UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
„CAROL DAVILA“ DIN BUCUREȘTI

Str. Dionisie Lupu 37, sector 2, București, 020021, România, www.umfcd.ro, email: rectorat@umfcd.ro

UNIVERSITY OF MEDICINE AND PHARMACY
“CAROL DAVILA” BUCHAREST
DOCTORAL SCHOOL
FIELD OF MEDICINE

AMYLOIDOGENIC PRODUCTS OF THE GUT MICROBIOTA, INTESTINAL BARRIER PERMEABILITY, AND GASTROINTESTINAL INFLAMMATION IN SPORADIC PARKINSON'S DISEASE

ABSTRACT OF THE PHD THESIS

PhD Supervisor:
PROF. UNIV. DR. BOGDAN OVIDIU POPESCU

PhD Student:
DUMITRESCU LAURA

2023

Table of Contents of the PhD Thesis

List of Publications

In extenso ISI papers I published from my doctoral research theme (main author):

1. Dumitrescu L, Popescu-Olaru I, Cozma L, Tulbă D, Hinescu ME, Ceafalan LC, Gherghiceanu M, Popescu BO. Oxidative Stress and the Microbiota-Gut-Brain Axis. Oxidative Medicine and Cellular Longevity 2018, 2018:2406594, doi:10.1155/2018/240659, https://www.hindawi.com/journals/omcl/2018/2406594/, IF 4.86 (2018). Part of Chapter 1, Subchapter 1.2, Section 1.2.3, pages 24 - 28.

2. Manole E[#], **Dumitrescu L**[#], Niculițe C, Popescu BO, Ceafalan LC. Potential Roles of Functional Bacterial Amyloid Proteins, Bacterial Biosurfactants and Other Putative Gut Microbiota Products in the Etiopathogeny of Parkinson's Disease. Biocell 2021, 45(1): 1-16, doi:10.32604/biocell.2021.013452, https://www.techscience.com/biocell/v45n1/41395, IF 1.1 (2021). Part of Chapter 2, pages 31 -37.

3. Dumitrescu L, Marta D, Dănău A, Lefter A, Tulbă D, Cozma L, Manole E, Gherghiceanu M, Ceafalan LC, Popescu BO. Serum and Fecal Markers of Intestinal Inflammation and Intestinal Barrier Permeability are Elevated in Parkinson's Disease, Frontiers in Neuroscience 2021, 15, 689723, doi:10.3389/fnins.2021.689723, https://www.frontiersin.org/articles/10.3389/fnins.2021.689723/full, IF 5.15 (2021). Part of Chapter 5, pages 75 – 113.

Abstract of the PhD Thesis

Introduction

The thesis presents the three studies I conducted as part of my doctoral research, under the supervision of Prof. Bogdan O. Popescu. My overall aim was to better characterize the relations between markers of bacterial endotoxin exposure (i.e., serum liposaccharide (LPS) and LPS-binding protein (LBP)), intestinal barrier permeability (i.e., serum and fecal zonulin), and gastrointestinal inflammation (i.e., serum and fecal calprotectin) in people with sporadic probably multifactorial Parkinson's disease (PD). In the first study I evaluated the correlations between the above markers and the markers of peripheral inflammation routinely tested in clinical practice in patients with sporadic probably multifactorial PD. In the second study I evaluated the levels of the above markers in patients with sporadic probably multifactorial PD compared to controls without PD. In the third study I evaluated the same markers in patients with sporadic probably multifactorial PD compared to patients with early-onset familial PD (i.e., probably monogenic). The research theme is important, these being potentially modifiable risk factors for the development and progression of multifactorial PD (and possibly of monogenic PD), as well as potentially useful markers for the diagnosis of prodromal PD.

The studies included a total of 61 participants, of which 28 patients with sporadic probably multifactorial PD (dataset used in all three studies), 28 controls without PD (second study), and 5 controls with early-onset familial PD (third study). In the first study I found that beta2-microglobulin and monocyte to lymphocyte ratio (MLR) correlated significantly to serum calprotectin. In the second study I found significantly lower levels of serum LBP in the sporadic probably multifactorial PD group compared to controls without PD, and significantly higher levels of serum and fecal zonulin and calprotectin. In the third study, I found higher levels of serum LPS and fecal zonulin and lower levels of serum LBP in the patients with sporadic probably multifactorial PD compared to controls with early-onset familial PD. The literature on the topic has been rapidly expanding over the past years, but none of the available studies tested all these markers together nor their correlations with the markers of peripheral inflammation routinely tested in clinical practice. Moreover, no study tested serum calprotectin or any of the other specific markers in patients with probably monogenic PD.

The results of my doctoral research add to the evidence that chronic endotoxin exposure or an altered response to endotoxin, increased intestinal barrier permeability, and gastrointestinal inflammation occur in sporadic multifactorial PD and might play a role in its etiopathogenesis.

I. The Current State of Knowledge

1. Sporadic Parkinson's Disease

PD is a highly disabling multisystem progressive alpha-synucleinopathy with no preventive or disease-modifying treatment. It affects more than 8.5 million people worldwide, its burden increasing faster than for any other neurological disorder (WHO, 2023). Most cases are sporadic and multifactorial, one or more environmental factors as well as polygenic predisposition playing a role in their etiopathogenesis. Monogenic PD is rare, typically familial, and with an earlier clinical onset. Lifestyle and other potentially modifiable environmental factors are of special interest since they could offer the means for preventive or disease modifying interventions (Ascherio and Schwarzschild, 2016).

Neuropathologically, PD is defined by the presence of selective neurodegeneration with intraneuronal accumulation of insoluble aggregates comprising mainly of misfolded alpha-synuclein, the so-called Lewy bodies and Lewy neurites (Mezey et al., 1998). Lewy pathology is present in 95% to 99% of the cases of PD, including most monogenic forms (Balestrino and Schapira, 2020; Berg et al., 2014; Braak et al., 2003a; Coughlin et al., 2019). Low grade neuroinflammation and selective neuronal dysfunction are also found, involving both the central and the peripheral nervous systems (Balestrino and Schapira, 2020; Braak et al., 2003a; Coughlin et al., 2019).

In most cases the pathology begins in the gut, subsequently spreading to the central nervous system via the neural gut-brain axis, probably by a prion-like mechanisms. This view is included in the dual hit theory of Braak (i.e., the "hit" from the gut) (Braak and Del Tredici, 2017; Hawkes et al., 2007; Hawkes et al., 2009), and corresponds to the more recently proposed alpha-synuclein origin site and connectome model, which states the existence of a body-first (or gut-first) PD subtype, and a brain-first subtype (Borghammer, 2021; Borghammer and Van Den Berge, 2019; Horsager et al., 2020; Horsager et al., 2022). The upstream events that trigger the overexpression and amyloid transformation of alpha-synuclein, and the mechanistic link between the latter and the selective neurodegeneration characteristically seen in PD, require further clarifications. The recent failure of two monoclonal antibodies that bind alpha-synuclein aggregates to decrease PD progression (Lang et al., 2022; Pagano et al., 2022) seems to argue against a pathogenic role of the already formed alpha-synuclein aggregates but does not preclude a pathogenic role of the amyloidogenic process. On the other hand, the long latency between the biological onset of PD and the clinical presentation offers a window of opportunity for interventions that might delay or stop the progression of the disease.

