclonal)	1:1 000	Santa Cruz Biotechnology	Sc-374650

Table 7.3. Commercial Kits and reagents

Name	Supplier	Method	Catalog number
Clarity Western ECL Substrate	Bio-Rad	Dot blot	170-5061
MACH 4 universal HRP-Polymer	BioCare Medicl	IHC	BRI4012L
Poly (ADP-Ribose) ELISA Kit	Cell Biolabs	ELISA	XDN-5114

Table 7.4. Membranes

Membrane Type	Supplier	Catalog number
Nitrocellulose Blotting Membrane	Amersham™Protran™0.45µm NC	10600002
Hybond N+ Nylon Transfer Membrane	Amersham Biosciences	RPN303B
Mini-PROTEAN TGX Gels	Bio-Rad	4569036

Trans-Blot Turbo Transfer Pack	Bio-Rad	1704156

Chemicals and Buffers

Table 7.5 Chemicals

Name	Supplier	Catalog number
Tacha's Automated Hematoxylin	Biocare medical	NM-HEM-M
Da Vinci Green Diluent	Biocare medical	PD900M
Peroxidazed 1	Biocare medical	PX968M
Betazoid DAB Substrate Buffer	Biocare medical	DS900L
MACH 4 Mouse Probe	Biocare medical	UP534L
MACH 4 HRP Polymer	Biocare medical	MRH534L
DAB	Biocare medical	BDB900F
Tissue Membrane Pen (A- PAP-penn)	Histolab	08045N
PT-Link Buffer	Dako	K8005
(96% EtOH)	Antibac	Art.nr 600051
(100% EtOH)	Antibac	Art.nr 600068
Xylene	Technical	28973.363

Table 7.6 Buffers and working solutions.

Name	Component	Concentration	Supplier	Catalog number	Method
1xPBS solution (pH7.4)	NaCl	137mM	Gibco	18912-014	ELISA
	KCl	2.68mM			
	KH_2PO_4	10mM			
	Na_2HPO_4	1.8mM			
TBS (pH7.4)	TrisHCl	50mM			Dot blot and WB
	NaCl	150mM			
TBST	Tween20	0.1%			Dot blot and WB
	TBS	1x			
EveryBlot Blocking Buffer pH7.4	pH7	1x	Bio-Rad	12010020	Dot blot
Bovine Serum Albumin (BSA)				SLBX4875	Dot blot
TBS Auto Wash Buffer 40x		40x	Biocare medical	TWB946L2J	IHC

Tris/Glycine/SDS Buffer	1x concentrations: 25mM Tris, 192mM glycine and 0.1% SDS.	10x	Bio-Rad	161-0732	WB
Precision Plus Protein Dual Color Standards	SDS marker		Bio-Rad	1610374	WB
Instant skimmed milk powder			FREMA		WB
75% EtOH			Antibac		

Table 7.7 Instruments and software

Name	**Supplier**	**Instrument or software**	**Method**
PowerPac 1000 Electrophoresis Power Supply	Bio-Rad	Instrument	WB and SDS-page
Trans-Blot Turbo	Bio-Rad	Instrument	WB
ChemiDoc™ XRS+	Bio-Rad	Instrument	Dot Blot and WB
Image Lab Software	Bio-Rad	Software	Dot Blot and WB
Infinite F50 microplate reader	Tecan	Instrument	ELISA
Magellan™ data analysis	Tecan	Software	ELISA
PT Link (Pre-treatment Module for Tissue Specimens	Agilent Dako	Instrument	IHC

8. Results

The main goal of this project was to investigate the levels of poly-ADP-ribose (PAR) in the cerebrospinal fluid (CSF) of individuals with Parkinson`s disease and neurological healthy controls, in order to establish whether PAR in CSF could be used as a potential biomarker for PD. To this end, a commercially available ELISA kit for PAR detection was employed.

First, the method had to be established and evaluated whether it was suitable for PAR detection in CSF. Thus, a pilot experiment was carried out, using two test CSF samples, and following the manufacturer`s instruction precisely with regard to the standard series and incubation times. The standard series included samples ranging from 5-0.0019 µM PAR.

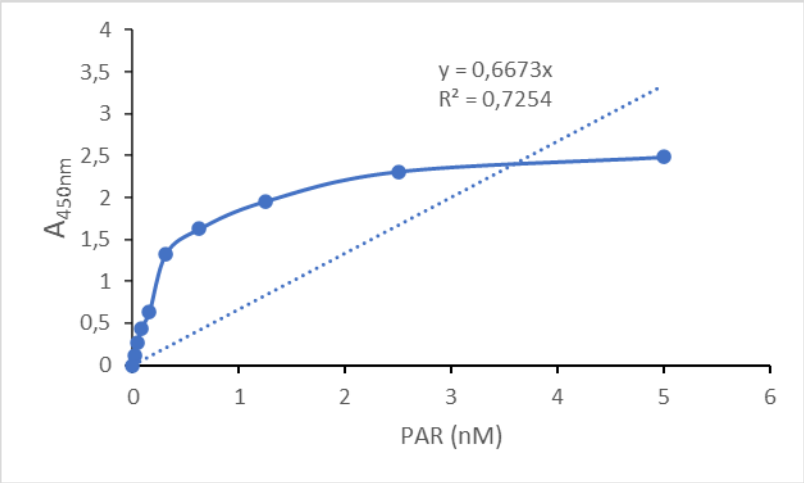


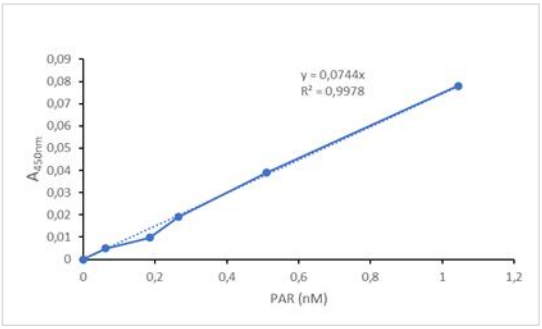
Figure 8.1. Pilot ELISA according to manufacturer's recommendation.
The plot shows relative absorbance values obtained from the indicated concentrations of PAR standard after 5 minutes of reaction time. Using this setup cerebrospinal fluid (CSF) samples were below the detection limit of the assay.

However, the color reaction in the standard samples was very quick and reached saturation in the highest concentrated samples already after a few minutes. As shown in Figure 4.1, after 5 min incubation time (normal range was indicated between 2-30 min) several standard samples were already saturated. In contrast, at that time, the unknown CSF samples were still not showing a detectable color reaction and were thus under the detection limit under these conditions. It was therefore concluded to adjust the experimental setup in order to increase sensitivity by diluting the standards further, and increasing the reaction time until CSF samples were also over the detection limit.

A

Sample	A_{450nm}	Normalized A_{450nm}	PAR concentration (nM)
1	1,30405	1.044	0.078
2	0,77025	0.5102	0.039
3	0,52525	0.2652	0.019
4	0,445	0.18495	0.00975
5	0,3222	0.06215	0.004875
Control	0,26005	0	0
CSF1	0,36335	0,1033	**0,00768552**
CSF2	0,63295	0,3729	**0,02774376**

B



C

Figure 8.2: Pilot ELISA under adjusted conditions yielded positive PAR detection in CSF samples.
A. The table presents the raw absorbance values from both PAR standard solutions and CSF test samples under adjusted conditions. Both CSF samples yielded positive detection above the limit of the assay.
B. The plot shows the standard curve of the relative absorbance values obtained from the indicated concentrations of PAR standard after 16 minutes of reaction time. The equation to calculate unknown samples is indicated. Using this setup Cerebrospinal fluid (CSF) samples were above the detection limit of the assay.
C. Representative image of the color reaction after termination. Column 1-2 were duplicates of both the standards (row 1-6 from highest (top) to lowest (bottom)) and the 2 CSF samples (row 7-8).

Figure 8.2 shows the final experimental setup and standard concentrations that were determined after several tests to be best suited for this experiment. Here, two CSF samples were tested in parallel, and the PAR signal was above the detection limit. PAR concentrations were calculated from the linear regression equation of the graph, yielding ~7.6 and 27.7nM for sample 1 and 2, respectively.

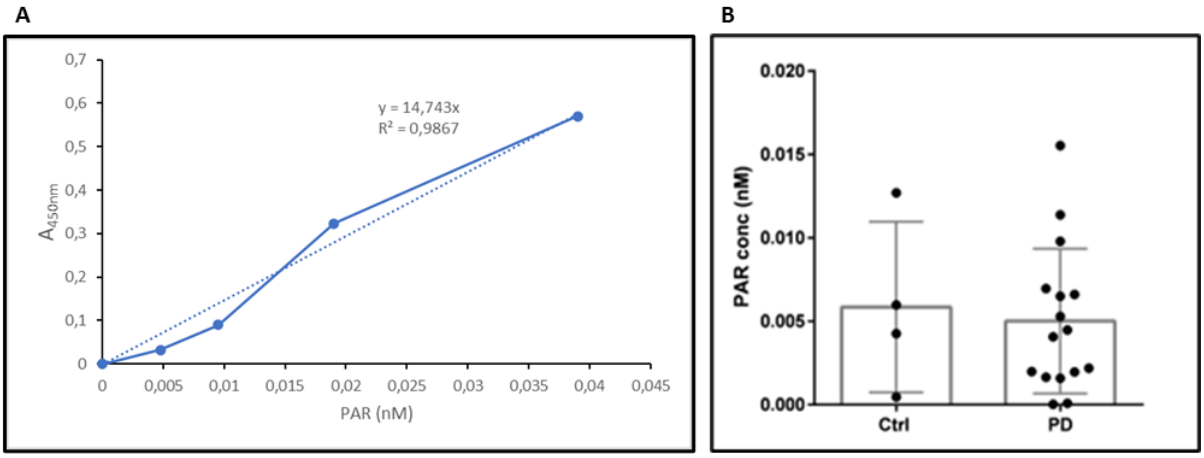


Figure 8.3. PAR levels in CSF from individuals with PD and controls detected by ELISA.
A. The plot shows the standard curve with relative absorbance values obtained from the indicated concentrations of PAR standard after 28 minutes of reaction time. **B**. CSF samples from individuals with PD and controls were analyzed using the adjusted ELISA protocol. Bar plots show the average concentration of PAR in CSF, black dots show the individual values. Error bars indicate standard deviation.

After the method was established, the ELISA assay was applied to detect and quantify PAR in CSF of individuals with PD and neurologically healthy controls. 29 PD CSF samples and 10 control CSF samples were tested. A second set of 25 PD and 15 control samples was unfortunately not possible to analyze due to a technical failure during the experiment.
Surprisingly, of those samples that could be analyzed, about 55% of the PD samples (16 out of 29) and 40% of the control samples (4 out of 10) were below the detection limit of the assay. However, those samples that showed a positive signal in the PAR ELISA (16 PD and 4 control samples), were used for quantitative analysis. As shown in Figure 8.3B, the average PAR concentration was 0.00501±0.00434 nM in the PD group and 0.00585±0.00511 in the control group. Both groups showed substantial individual variation, which resulted in the high standard deviation in both PD and control groups. Statistical analysis using student t-test showed that there was no statistically significant difference between PD and control groups.
In conclusion, PAR detection using the commercial ELISA kit showed that, when detectable, PAR levels in CSF from individuals with PD did not differ significantly from control values.

Immunodetection of PAR by Western blotting is not suitable in CSF and standard solutions.

Since most of the CSF samples showed PAR levels around the detection limit of the ELISA method, it was desirable to apply another method to further confirm and extend the results obtained from the ELISA analyses. One common method to detect PAR in cell lysates and tissue samples is Western blotting (WB). It was therefore tested whether this could be applied for CSF samples and PAR standards as well. Unfortunately, despite several attempts using various standard concentrations and amounts of CSF, the achieved results were negative as shown in Figure 8.4. A positive control (cell lysate overexpressing a constitutively active PARP construct resulting in strongly detectable PAR signal), 2 types of high concentrated PAR standard concentrations and 2 CSF samples were tested several times under different conditions. Only the positive control showed the expected smear of a positive PAR signal, while the other CSF samples and standard samples showed no signal even after very long exposure. Therefore, WB was declined as another method of detecting PAR in CSF samples.