The gut microbiome is a complex ecosystem found in the gastrointestinal lumen and on the gastrointestinal mucosa. There is a high prevalence of gut dysbiosis in people with PD, even in the early stages of the disease (Baldini et al., 2020; Bedarf et al., 2017; Cirstea et al., 2020; Dumitrescu et al., 2018; Heintz-Buschart et al., 2018; Hill-Burns et al., 2017; Houser et al., 2018; Houser and Tansey, 2017; Nishiwaki et al., 2020; Nuzum et al., 2020), and experimental studies show that the gut microbiota is required for the development of Lewy pathology and motor impairment in alpha-synuclein overexpressing mice (Sampson et al., 2016). Among the consequences of gut dysbiosis, the potential overexpression of bacterial amyloidogenic products is of particular importance, since these are potential triggers of the initial alpha-synuclein misfolding events in the body(gut)-first subtype of PD.

2. Amyloidogenic Products of the Gut Microbiota and Alpha-Synuclein Cross-Seeding

The gastrointestinal tract has a large surface where alpha-synuclein might be exposed to the environment, especially in the presence of increased intestinal barrier permeability (allowing a closer contact between the potentially amyloidogenic xenobiotics of the gastrointestinal lumen and the alpha-synuclein of the enteric neurons), and gastrointestinal inflammation (inducing overexpression of alpha-synuclein in the enteric neurons). Alpha-synuclein amyloidogenesis (i.e., alpha-synuclein misfolding, self-aggregation with formation of insoluble aggregates, and seeding) is the core pathogenic feature in sporadic PD. Gastrointestinal inflammation and intestinal barrier dysfunction, related to gut dysbiosis or to other causes, occur in sporadic PD, and are potential risk factors for its development, creating a gastrointestinal milieu that may promote alpha-synuclein amyloidogenesis and increase the neuronal susceptibility to neurodegeneration (Manole et al., 2021).

Experimental evidence suggests that the presence of certain amyloidogenic products of the gut microbiota, such as the bacterial endotoxin LPS, the functional bacterial amyloid protein curli, and the bacterial biosurfactant rhamnolipid, as well as the presence of other luminal amyloidogenic xenobiotics (e.g., from environmental exposure or alimentary sources), may trigger or enhance alpha-synuclein amyloidogenesis (Brown, 2019; Brown et al., 2023; Clunn et al., 2000; Lange et al., 2003; Lerner, 2022; Manole et al., 2021; Niehaus and Lange, 2003). Thus, the presence of an amyloidogenic gut microbiota could trigger or exacerbate alpha-synuclein amyloidogenesis (in the presence of increased intestinal barrier permeability and gastrointestinal inflammation), being a potential risk factor for sporadic body(gut)-first PD.

II. Personal Contributions

3. Research Concept and Methodology

Most people with sporadic PD develop gastrointestinal symptoms early in the course of the disease, constipation preceding parkinsonism by more than a decade (Postuma et al., 2015). The gastrointestinal symptoms are thought to be related to the PD pathology that develops in the enteric nervous system before spreading to the central nervous system via the vagus nerve and the sympathetic connectome (Borghammer, 2021; Braak et al., 2006; Devos et al., 2013).

Several peripheral inflammation markers are routinely tested in clinical practice, most being cheap and readily available. These include erythrocyte sedimentation ratio (ESR), fibrinogen, C reactive protein (CRP), beta2-microglobulin, neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR), and platelet to lymphocyte ratio (PLR).

LPS, the endotoxin produced by Gram-negative bacteria, is widely used to induce PD-like pathology in animal models (Deng et al., 2020; Dutta et al., 2008; Gao et al., 2011a; Kelly et al., 2014). Different bacteria species produce LPS variants with different capacity to induce neuroinflammation and toxicity (Raetz and Whitfield, 2002; Vatanen et al., 2016). In the absence of bacterial infections, the main sources of circulating LPS in humans (i.e., endotoxinemia) are the Gram-negative bacteria of the gut microbiome (Bhattacharyya and Bhunia, 2021; Shannon, 2022). The gut microbiome of patients with PD has higher counts of Gram-negative bacteria that produce proinflammatory LPS, while the gut microbiome of controls without PD has higher counts of Gram-positive bacteria and anti-inflammatory LPS-producing Gram-negative bacteria (Gorecki et al., 2019; Scheperjans et al., 2015; Scheperjans et al., 2016; Yan et al., 2021). Dietary contaminants can also contribute to the endotoxinemia, and transient rises in blood LPS were found after high-fat meals (Erridge et al., 2007). Periodontitis is associated with a higher risk of PD, and with higher levels of circulating LPS (Chen et al., 2018; Chen et al., 2017; Olsen et al., 2020). Increased intestinal barrier permeability, obesity, and liver disease are also associated with higher levels of circulating LPS (Hersoug et al., 2016; Kitabatake et al., 2017). Several methods can be used to assess serum or plasma LPS, the most widely used being ELISA. However, the LPS levels are notoriously unreproducible (Citronberg et al., 2016; Forsyth et al., 2011; Hasegawa et al., 2015; Loffredo et al., 2020; Pal et al., 2015; Wijeyekoon et al., 2020; Yao et al., 2016). In humans, the presence of LPS induces the synthesis of LBP, which is considered a better marker of endotoxin exposure because of its longer half-live (Schumann, 2011). However, circulating LBP shows only moderate test-retest reliability (Citronberg et al., 2016). Moreover, at low levels it

enhances and at high levels it diminishes the activation of the immune system induced by LPS (Gutsmann et al., 2001), making the interpretation of the results not so straight forward (Forsyth et al., 2011; Zhao et al., 2023).

The presence of increased intestinal barrier permeability in PD was demonstrated by highly specific methods (Forsyth et al., 2011; Perez-Pardo et al., 2019). Zonulin is part of the tight junctions of the intestinal barrier, modulating its permeability by promoting the disassembly of zonula occludens (Ajamian et al., 2019; Fasano, 2012; Ohlsson et al., 2017b). Its release is triggered by bacteria, but also by some dietary components, higher levels resulting in increased barrier permeability (Fasano, 2012). It can be measured by ELISA, its fecal levels being considered a marker of intestinal barrier permeability (Aho et al., 2021; Boncuk Ulas et al., 2023; Loffredo et al., 2020; Mulak et al., 2019; Schwiertz et al., 2018). The reliability of serum zonulin as a marker of intestinal barrier permeability is still debated (Ajamian et al., 2019; Fasano, 2021; Massier et al., 2021; Ohlsson et al., 2017a; Talley et al., 2020).