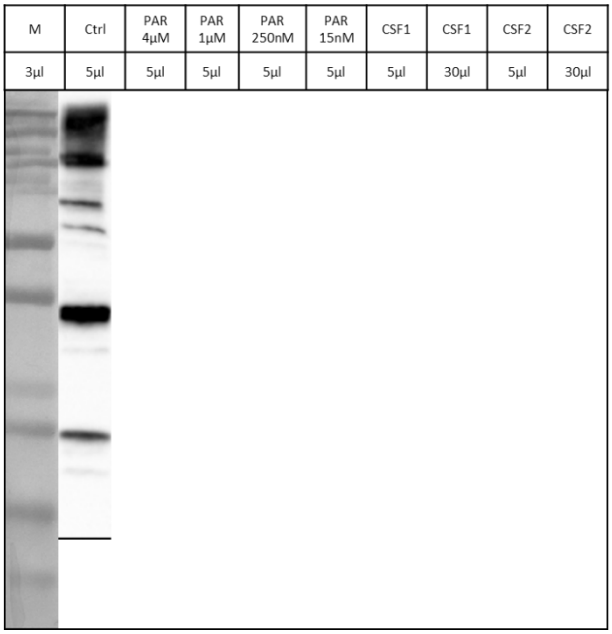


Figure 8.4. PAR detection by immunoblotting after SDS gel electrophoresis is not suitable for cerebrospinal fluid (CSF) or PAR standards.

A positive control (Ctrl), 2 CSF samples and several PAR standard samples were prepared for SDS gel electrophoresis using SDS sample buffer, separated by SDS PAGE, blotted onto a nitrocellulose membrane, and developed using a monoclonal antibody against PAR. The positive control shows a regular PAR signal of a smear ranging over 10-50kD in size due to the heterogenous length and branching of the molecule. PAR standards (lanes 2-5) and CSF samples (6-9) were loaded in various amounts as indicated but yielded no positive signal.

Dot blotting allows for PAR quantification in CSF and reveals no significant differences between individual with PD and controls.

Since immunodetection by Western blotting was not suitable for this project, next the possibility of dot blotting was evaluated. In dot blot, samples are directly dotted onto the membrane instead of separation on an SDS-gel and subsequent transfer. Then, the procedure of immunodetection is similar to other antibody-based methods including Western blotting (see methods for details). Again, a monoclonal antibody was used to detect PAR molecules. In order to be able to estimate the PAR concentration in the CSF, a PAR standard serial dilution series with known concentrations was dotted on the same membrane. To mimic CSF composition, PAR standard was prepared and diluted in a 1% BSA solution. Moreover, the CSF samples were dotted both undiluted and in a 1:2 and 1:4 dilution (dilutions were prepared in the same dilution buffer as the standards). Figure 8.5A-D shows that dot blotting was successful in detecting PAR in the CSF, both undiluted and diluted. This was encouraging, because the evident CSF levels were not at the limit of detection as in the ELISA approach but allowed for robust detection of PAR even in diluted samples. On each membrane shown in Figure 8.5A-D, the upper row on each panel showed the PAR standard (PAR STD), ranging from 5-0.315nM. Control and PD samples were randomly distributed over all membranes to avoid batch effects. Importantly, all 23 control and 54 PD samples could be analyzed using the dot blot approach. Visually, there was no difference observed between CSF samples from individuals with PD and controls, both in the undiluted and in the diluted samples. For determination of the PAR concentration in the CSF samples, the signal intensities were quantified for each dilution and the original concentration determined using the specific dilution factors. The average of all dilutions was then calculated. The PAR levels in CSF were performed twice using dot blotting. Therefore, the analysis is from the average of the 2 experiments. As shown in Figure 8.5E, The PAR levels of both controls and PD samples varied within the groups and were largely overlapping among the groups. Consistent with this, statistical analysis showed that there was no significant difference between PD and control samples.

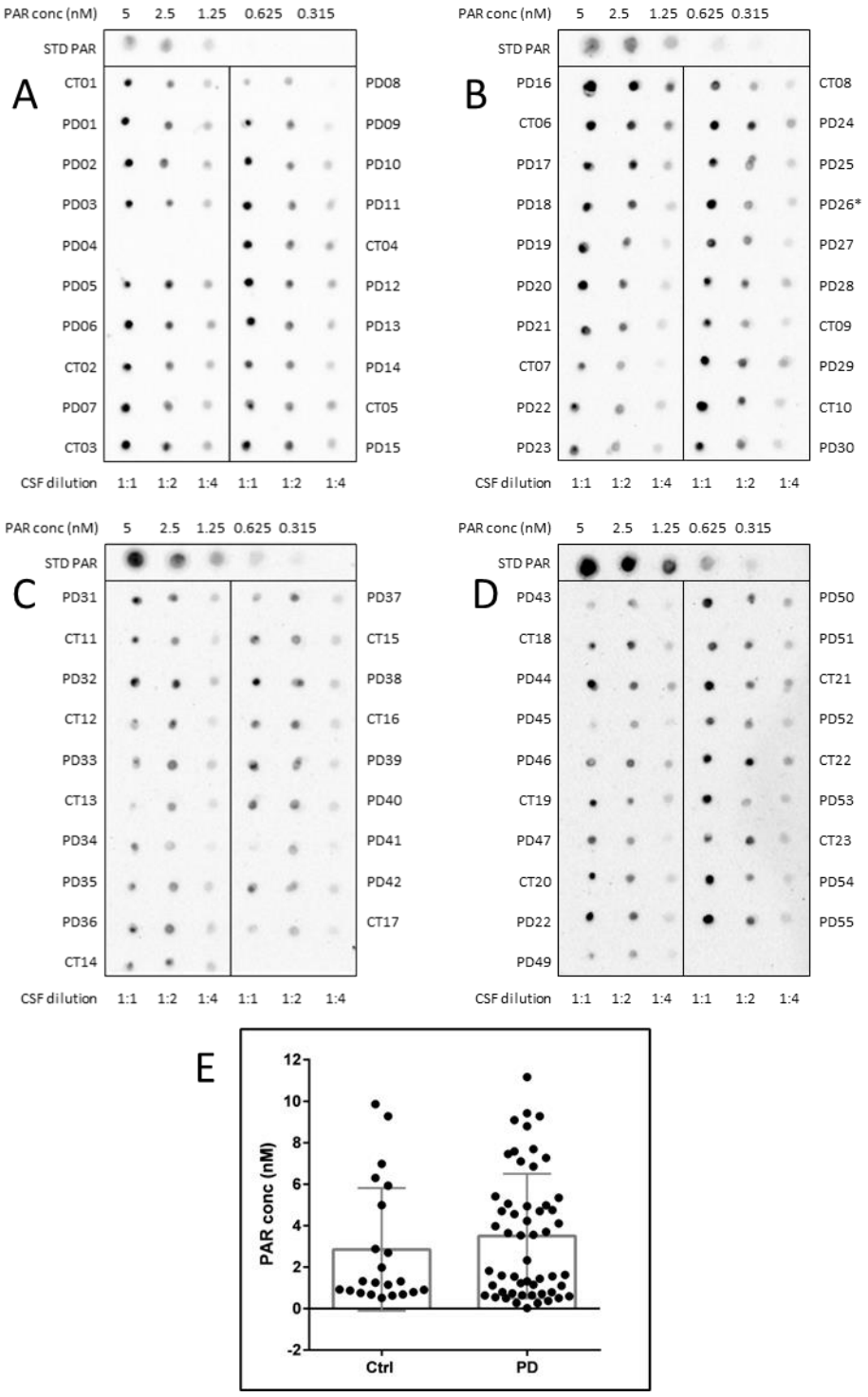


Figure 8.5: PAR levels in CSF detected by dot blotting were not elevated in PD.
A-D. The images show dot blots for all analyzed samples, where 2µl of the indicated sample dilution (undiluted (1:1) and (1:2)- and (1:4)-dilutions) were dotted on nitrocellulose membrane and developed with a monoclonal anti PAR antibody. A dilution series of poly ADP-ribose (PAR) standard solution (STD PAR) with the indicated concentrations was dotted in parallel on each membrane as quantification reference (2µl each). Individual with Parkinson's disease (PD01, PD02 etc.) and control (CT01, CT02, etc.) samples are indicated. Representative images of two independent experiments are shown. * indicates one sample (PD26) from an individual whose diagnose was changed from idiopathic PD to a different form of parkinsonism after the experiment was carried out. This sample was excluded from analysis. **E)** The bar plot shows the averages PAR concentration in CSF of 23 controls and 54 individuals with PD samples, analyzed in two independent experiments. Individual values are indicated as black dots. Error bars indicate standard deviation.

Nicotinamide riboside (NR) treatment did not elevate PAR in CSF of individuals with PD.

Supplementation with NR, which is an NAD^+ precursor, is being explored as a possible treatment for PD. However, PAR is synthesized from NAD+, and if PAR would be an assumed biomarker for PD, then NAD replenishment could have an enhancing effect by over-synthesizing PAR due to the availability of its substrate (NAD^+), and could worsen the disease, instead of being a possible treatment.

To investigate whether the replenishment of NR could result in increased levels of PAR, CSF samples from the NADPARK study, a phase 1 clinical trial of NR supplementation in individuals with PD (33) were examined. Samples from 11 participants that received NR and 8 participants from the placebo group were analyzed, both from before and after treatment for one month.

Initially, the samples were attempted to be analyzed by ELISA to determine PAR concentrations in CSF before and after NR vs placebo treatment. However, only 6 samples in total (4 of the 22 NR samples and 2 of 16 placebo samples exhibited positive signal, while the majority of samples produced negative results. All samples were from the same three individuals, allowing for a comparison between before and after treatment (Figure 8.6). However, as both control and PD samples showed an increase in PAR and due to the low number of positive values, these results were considered uninterpretable.

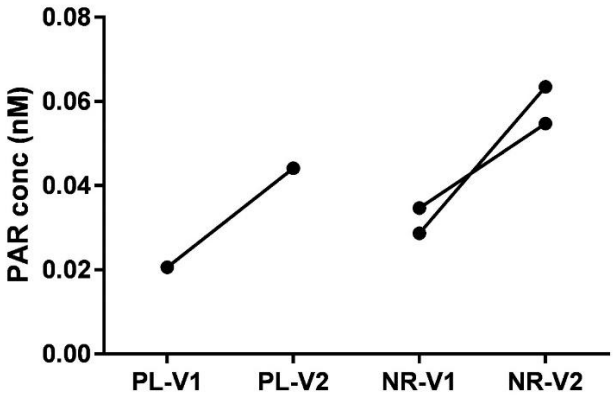


Figure 8.6. ELISA assay for detection of PAR in CSF before and after treatment with NR or placebo. The plot shows the PAR concentration in CSF of placebo (PL) or NR treated individuals with PD before (visit 1, V1) and after (visit 2, V2) treatment.