Calprotectin is a pleiotropic cytokine-like protein with bacteriostatic and fungistatic effects (Walsham and Sherwood, 2016). It is released by activated neutrophils, monocytes, and endothelial cells at the site of inflammation, its levels raising rapidly in the presence of bacteria (Dhaliwal et al., 2015; Diamanti et al., 2010; Jensen et al., 2011; Kowalski and Mulak, 2019; Moein et al., 2017). Due to its stability, it can be easily measured using ELISA, fecal calprotectin being a well-established marker of intestinal inflammation (Mumolo et al., 2018). However, high levels of fecal calprotectin are not specific for IBD, fecal calprotectin also increasing in other circumstances (Khaki-Khatibi et al., 2020). The reliability of serum calprotectin as a marker of gastrointestinal inflammation remains under debate.

The main objectives of my doctoral research are:

1. to evaluate serum markers of bacterial endotoxin exposure (i.e., serum LPS, and serum LBP), serum and fecal markers of intestinal barrier permeability (i.e., serum and fecal zonulin), and serum and fecal markers of gastrointestinal inflammation (i.e., serum and fecal calprotectin) in people with sporadic probably multifactorial PD compared to controls without PD, and with early-onset familial PD (i.e., probably monogenic).

2. to evaluate the corelations between the markers of peripheral inflammations that are routinely tested clinical practice (i.e., peripheral blood ESR, fibrinogen, CRP, beta2-microglobulin, procalcitonin, and NLR, MLR, and PLR, calculated based on CBC with DLC) and the markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation, in patients with sporadic probably multifactorial PD.

The secondary objectives of the doctoral research are:

1. to evaluate the usefulness of the serum markers of bacterial endotoxin exposure, serum and fecal markers of intestinal barrier permeability, and serum and fecal markers of gastrointestinal inflammation in predicting the risk or likelihood of having a diagnostic of sporadic probably multifactorial PD (versus not having clinical PD).

2. to evaluate the correlations between the serum markers of bacterial endotoxin exposure (i.e., serum LPS, and serum LBP), serum and fecal markers of intestinal barrier permeability (i.e., serum and fecal zonulin), and serum and fecal markers of gastrointestinal inflammation (i.e., serum and fecal calprotectin) and PD-related characteristics in people with sporadic probably multifactorial PD.

The research questions are:

1. Are there any significant differences between the serum levels of LPS and LBP (i.e., markers of bacterial endotoxin exposure) in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD?

2. Are there any significant differences between the serum and fecal levels of zonulin (i.e., markers of intestinal barrier permeability) in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD?

3. Are there any significant differences between the serum and fecal levels of calprotectin (i.e., markers of gastrointestinal inflammation) in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD?

4. Do the markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation, alone or in combination, predict the risk or likelihood of having a diagnostic of sporadic probably multifactorial PD (versus not having clinical PD)?

5. Do the markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation correlate with PD-related characteristics in patients with sporadic probably multifactorial PD?

6. Are there any significant correlations between the markers of peripheral inflammation that are routinely tested in the peripheral blood in clinical practice and the markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation in patients with sporadic probably multifactorial PD?

The research hypotheses are:

1. The mean serum levels of LPS are higher in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD. The serum LPS levels do not predict the risk/ likelihood of having sporadic probably multifactorial PD (compared to not having clinical PD), and are not associated with the duration, stage, and severity of the disease. *[related to the 1st, 4th, and 5th research questions]*

2. The mean serum levels of LBP are lower in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD. Lower serum LBP levels are associated with a higher risk / likelihood of having sporadic probably multifactorial PD (compared to not having clinical PD), and with the duration, stage, and severity of the disease. *[related to the 1st, 4th, and 5th research questions]*

3. The mean serum and fecal zonulin levels are higher in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD. Higher serum and fecal zonulin levels are associated with a higher risk or likelihood of having sporadic probably multifactorial PD (compared to not having clinical PD), and with the duration, stage, and severity of the disease. *[related to the 2nd, 4th, and 5th research questions]*

4. The mean serum and fecal calprotectin levels are higher in patients with sporadic probably multifactorial PD compared to controls without PD, and compared to controls with early-onset familial PD. Higher serum and fecal calprotectin levels are associated with a higher risk or likelihood of having sporadic probably multifactorial PD (compared to not having clinical PD), and with the duration, stage, and severity of the disease. *[related to the 3rd, 4th, and 5th research questions]*

5. The markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation used in various combinations are better predictors then when used alone of the likelihood of having sporadic probably multifactorial PD (versus not having clinical PD). *[related to the 5th research question]*

6. There are no significant correlations between the markers of peripheral inflammations that are routinely tested in the peripheral blood in clinical practice and the markers of bacterial endotoxin exposure (i.e., serum LPS and LBP), intestinal barrier permeability (i.e., serum and fecal zonulin), and gastrointestinal inflammation (i.e., serum and fecal calprotectin) in patients with sporadic probably multifactorial PD. *[related to the 6th research question]*

Design of the Studies

The first study has a cross-sectional design with correlation analysis. It includes patients with sporadic probably multifactorial PD that comply with the inclusion and exclusion criteria detailed below. The study evaluates the prevalence of increased levels of peripheral inflammation markers that are routinely tested in the peripheral blood in clinical practice (obtained from recent medical records, if available) in patients with sporadic probably multifactorial PD and the correlations between the levels of these markers and the levels of serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin, respectively, as well as between the levels of the markers and PD-related characteristics. The first study serves the 2nd main objective and the 2nd secondary objective, answering the 6th and the 5th research questions, and testing the 6th hypothesis, and part of the first 4 hypotheses.

The second study has a case-control design. It evaluates serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin in patients with sporadic probably multifactorial PD compared to controls without PD. The second study serves the 1st main objective and the 1st secondary objective, answering the first 4 research questions, and testing the first 5 hypotheses. The inclusion and exclusion criteria for the two groups, with sporadic probably multifactorial PD, and without PD, are detailed below.

The third study also has a case-control design, evaluating serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin in patients with sporadic probably multifactorial PD compared to patients with early-onset familial PD. The inclusion and exclusion criteria for the sporadic PD group are the same as for the second study. The inclusion and exclusion criteria for the early-onset familial PD group are detailed below. The third study serves the 1st main objective, answering the first 3 research questions, and testing part of the first 4 hypotheses.

Research Setting, Recruitment, Enrolment, and Evaluation

The research was conducted at the "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania, in collaboration with the Neurology Departments at Colentina Clinical Hospital, and the "Victor Babeș" National Institute of Pathology. Enrolment was based on predefined inclusion and exclusion criteria. We evaluated the participants clinically and collected data from the available medical records. We also collected serum and fecal samples, and measured the levels of LPS, LBP, zonulin, and calprotectin, using commercially available ELISA kits.

Inclusion criteria for the first study: patients fulfilling the MDS Clinical Diagnostic Criteria for clinically established or clinically probable PD (Postuma et al., 2015), with the onset of the motor symptoms after the age of 50 years (i.e., 50 years and older at onset), no family history of PD (first or second degrees), and no other evidence suggestive of monogenic PD, that were tested within a month of study enrolment, as part of their routine medical care, for at least one of the peripheral blood inflammation markers assessed by the study.