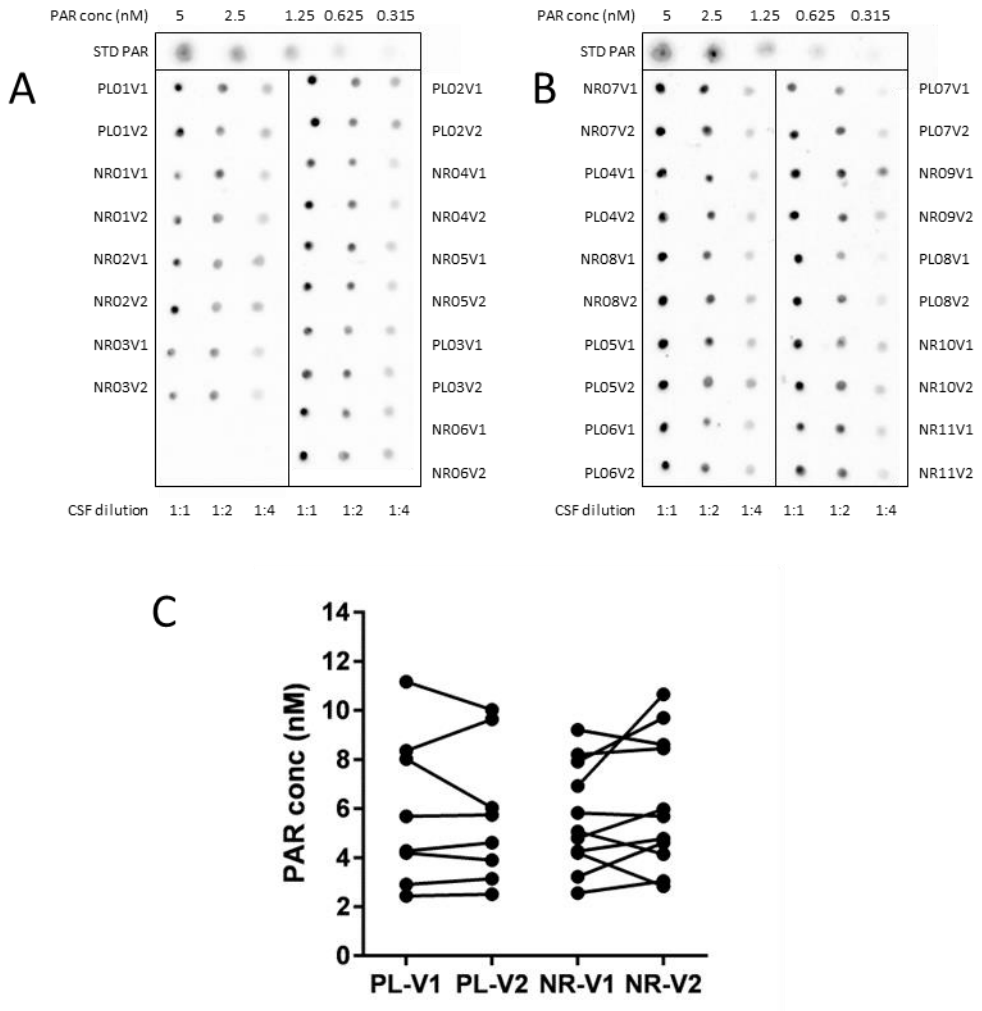


Figure 8.7. NR treatment does not elevate PAR levels in the CSF.

A-B) The images show dot blots for all analyzed samples, where 2µl of the indicated sample dilution (undiluted (1:1) and (1:2) and (1:4) dilutions) were dotted on nitrocellulose (NC) membrane and developed with a monoclonal anti PAR antibody. A dilution series of poly ADP-ribose (PAR) standard solution (STD PAR) with known concentration was dotted in parallel on each membrane as quantification reference (2µl each). Individuals in the NR group (NR01, NR02 etc.) and the placebo group (PL01, PL02 etc.) donated CSF before (visit 1, V1) and after (visit 2, V2) treatment. Representative images of two independent experiments are shown. **C)** The plots show the PAR levels in CSF before and after treatment (dots). Samples from the same individual before and after treatment are connected with a black line Pairwise t-test (Mann Whitney test) revealed showed no significant difference between after and before treatment in either NR or Placebo group (Placebo: $p>0.999$; NR: $p=0.2783$)

Since the ELISA results were not definite, dot blot experiments were carried out also for these CSF samples. As shown in Figure 8.7, all samples gave a robust PAR signal both undiluted and in several dilutions. Since all participants were individuals with PD, only comparison of the changes of PAR from before and after the different treatments could be carried out. Individuals in the NR group showed on average change of 0.477nM (from -1.6 to 3.7nM), while individuals in the placebo group showed on average a change of -0,14nM PAR (from -

2.0 to 1.3nM). Relative changes were on average 7.3% in the NR group, and -1.97% on average in the placebo group. However, individual changes were similar in both treatment groups, ranging from -26% to 54 % change in the NR and -25% to 15% in the placebo group. The pairwise comparison (paired t-test) revealed that neither the NR nor the placebo group showed statistically significant changes after treatment (Placebo: p>0.999; NR: p=0.2783).

Expression of CD38, an NAD glycohydrolase, is not affected in PD

Assumed changes in PAR levels in the CSF could also be results of expression changes in several PAR and NAD-related proteins. For example, if PARP1, the main PAR synthesizing enzyme, was elevated in the PD brain, this could lead to increased PAR detection. On the other hand, if the expression of an NAD degrading enzyme such as CD38, which has been described to increase with age and to be responsible for NAD decline with age, was changed in PD, this could affect PAR formation due to changes in the NAD level and thus substrate availability for PAR synthesis. Thus, in order to determine whether PAR level changes could be due to expressional changes in these enzymes, it was sought to investigate expression of PARP1 and CD38 in the frontal cortex and substantia nigra of PD and control individuals by immunohistochemistry analysis.

For detection of PARP1, two different monoclonal antibodies were tested. While the first antibody did not give a positive staining at all, the second antibody gave a strong and neuron specific signal in the tested sections of frontal cortex (Figure 8.8A) Unfortunately, the staining resulted in a signal in the whole cell and was not nuclear specific as expected from the known subcellular localization of PARP1. Further titration of the antibody resulted in ever reduced signal (Figure 8.8B) but did not lead to higher specificity for nuclear stain. Thus, the expression analysis had unfortunately to be abandoned at this stage due to time limitations.

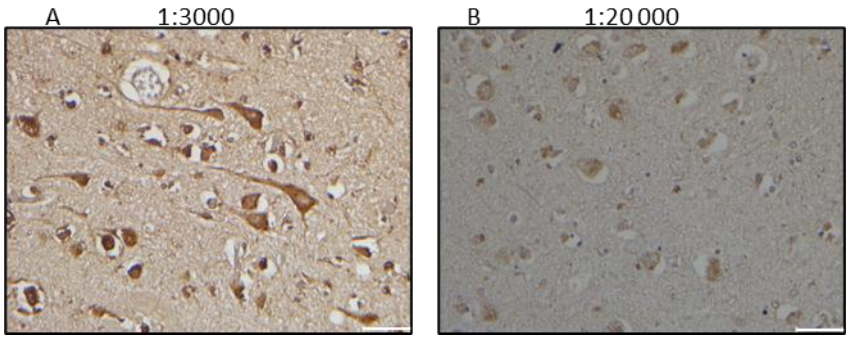


Figure 8.8. Representative images of PARP1 antibody in frontal cortex.
A. Immunohistochemistry images obtained with a PARP1 antibody at a dilution of 1:3000 (A) and 1:20000 (B) in section of the frontal cortex. The staining was performed using DAB and observed under a 20x magnification. Cell specific signal was observed throughout the cell body and axons, instead of an expected nuclear signal, in all dilutions.

The CD38 antibody gave a positive cellular stain in frontal cortex test sections and could thus be applied to investigate expression differences between PD and control. Sections from the frontal cortex and the substantia nigra from 3 individuals with PD and 3 neurological healthy control were stained and analyzed. In the frontal cortex, both grey and white matter were analyzed (Figure 8.9 and 8.10) and showed that there was no detectable difference in the expression level between PD and control samples. Also, the results from CD38 staining in the substantia nigra indicated that there were no significant differences observed in CD38 expression between individuals with PD and controls (Figure 8.11). In dopaminergic neurons of the substantia nigra, light brown staining of CD38 signal was detectable in addition to the dark brown vesicular signal of neuromelanin, a characteristic feature of dopaminergic neurons. These results suggest that CD38 expression is not altered in frontal cortex and substantia nigra of individuals with PD compared to controls.

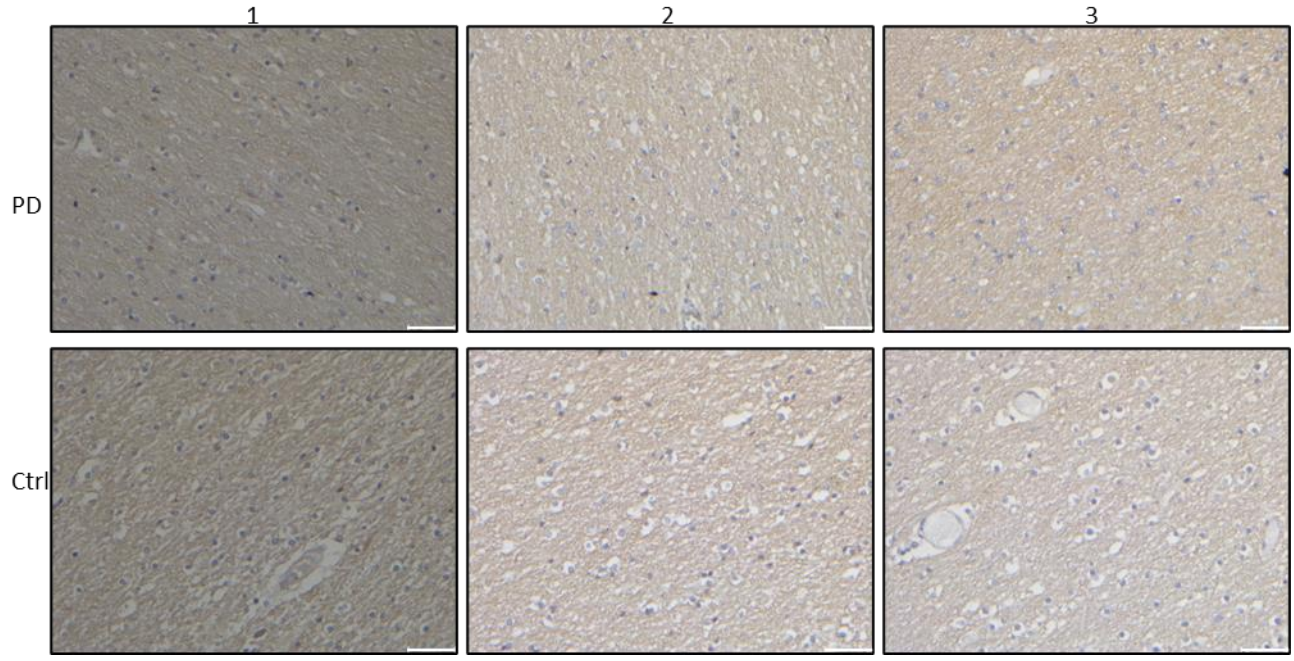


Figure 8. 9 CD38 detection in the white matter of the frontal cortex.
Images from three individuals with PD and three controls are shown. The staining was performed using DAB and observed under a 20x magnification.

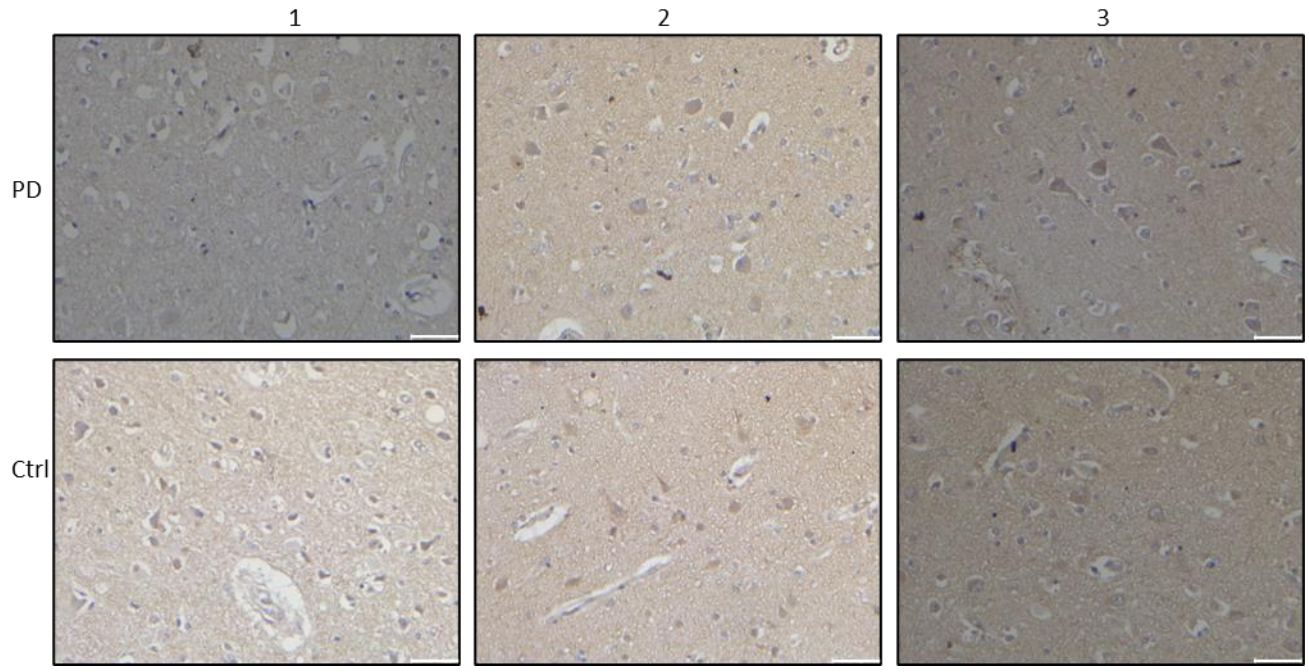


Figure 8.10 CD38 detection in the grey matter of the frontal cortex.
Images from three individuals with PD and three controls are shown. Neuronal stain is detectable in all sections, in addition to surrounding tissue. The staining was performed using DAB and observed under a 20x magnification.