Inclusion criteria for the case group in the second and third studies (i.e., sporadic probably multifactorial PD): patients fulfilling the MDS Clinical Diagnostic Criteria for clinically established or clinically probable PD (Postuma et al., 2015), with the onset of their motor symptoms after the age of 50 years (i.e., 50 years and older at onset), no family history of PD in first or second degree relatives, and no other evidence suggestive of monogenic PD (i.e., results of genetic testing, if available).

Inclusion criteria for the control group in the second study (i.e., without PD): adults not meeting diagnostic criteria for PD, without clinical motor or nonmotor markers of prodromal PD (Berg et al., 2015; Heinzel et al., 2019), except for isolated constipation (not mandatory, but permitted).

Inclusion criteria for the control group in the third study (i.e., early-onset familial PD): patients fulfilling the MDS Clinical Diagnostic Criteria for clinically established or clinically probable PD (Postuma et al., 2015), with the onset of their motor symptoms before the age of 50 years and history of PD in first or second degree relatives, with or without other evidence suggestive of monogenic PD (i.e., results of genetic testing, if available).

Exclusion criteria for all studies and groups: recent (less than 6 months) or concurrent / ongoing diagnosis of gastrointestinal or systemic conditions (unrelated to PD) that could interfere with the laboratory results; recent stroke or other recent or ongoing neurologic disorders or severe disability that may interfere with the study evaluation procedures or results; antibiotic treatment within the past 3 months prior to inclusion; use within the past month of other drugs or supplements that may interfere with the lab test results.

Biological Sample Collection, Processing, and Storage

Peripheral venous whole blood samples were collected à jeun, the day after the clinical evaluation or during the same day. Stool samples were collected directly by the study participants, were kept at room temperature for as short as possible, then refrigerated at 4-8 °C, for a maximum of 24 hours, then preprocessed using commercially available preparation and extraction tubes (K 6998SAS, K 6999, Immundiagnostik AG, Germany).

Laboratory Methods

The endotoxin exposure markers, intestinal barrier permeability markers, and gastrointestinal inflammation markers were measured at the "Victor Babeș" National Institute of Pathology, Bucharest, Romania, using commercially available ELISA kits. The testing was performed according to the protocols that came with each kit. The following kits were used: the Human Lipopolysaccharides ELISA kit CSB-E09945h (Cusabio, China), the Human Lipopolysaccharides Binding Protein ELISA kit CSB-E09629h (Cusabio, China), the IDK® Zonulin ELISA K 5601 (Immundiagnostik AG, Germany), the IDK® Zonulin ELISA K 5600 (Immundiagnostik AG, Germany), the IDK® Calprotectin ELISA K 6935 (Immundiagnostik AG, Germany), and the IDK® Calprotectin ELISA K 6927 (Immundiagnostik AG, Germany).

Statistical Analysis

For statistical analysis I used IBM® SPSS® Statistics version 29. For power analysis I used G*Power version 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf) (Faul et al., 2007).

Ethics and Personal Data Protection

The research protocol is compliant with the Declaration of Helsinki and was approved by the Colentina Hospital Ethics Committee (EMI-BPs, 3/16.04.2019, with subsequent reapproval in 2021). Written informed consent was obtained from all participants prior to study enrolment. Processing of personal data was performed in agreement with the European General Data Protection Regulation (GDPR) 2016/679 and local practice. The collected data were pseudonymised, and then anonymised, prior to statistical analysis.

Funding

The evaluation of the markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation was funded by the Ministry of Research and Innovation in Romania (currently the Ministry of Research, Innovation and Digitalisation), through the grant PN 19.29.02.01/2019, Programme 1 – Improvement of the National System of Research and Development, Subprogramme 1.2 – Institutional Excellence - Projects of Excellence Funding in RDI, as part of the second and third phases of the project "The Effects of the Intestinal Microbiota Products and Functional Amyloid Protein on the Pathological Aggregation of Proteins and on the Propagation of Pathology in a Prion-Like Manner in Neurodegeneration Models", conducted at the "Victor Babeș" National Institute of Pathology, Bucharest, Romania, under the coordination of Prof. Dr. Bogdan O. Popescu, who is also the PhD supervisor. The grant was awarded by competition.

4. The First Study: Markers of Peripheral Inflammation and Their Correlations with Markers of Bacterial Endotoxin Exposure, Intestinal Barrier Permeability, and Gastrointestinal Inflammation in Patients with Sporadic Parkinson's Disease

In the first study I assessed the correlations between the peripheral inflammation markers routinely tested in clinical practice, and the levels of serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin, in patients with sporadic probably multifactorial PD. The study enrolled 28 patients with sporadic probably multifactorial PD, 19 males and 9 females. The mean age was 66.46 years old.

I found statistically significant results for the correlation between serum beta2-microglobulin and serum calprotectin ($r_s = 0.534$, N = 22, p = 0.01), and between the MLR and serum calprotectin ($r_s = 0.499$, N = 27, p = 0.008). Noteworthy, beta2-microglobulin was above the upper reference limit in 39.13% of the patients.

The levels of serum LPS showed a strong direct correlation with serum LBP ($r_s = 0.878$, N = 27, $p < 0.0005$). The levels of fecal zonulin correlated indirectly with fecal calprotectin ($r_s = - 0.526$, N = 23, p = 0.01), and serum calprotectin corelated with the age ($r_s = 0.441$, N = 27, p = 0.021), and with the age at onset ($r_s = 0.398$, N = 27, p = 0.040). Interestingly, I also found a significant inverse corelation between the serum levels of LPS and the serum levels of uric acid, a potent antioxidant ($r_s = -0.405$, N = 26, p = 0.040).

5. The Second Study: Markers of Bacterial Endotoxin Exposure, Intestinal Barrier Permeability, and Gastrointestinal Inflammation in Patients with Sporadic Parkinson's Disease Compared to Controls without Parkinson's Disease

In the second study I assessed the levels of serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin in patients with sporadic probably multifactorial PD compared to controls without PD. The study enrolled 56 participants, 28 patients with sporadic PD (19 males, 9 females), and 28 unmatched controls (15 males, 13 females). The sporadic PD group completely overlaps with the population of the first study.

Mean serum LBP levels were significantly lower in the sporadic PD group than in controls (6.82 mcg/ml versus 17.28 mcg/ml, p = 0.001), with similar serum LPS levels (406.17 pg/ml versus 407.63 pg/ml, p = 0.975). Serum LBP levels below 15 mcg/ml were associated with a 15 times higher risk of having PD (OR 15, 95% CI: 2.972 – 75.695, $p < 0.001$), with a sensitivity of 92.86%, and a specificity of 53.57%.