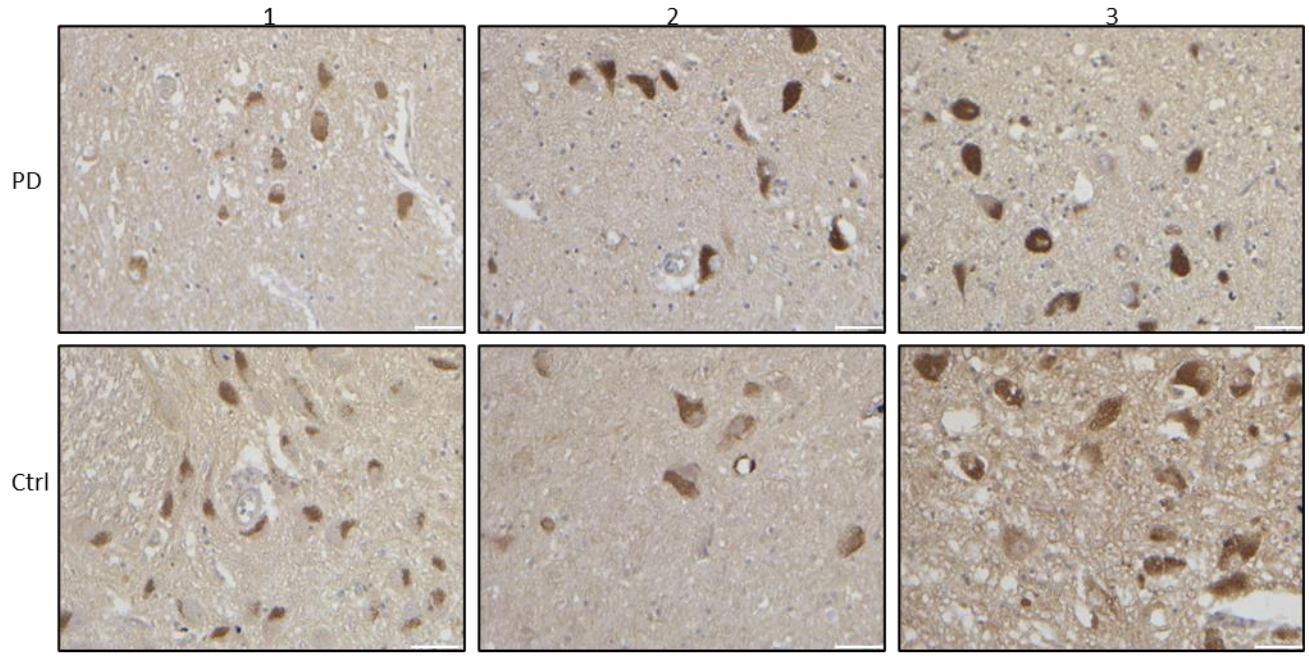


Figure 8.11 CD38 detection in the substantia nigra.
Images of three individuals with PD and three controls are shown. The very dark brown stain stems from intracellular vesicle structures containing neuromelanin, a characteristic of dopaminergic neurons in the substantia nigra. The lighter brown staining stems from CD38 detection

9. Discussion

In this study, the possibility of using immunodetection of poly-ADP-ribose as potential biomarker in PD was investigated. CSF samples of individuals with PD and neurological healthy controls were analyzed using a commercial ELISA setup and by dot blotting. The results of this study showed that PAR levels in CSF of individuals with PD were similar to those of controls. NR treatment, which increases cerebral levels of NAD, the substrate for PAR formation, did not affect the level of PAR in the CSF. Moreover, the expression of CD38, an NAD degrading enzyme, was found similar in control and PD samples.

PAR levels in CSF are not elevated in PD and therefore not a suitable biomarker

In this study, PAR levels in individuals in the PD group showed on average PAR levels of 3.49 ± 3.00 nM, while individuals in the control group showed on average 2.93 ± 2.92 nM. The statistical analysis of PAR in PD vs controls yielded a P-value of 0.5203. Although the PD group seemed to have a slightly higher PAR level on average compared to control, this difference was not statistically significant. Moreover, the individual values of each group were heterogenous, spread over a great range and largely overlapping between groups. Therefore, there is no significant difference in PAR levels between PD and controls.

This thesis was inspired by a previous study (21) that indicated higher PAR levels in individuals with PD compared to healthy controls and suggested that PAR could be a potential biomarker for PD. Kam et al presented results from 2 different cohorts, of which one showed a clearer difference between PD and controls than the other. However, in both cohorts, the individual results were heterogenous and showed extensive overlap of PAR between PD and control samples. The cohort in this study also showed varying results among individuals, both in the PD group and the controls. While there was a trend towards a slight increase in PAR in PD vs controls, this did not reach significance. It is possible that a larger cohort would result in a slightly different observation, and thus, it may be beneficial to increase the study group. However, a great concern for a reliable diagnostic biomarker is the large interindividual variability both within the groups, and also among PD and control samples, which largely overlapped in the three cohorts (two in the previous study by Kam et al, and one in the present study) studied so far. In order to be suitable for diagnostics, a biomarker should be characterized by a robust and clear difference between the groups.

The previous study also highlighted that the PAR increase in PD was due to excessive PAR synthesis, and that PAR levels increased the neurotoxicity of pathological forms of a-synuclein. In experiments with preformed fibrils of alpha synuclein, which have a neurotoxic effect on cultured cells and when injected in animal models, it was found that the presence of PAR increased the neurotoxic effect of the preformed fibrils compared to PARP1 knockout models. The interaction between PAR and a-synuclein fibrils resulted in neuronal parthanatos, the PAR mediated cell death pathway. Thus, while the investigation of PAR in CSF did not support the potential as a biomarker for PD; PAR metabolism is still an important area of research also in the PD field. The understanding of a potential mechanism that mediates neuronal death, and in turn, intervention with such mechanism in order to potentially halt neuronal death could help with the task to find a treatment for PD.

Nicotinamide riboside treatment did not elevate PAR in CSF of individuals with PD

As mentioned above, NAD replenishment therapy, primarily by supplementation of nicotinamide riboside (NR) is explored as a possible treatment for PD. If the working hypothesis of this study was true, namely that individuals with PD had higher levels of PAR in their CSF and thus likely also in the brain, it would have been likely that PAR formation was involved in the progression and pathology of the disease. Since PAR is synthesized of NAD^+ increasing the substrate of PAR formation as a recognized therapeutic approach could have severe effects on the disease state. In other words, NAD replenishment could worsen the disease by over-synthesizing PAR due to the availability of its substrate instead of treating the disease.