The mean levels of serum and fecal zonulin were significantly higher in the sporadic PD group than in controls (26.6 ng/ml versus 21.5 ng/ml, p = 0.018, respectively 94.72 ng/ml

versus 38.6 ng/ml, $p < 0.001$). None of the participants had serum zonulin levels above the upper reference limit, but 18.51% of the patients with sporadic PD and 54.17% of the controls had levels below the lower reference limit. Fecal zonulin levels above the upper reference limit were found in 33.33% of the sporadic PD patients and in none of the controls. Serum zonulin levels above 20 ng/ml were associated with a 4.4 times higher risk of having sporadic PD (OR 4.4, 95% CI: 1.297 – 3.092, $p = 0.023$), with a sensitivity of 81.48%, and a specificity of 50%. Concurrently, fecal zonulin levels above 50 ng/ml were associated with an 8.4 times higher risk of having sporadic PD (OR 8.4, 95% CI 2.119 – 33.292, $p = 0.002$), with a sensitivity of 75%, and a specificity of 73.68%.

The levels of serum and fecal calprotectin were significantly higher in the sporadic PD group than in controls (13.77 mcg/ml versus 6.88 mcg/ml, $p = 0.004$, and 187.16 mcg/ml versus 57.94 mcg/ml, $p < 0.001$). Serum calprotectin levels above the upper reference limit were found in 88.89% of the sporadic PD patients and in 55.56% of the controls. Fecal calprotectin levels above the upper reference limit were found in 91.3% of the patients with sporadic PD and in 33.33% of the controls. Noteworthy, fecal calprotectin levels over 100 mcg/ml were found in 56.5% of the patients with sporadic PD and in 11.1% of the controls, while very high fecal calprotectin levels, above 250 mcg/ml, were found in 26.1% of the patients with sporadic PD and in none of the control. Serum calprotectin levels above 2.5 mcg/ml were associated with a 10 times higher risk of having PD (OR 10, 95%CI: 1.963 – 50.940, $p = 0.004$), with a sensitivity of 92.6%, and a specificity of 44.44%, and fecal calprotectin levels above 100 mcg/ml were associated with a 10.4 higher risk of having PD (OR 10.4, 95% CI: 1.928 – 56.102, $p = 0.004$), with a sensitivity of 56.52%, and a specificity of 88.89%.

The study included multiple binary logistic regression models, all showing that higher LBP levels are associated with a significantly lower likelihood of having sporadic PD, while higher fecal zonulin and calprotectin levels are associated with a higher likelihood of having PD. The binary logistic regression model that determined the effects of serum LBP, fecal zonulin, fecal calprotectin, age, and sex on the likelihood of having sporadic PD was the most performant, being statistically significant, with a specificity of 94.4%, and a sensitivity of 100%. Within the model, only serum LBP and fecal calprotectin were significant, higher LBP levels being associated with a lower likelihood of having sporadic PD (OR = 0.700, 95% CI: 0.497 – 0.987, $p = 0.042$), and higher fecal calprotectin levels being associated with a higher likelihood of having sporadic PD (OR = 1.022, 95% CI: 1.003 – 1.042, $p = 0.022$).

The serum levels of LPS were assessed in five studies of idiopathic / sporadic PD, including the present study, three of them finding higher levels in people with PD than in

controls (de Waal et al., 2018; Loffredo et al., 2020; Wijeyekoon et al., 2020). Another very small study (Forsyth et al., 2011), as well as the present study, found no significant differences. Wijeyekoon et al. found that the increased serum levels of bacterial LPS were accompanied by innate immune changes, suggesting that LPS may be playing a critical role in driving the pathology of PD in a subgroup of patients (Wijeyekoon et al., 2020).

Six cross-sectional case-control studies, including the present study, evaluated the circulating levels of LBP in patients with sporadic / idiopathic PD compared to controls. All six of them found significantly lower levels of LBP in sporadic / idiopathic PD (Chen et al., 2021; Forsyth et al., 2011; Hasegawa et al., 2015; Pal et al., 2015; Perez-Pardo et al., 2019). On the contrary, a recently published case-control study nested within a large European prospective cohort found that higher pre-diagnostic plasma levels of LBP might be associated with a higher risk of developing PD (Zhao et al., 2023). These apparently contradictory results support the role of endotoxinemia in the pathogenesis of sporadic PD but mandate the need for further investigation on the topic. The finding of lower circulating levels of LBP in patients with PD is not fully understood. Acute LPS exposure induces the release of LBP which opsonizes the circulating LPS molecules, facilitating their recognition by macrophages, and enhancing the immune response to LPS (Ding and Jin, 2014; Martin et al., 1992; Meng et al., 2021). On the contrary, chronic LPS exposure might decrease the production of LBP (Gutsmann et al., 2001). Increased age, being overweight or obese, having hyperlipidaemia or metabolic syndrome, and smoking, correlate with higher circulating levels of LBP (Gonzalez-Quintela et al., 2013). Chen et al. proposed that the reduced levels of LBP might also reflect the internalization of LBP and LPS in the gastrointestinal wall (Chen et al., 2021).

Increased intestinal permeability was reported in PD patients (Forsyth et al., 2011). Three studies, including the present study (partially published results), evaluated the serum levels of zonulin in idiopathic / sporadic PD, finding them to be higher compared to controls, suggesting increased permeability of the intestinal mucosa (Boncuk Ulas et al., 2023; Dumitrescu et al., 2021; Loffredo et al., 2020). The fecal zonulin levels were evaluated in four idiopathic / sporadic PD studies (Aho et al., 2021; Dumitrescu et al., 2021; Mulak et al., 2019; Schwiertz et al., 2018), including the present study (partially published results). All these studies found higher levels of fecal zonulin in people with PD compared to controls, despite not all being statistically significant.

No other studies evaluated serum calprotectin. Seven studies, including the present study (partially published results), evaluated the levels of fecal calprotectin, a well-established marker of gastrointestinal inflammation, in people with sporadic/idiopathic PD. All the studies

found that fecal calprotectin is higher in patients with PD than in controls (Aho et al., 2021; Augustin et al., 2023; Dumitrescu et al., 2021; Hor et al., 2022; Mulak et al., 2019; Schwiertz et al., 2018; Weis et al., 2019).

6. The Third Study: Markers of Bacterial Endotoxin Exposure, Intestinal Barrier Permeability, and Gastrointestinal Inflammation in Patients with Sporadic Parkinson's Disease Compared to Patients with Early-Onset Familial Parkinson's Disease

In the third study I assessed the levels of serum LPS and LBP, serum and fecal zonulin, and serum and fecal calprotectin in patients with sporadic probably multifactorial PD compared to controls with early-onset familial PD. The study enrolled 33 participants, 28 patients with sporadic probably multifactorial PD (19 males, 9 females), and 5 unmatched controls with early-onset familial PD (3 males, 2 females). The sporadic PD group completely overlaps with the population of the first two studies.

The mean serum LPS levels were higher in the sporadic PD group than in the familial PD group (406.17 mcg/ml versus 209.41 pg/ml), while being similar between the sporadic PD group and the controls without PD that were evaluated in the second study of my doctoral research. The mean serum LBP levels were lower in the sporadic PD group than in the familial PD group (6.82 mcg/ml versus 12.50 mcg/ml), but they were also lower in the familial PD group compared to the controls without PD (12.5 mcg/ml versus 17.28 mcg/ml). The mean serum levels of zonulin were similar in the sporadic PD group and the familial PD group (26.6 ng/ml versus 27.78 ng/ml), being somewhat higher than in the controls without PD (21.5 ng/ml). The mean fecal levels of zonulin were higher in the sporadic PD group compared to the familial PD (94.72 ng/ml versus 25.86 ng/ml); interestingly they were somewhat lower in the familial PD group than in the controls without PD (25.86 ng/ml versus 38.6 ng/ml). The mean levels of serum calprotectin were highest in the sporadic PD group and lowest in the controls without PD (13.77 mcg/ml versus 10.38 mcg/ml versus 6.88 mcg/ml). The mean levels of fecal calprotectin were also the highest in the sporadic PD group, and lowest in the controls without PD, with a trend towards higher values in the familial PD group compared to the controls without PD (187.16 mcg versus 145.92 mcg/ml versus 57.94 mcg/ml).

In this study I found higher levels of serum LPS and fecal zonulin and lower levels of serum LBP in the patients with sporadic probably multifactorial PD compared to controls with early-onset familial PD. The results of the study suggest that serum LPS levels are low in early-onset familial PD (i.e., probably monogenic), arguing against a strong involvement of endotoxin exposure in this group of patients – however, in the presence of pathogenic gene

variants, lower amounts of LPS could be needed. Noteworthy, a trend towards higher gastrointestinal inflammation markers, especially fecal calprotectin, is also found in the early-onset familial PD group, suggesting that gastrointestinal inflammation might also occur in monogenic PD (either as contributor to the pathology, or as consequence, or both).

7. Concluding Remarks and Summary of the Personal Contributions

7.1. Concluding Remarks

My doctoral research includes three studies aiming to better characterize the relations between bacterial endotoxin exposure (LPS being, among others, amyloidogenic), intestinal barrier permeability, and gastrointestinal inflammation in sporadic PD.

My main results are in line with the available literature.

Despite the obvious limitations of my studies, mainly related to the small sample size, the cross-sectional nature of the design, and the debatable sensitivity and specificity of the laboratory markers for bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation, my doctoral research adds to the evidence that chronic endotoxin exposure, intestinal barrier dysfunction, and gastrointestinal inflammation occur in sporadic multifactorial PD, and might play a role in its development and/or progression. The coexistence of an amyloidogenic microbiota with intestinal barrier dysfunction and gastrointestinal inflammation could create a gut environment that might trigger or promote the development of body(gut)-first sporadic multifactorial PD in genetically susceptible individuals.

Further studies on larger prospective cohorts, are needed to confirm and expand the current results, and to find the best means for translating these results into the clinical practice. These studies should be focused multimodal evaluation of the gut microbiome, intestinal barrier, and gastrointestinal inflammation.

7.2. Summary of the Personal Contributions

My doctoral research contributes to a better understanding of the relations between bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation in sporadic PD, as follows:

1. The first study (Chapter 4) provides the results of the first and only available study to date on the correlations between peripheral inflammation markers routinely tested in clinical practice (which are cheap and readily available), and laboratory markers of bacterial endotoxin exposure (i.e., serum LPS and LBP), intestinal barrier permeability (i.e., serum and fecal zonulin), and gastrointestinal inflammation (i.e., serum and fecal calprotectin). In this study I

found that serum beta2-microglobulin and MLR correlate directly with the levels of serum calprotectin. These correlations were not reported to date but are biologically plausible.

2. The second study (Chapter 5) provides the results of the first and only available study to date that evaluates the laboratory markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation in patients with sporadic probably multifactorial PD compared to controls without PD. In this study I found that serum LBP is significantly lower in patients with sporadic probably multifactorial PD than in controls without PD, despite similar levels of serum LPS. Lower LBP levels have been consistently reported in sporadic PD, possibly reflecting higher chronic exposure to LPS or an altered response to LPS. Concurrently, I found that serum and fecal levels of zonulin and calprotectin are significantly higher in patients with sporadic probably multifactorial PD compared to controls without PD, indicating the coexistence of intestinal barrier dysfunction and gastrointestinal inflammation. The multiple binary logistic regression models I performed showed that higher levels of LBP are significantly associated with a lower likelihood of sporadic PD, while higher fecal levels of zonulin and calprotectin are significantly associated with higher likelihoods of sporadic PD.

3. The third study (Chapter 6) provides the results of the first and only study available to date that evaluates the laboratory markers of bacterial endotoxin exposure, intestinal barrier permeability, and gastrointestinal inflammation in patients with sporadic probably multifactorial PD compared to controls with early-onset familial PD (i.e., probably monogenic PD). The sample size of the control group was very small, but the results of the study suggest that further evaluation of fecal and serum calprotectin and bacterial endotoxin exposure markers is warranted in patients with monogenic PD compared to controls with sporadic multifactorial PD and controls without PD.

Selected References

Aho, V.T.E., Houser, M.C., Pereira, P.A.B., Chang, J., Rudi, K., Paulin, L., *et al.* (2021). Relationships of gut microbiota, short-chain fatty acids, inflammation, and the gut barrier in Parkinson's disease. Mol Neurodegener *16*, 6.

Ajamian, M., Steer, D., Rosella, G., and Gibson, P.R. (2019). Serum zonulin as a marker of intestinal mucosal barrier function: May not be what it seems. PLoS One *14*, e0210728.

Ascherio, A., and Schwarzschild, M.A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. Lancet Neurol *15*, 1257-1272.

Augustin, A., Guennec, A.L., Umamahesan, C., Kendler-Rhodes, A., Tucker, R.M., Chekmeneva, E., *et al.* (2023). Faecal metabolite deficit, gut inflammation and diet in Parkinson's disease: Integrative analysis indicates inflammatory response syndrome. Clin Transl Med *13*, e1152.

Balestrino, R., and Schapira, A.H.V. (2020). Parkinson disease. European journal of neurology *27*, 27-42.

Baldini, F., Hertel, J., Sandt, E., Thinnes, C.C., Neuberger-Castillo, L., Pavelka, L., *et al.* (2020). Parkinson's disease-associated alterations of the gut microbiome predict disease-relevant changes in metabolic functions. BMC Biol *18*, 62.

Bedarf, J.R., Hildebrand, F., Coelho, L.P., Sunagawa, S., Bahram, M., Goeser, F., *et al.* (2017). Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naive Parkinson's disease patients. Genome Med *9*, 39.

Berg, D., Postuma, R.B., Bloem, B., Chan, P., Dubois, B., Gasser, T., *et al.* (2014). Time to redefine PD? Introductory statement of the MDS Task Force on the definition of Parkinson's disease. Mov Disord *29*, 454-462.

Berg, D., Postuma, R.B., Adler, C.H., Bloem, B.R., Chan, P., Dubois, B., *et al.* (2015). MDS research criteria for prodromal Parkinson's disease. Mov Disord *30*, 1600-1611.

Bhattacharyya, D., and Bhunia, A. (2021). Gut-Brain axis in Parkinson's disease etiology: The role of lipopolysaccharide. Chem Phys Lipids *235*, 105029.