It was therefore investigated whether the supplementation with NR resulted in increased levels of PAR. CSF samples from the NADPARK study, a phase 1 clinical trial of NR supplementation in individuals with PD (33) were examined, including 11 samples from participants that received NR and 8 participants from the placebo group, both from before and after treatment for one month. Interestingly, the results did not reveal any significant difference between NR treated individuals and the placebo group. This is interesting and reassuring for several reasons. The aim of NAD replenishment therapy is to repair and improve cellular functions rather than causing harm. NAD^+ is an important metabolite involved in various cellular activities, including mitochondrial homeostasis and cell survival. Depletion of NAD^+ has been linked to neurodegenerative diseases including PD and replenishing NAD^+ has the potential of a neuroprotective effect. However, NAD^+ is also a substrate for the formation of PAR, and supplementation of high levels of its substrate could in principle increase PAR

formation substantially, at least for a certain period of time, and under the assumption that PARP1 and other PARPs are activated. The usually short-lived polymer is efficiently removed by PAR glycohydrolase (PARG), specifically also because prolonged elevated levels of PAR can lead to cell death. Our results indicate that, while PARP activity and PAR metabolism may still play an important role in PD, the "safety mechanism" of efficient removal of PAR is still in place. It also suggests that NAD replenishment therapy does not put the receiving individual at risk of a higher PAR synthesis and thus an increased possibility of neuronal cell death by parthanatos.

However, the samples from this study were from individuals that had taken NR for one month. It may be beneficial to also investigate PAR levels in CSF of participants of the ongoing phase II study (NOPARK), where individuals are treated with NR for a year. If no increase in PAR is still detected, this would corroborate the findings presented here.

CD38 expression is not changed in PD

Immunohistochemistry (IHC) analysis was used in order to evaluate the expression levels of PARP1 and CD38 in brain sections from individuals with PD and control subjects. Again, if the working hypothesis of elevated PAR in the CSF of individuals with PD was true, these experiments could help to interpret and understand potential reasons behind this.

Increased PARP1 expression is more commonly known in malignant transformation, for example in breast cancer when other DNA repair mechanisms fail (34). Also in neurodegeneration DNA damage is an important factor (34). In Alzheimer`s disease expression of PARP1 has been described to be elevated (34)

Unfortunately, the PARP1 antibodies used here did not lead to the expected nuclear localized signal, despite, in case of one of the antibodies, exhibiting a strong and cell-specific signal. It will be important to continue these investigations, even though the main question of elevated PAR in the CSF did not yield positive answers. An overexpressed, or hyperactivated PARP1 in neurons or other cell types in the PD brain may still be involved in the underlying pathogenic mechanisms of PD. The fact that interaction with PAR increased the neurotoxicity of pathogenic alpha-synuclein indicates that PAR metabolism may be strongly involved in PD. If PARP1 indeed showed increased expression in PD; combination therapies including PARP inhibitors would be a possibility for treatment.

The CD38 antibody successfully detected CD38 expression in both the substantia nigra and frontal cortex. However, the results in this study indicated no significant changes in CD38 expression in the frontal cortex and substantia nigra in the PD brain. It is known that CD38

expression tends to increase with age, which has been implemented in the observed NAD decrease with age, and since aging is the primary risk factor for PD, it is valid to investigate the potential involvement of CD38 in PD. The fact that no differences were observed seems to disprove this possibility. However, it is still possible that the small sample size limited the observation, and it would be interesting to increase the samples size, and potentially extend the scope to other brain areas as well.

10. Limitation of the study

Sensitivity of the commercial ELISA

The commercial PAR ELISA had limited sensitivity in CSF samples, which caused major challenges with regard to the detection limit. This was rather unexpected as the range of this ELISA was described to be similar to the values observed in CSF in previous studies. It may be that the antibody used in this kit was specific towards certain forms of PAR (for example non-protein bound, or specific levels of branching etc.) and that this affected its sensitivity in the CSF samples, while it was able to detect the PAR standards samples below nanomolar range. If PAR detection in CSF and other body fluids was further desirable, it would probably help to develop an advanced ELISA such as "self-made" ELISA could for example apply the monoclonal antibody used in this study for dot blotting, which seemed to exhibit an excellent sensitivity towards PAR in CSF. This would allow for a more robust and reproducible quantification in a plate reader format and help to further interpret PAR metabolism in the context of PD and other neurodegenerative diseases.

PARP1 antibodies

As mentioned above, the PARP1 antibodies were repeatedly tested but did not provide accurate staining results in the tested brain sections. While one antibody did not result in a detectable signal at high concentrations, another yielded strong and cell specific signal, however distributed throughout the whole cell, in contrast to the expected nuclear localization. Due to the time limits of the study, these investigations had to be abandoned. However, testing several more antibodies suitable for IHC analysis of PARP1 expression should be pursued. Once a suitable candidate antibody is found, the analysis of PARP1 expression in different areas of the PD brain will be intriguing, and hopefully shed more light on the involvement of PAR metabolism in PD.

11. Conclusion and future perspectives

In conclusion, this study indicates that PAR should not be considered as a diagnostic biomarker for PD. NR treatment did not have a detectable effect on PAR levels, and CD38 expression does not exhibit significant differences in PD patients compared to controls.

However, it will be important to conduct further research and consider additional regions and factors to gain a more comprehensive understanding of PAR metabolism and its potential implications in PD.

For example, the involvement of PAR in mediating the neurotoxicity of alpha synuclein requires further interpretation. Thus, exploring PARP1 activity and PAR metabolism in PD will be worth investigating. PARP1 is found in nearly all types of cells, making it worthwhile to compare PAR levels and PARP1 activity in other cell types besides dopaminergic and other neurons, as this could potentially help explain the mechanisms involved in PD better. Investigating PAR levels in neurons and mitotic cells could also give useful insights. Conducting the investigation with a larger group of participants for more precise comparisons would be beneficial. Additionally, exploring PAR levels in the early stages and last/advanced stages of PD could be worth investigating.

Finally, the search for diagnostic biomarkers needs to continue. In a recent glimpse of hope, a new study suggested that CSF samples could be used to confidently identify individuals with PD by analyzing a-synuclein aggregation patterns after combining the CSF with recombinant alpha-synuclein (35). While this is very intriguing, further confirmation of these findings is necessary. Moreover, a biomarker that would allow diagnosis before the onset of motor symptoms would grant the affected individual several more years of potential treatment.

12. Sources

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