Boncuk Ulas, S., Guzey Aras, Y., Irmak Gozukara, S., Acar, T., and Acar, B.A. (2023). Correlates of Zonulin and Claudin-5, markers of intestinal and brain endothelial permeability, in Parkinson's Disease: A pilot study. Parkinsonism Relat Disord *110*, 105361.

Borghammer, P. (2021). The alpha-Synuclein Origin and Connectome Model (SOC Model) of Parkinson's Disease: Explaining Motor Asymmetry, Non-Motor Phenotypes, and Cognitive Decline. J Parkinsons Dis *11*, 455-474.

Borghammer, P., and Van Den Berge, N. (2019). Brain-First versus Gut-First Parkinson's Disease: A Hypothesis. J Parkinsons Dis *9*, S281-S295.

Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., and Braak, E. (2003a). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging *24*, 197-211.

Braak, H., and Del Tredici, K. (2017). Neuropathological Staging of Brain Pathology in Sporadic Parkinson's disease: Separating the Wheat from the Chaff. J Parkinsons Dis *7*, S71-S85.

Braak, H., de Vos, R.A., Bohl, J., and Del Tredici, K. (2006). Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neurosci Lett *396*, 67-72.

Brown, G.C. (2019). The endotoxin hypothesis of neurodegeneration. J Neuroinflammation *16*, 180.

Brown, G.C., Camacho, M., and Williams-Gray, C.H. (2023). The Endotoxin Hypothesis of Parkinson's Disease. Mov Disord *38*, 1143-1155.

Chen, C.K., Huang, J.Y., Wu, Y.T., and Chang, Y.C. (2018). Dental Scaling Decreases the Risk of Parkinson's Disease: A Nationwide Population-Based Nested Case-Control Study. Int J Environ Res Public Health *15*.

Chen, C.K., Wu, Y.T., and Chang, Y.C. (2017). Periodontal inflammatory disease is associated with the risk of Parkinson's disease: a population-based retrospective matched-cohort study. PeerJ *5*, e3647.

Chen, S.J., Chi, Y.C., Ho, C.H., Yang, W.S., and Lin, C.H. (2021). Plasma Lipopolysaccharide-Binding Protein Reflects Risk and Progression of Parkinson's Disease. J Parkinsons Dis *11*, 1129-1139.

Cirstea, M.S., Yu, A.C., Golz, E., Sundvick, K., Kliger, D., Radisavljevic, N., *et al.* (2020). Microbiota Composition and Metabolism Are Associated With Gut Function in Parkinson's Disease. Mov Disord *35*, 1208-1217.

Citronberg, J.S., Wilkens, L.R., Lim, U., Hullar, M.A., White, E., Newcomb, P.A., *et al.* (2016). Reliability of plasma lipopolysaccharide-binding protein (LBP) from repeated measures in healthy adults. Cancer Causes Control *27*, 1163-1166.

Clunn, G., Hughes, A., and Schachter, M. (2000). Endotoxin-lipoprotein hypothesis. Lancet *356*, 2097-2098

Coughlin, D.G., Petrovitch, H., White, L.R., Noorigian, J., Masaki, K.H., Ross, G.W., *et al.* (2019). Most cases with Lewy pathology in a population-based cohort adhere to the Braak progression pattern but 'failure to fit' is highly dependent on staging system applied. Parkinsonism Relat Disord *64*, 124-131.

de Waal, G.M., Engelbrecht, L., Davis, T., de Villiers, W.J.S., Kell, D.B., and Pretorius, E. (2018). Correlative Light-Electron Microscopy detects lipopolysaccharide and its association with fibrin fibres in Parkinson's Disease, Alzheimer's Disease and Type 2 Diabetes Mellitus. Sci Rep *8*, 16798.

Deng, I., Corrigan, F., Zhai, G., Zhou, X.F., and Bobrovskaya, L. (2020). Lipopolysaccharide animal models of Parkinson's disease: Recent progress and relevance to clinical disease. Brain Behav Immun Health *4*, 100060.

Devos, D., Lebouvier, T., Lardeux, B., Biraud, M., Rouaud, T., Pouclet, H., *et al.* (2013). Colonic inflammation in Parkinson's disease. Neurobiol Dis *50*, 42-48.

Dhaliwal, A., Zeino, Z., Tomkins, C., Cheung, M., Nwokolo, C., Smith, S., *et al.* (2015). Utility of faecal calprotectin in inflammatory bowel disease (IBD): what cut-offs should we apply? Frontline Gastroenterol *6*, 14-19.

Diamanti, A., Panetta, F., Basso, M.S., Forgione, A., Colistro, F., Bracci, F., *et al.* (2010). Diagnostic work-up of inflammatory bowel disease in children: the role of calprotectin assay. Inflamm Bowel Dis *16*, 1926-1930.

Ding, P.H., and Jin, L.J. (2014). The role of lipopolysaccharide-binding protein in innate immunity: a revisit and its relevance to oral/periodontal health. J Periodontal Res *49*, 1-9.

Dumitrescu, L., Marta, D., Danau, A., Lefter, A., Tulba, D., Cozma, L., *et al.* (2021). Serum and Fecal Markers of Intestinal Inflammation and Intestinal Barrier Permeability Are Elevated in Parkinson's Disease. Front Neurosci *15*, 689723.

Dumitrescu, L., Popescu-Olaru, I., Cozma, L., Tulba, D., Hinescu, M.E., Ceafalan, L.C., *et al.* (2018). Oxidative Stress and the Microbiota-Gut-Brain Axis. Oxid Med Cell Longev *2018*, 2406594.

Dutta, G., Zhang, P., and Liu, B. (2008). The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. Fundam Clin Pharmacol *22*, 453-464.

Erridge, C., Attina, T., Spickett, C.M., and Webb, D.J. (2007). A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr *86*, 1286-1292.

Fasano, A. (2012). Zonulin, regulation of tight junctions, and autoimmune diseases. Ann N Y Acad Sci *1258*, 25-33.

Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A. (2007). G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods *39*, 175-191.

Forsyth, C.B., Shannon, K.M., Kordower, J.H., Voigt, R.M., Shaikh, M., Jaglin, J.A., *et al.* (2011). Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. PLoS One *6*, e28032.

Gao, H.M., Zhang, F., Zhou, H., Kam, W., Wilson, B., and Hong, J.S. (2011a). Neuroinflammation and alpha-synuclein dysfunction potentiate each other, driving chronic progression of neurodegeneration in a mouse model of Parkinson's disease. Environ Health Perspect *119*, 807-814.

Gonzalez-Quintela, A., Alonso, M., Campos, J., Vizcaino, L., Loidi, L., and Gude, F. (2013). Determinants of serum concentrations of lipopolysaccharide-binding protein (LBP) in the adult population: the role of obesity. PLoS One *8*, e54600.

Gorecki, A.M., Preskey, L., Bakeberg, M.C., Kenna, J.E., Gildenhuys, C., MacDougall, G., *et al.* (2019). Altered Gut Microbiome in Parkinson's Disease and the Influence of Lipopolysaccharide in a Human alpha-Synuclein Over-Expressing Mouse Model. Front Neurosci *13*, 839.

Grand, A., Rochette, E., Dutheil, F., Gozal, D., Calcaterra, V., Berni Canani, R., *et al.* (2020). Body Mass Index and Calprotectin Blood Level Correlation in Healthy Children: An Individual Patient Data Meta-Analysis. J Clin Med *9*.

Gutsmann, T., Muller, M., Carroll, S.F., MacKenzie, R.C., Wiese, A., and Seydel, U. (2001). Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. Infect Immun *69*, 6942-6950.

Hasegawa, S., Goto, S., Tsuji, H., Okuno, T., Asahara, T., Nomoto, K., *et al.* (2015). Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. PLoS One *10*, e0142164.

Hawkes, C.H., Del Tredici, K., and Braak, H. (2007). Parkinson's disease: a dual-hit hypothesis. Neuropathol Appl Neurobiol *33*, 599-614.

Hawkes, C.H., Del Tredici, K., and Braak, H. (2009). Parkinson's disease: the dual hit theory revisited. Ann N Y Acad Sci *1170*, 615-622.

Heintz-Buschart, A., Pandey, U., Wicke, T., Sixel-Doring, F., Janzen, A., Sittig-Wiegand, E., *et al.* (2018). The nasal and gut microbiome in Parkinson's disease and idiopathic rapid eye movement sleep behavior disorder. Mov Disord *33*, 88-98.

Heinzel, S., Berg, D., Gasser, T., Chen, H., Yao, C., Postuma, R.B., *et al.* (2019). Update of the MDS research criteria for prodromal Parkinson's disease. Mov Disord *34*, 1464-1470.

Hersoug, L.G., Moller, P., and Loft, S. (2016). Gut microbiota-derived lipopolysaccharide uptake and trafficking to adipose tissue: implications for inflammation and obesity. Obes Rev *17*, 297-312.

Hill-Burns, E.M., Debelius, J.W., Morton, J.T., Wissemann, W.T., Lewis, M.R., Wallen, Z.D., *et al.* (2017). Parkinson's disease and Parkinson's disease medications have distinct signatures of the gut microbiome. Mov Disord *32*, 739-749.

Hor, J.W., Lim, S.Y., Khor, E.S., Chong, K.K., Song, S.L., Ibrahim, N.M., *et al.* (2022). Fecal Calprotectin in Parkinson's Disease and Multiple System Atrophy. J Mov Disord *15*, 106-114.

Horsager, J., Andersen, K.B., Knudsen, K., Skjaerbaek, C., Fedorova, T.D., Okkels, N., *et al.* (2020). Brain-first versus body-first Parkinson's disease: a multimodal imaging case-control study. Brain *143*, 3077-3088.

Horsager, J., Knudsen, K., and Sommerauer, M. (2022). Clinical and imaging evidence of brain-first and body-first Parkinson's disease. Neurobiol Dis *164*, 105626.

Houser, M.C., Chang, J., Factor, S.A., Molho, E.S., Zabetian, C.P., Hill-Burns, E.M., *et al.* (2018). Stool Immune Profiles Evince Gastrointestinal Inflammation in Parkinson's Disease. Mov Disord *33*, 793-804.

Houser, M.C., and Tansey, M.G. (2017). The gut-brain axis: is intestinal inflammation a silent driver of Parkinson's disease pathogenesis? NPJ Parkinsons Dis *3*, 3.

Jensen, M.D., Kjeldsen, J., and Nathan, T. (2011). Fecal calprotectin is equally sensitive in Crohn's disease affecting the small bowel and colon. Scand J Gastroenterol *46*, 694-700.

Kelly, L.P., Carvey, P.M., Keshavarzian, A., Shannon, K.M., Shaikh, M., Bakay, R.A., *et al.* (2014). Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. Mov Disord *29*, 999-1009.

Khaki-Khatibi, F., Qujeq, D., Kashifard, M., Moein, S., Maniati, M., and Vaghari-Tabari, M. (2020). Calprotectin in inflammatory bowel disease. Clin Chim Acta *510*, 556-565.

Kitabatake, H., Tanaka, N., Fujimori, N., Komatsu, M., Okubo, A., Kakegawa, K., *et al.* (2017). Association between endotoxemia and histological features of nonalcoholic fatty liver disease. World J Gastroenterol *23*, 712-722.

Kowalski, K., and Mulak, A. (2019). Brain-Gut-Microbiota Axis in Alzheimer's Disease. J Neurogastroenterol Motil *25*, 48-60.

Lang, A.E., Siderowf, A.D., Macklin, E.A., Poewe, W., Brooks, D.J., Fernandez, H.H., *et al.* (2022). Trial of Cinpanemab in Early Parkinson's Disease. N Engl J Med *387*, 408-420.

Lange, J.H., Niehaus, I., and Thomulka, K.W. (2003). Is endotoxin an environmental cause of Parkinson's disease? Neuroepidemiology *22*, 313; author reply 314.

Lerner, A. (2022). The intestinal luminal sources of alpha-synuclein: a gastroenterologist perspective. Nutr Rev *80*, 282-293.

Loffredo, L., Ettorre, E., Zicari, A.M., Inghilleri, M., Nocella, C., Perri, L., *et al.* (2020). Oxidative Stress and Gut-Derived Lipopolysaccharides in Neurodegenerative Disease: Role of NOX2. Oxid Med Cell Longev *2020*, 8630275.

Manole, E.[#], **Dumitrescu, L**.[#], Niculițe, C., Popescu, B.-O., and Ceafalan, L.-C. (2021). Potential roles of functional bacterial amyloid proteins, bacterial biosurfactants and other putative gut microbiota products in the etiopathogeny of Parkinson’s Disease. BIOCELL *45*, 1--16.

Martin, T.R., Mathison, J.C., Tobias, P.S., Leturcq, D.J., Moriarty, A.M., Maunder, R.J., *et al.* (1992). Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. Implications for cytokine production in normal and injured lungs. J Clin Invest *90*, 2209-2219.

Massier, L., Chakaroun, R., Kovacs, P., and Heiker, J.T. (2021). Blurring the picture in leaky gut research: how shortcomings of zonulin as a biomarker mislead the field of intestinal permeability. Gut *70*, 1801-1802.

Meng, L., Song, Z., Liu, A., Dahmen, U., Yang, X., and Fang, H. (2021). Effects of Lipopolysaccharide-Binding Protein (LBP) Single Nucleotide Polymorphism (SNP) in Infections, Inflammatory Diseases, Metabolic Disorders and Cancers. Front Immunol *12*, 681810.

Mezey, E., Dehejia, A.M., Harta, G., Tresser, N., Suchy, S.F., Nussbaum, R.L., *et al.* (1998). Alpha synuclein is present in Lewy bodies in sporadic Parkinson's disease. Mol Psychiatry *3*, 493-499.

Moein, S., Qujeq, D., Vaghari Tabari, M., Kashifard, M., and Hajian-Tilaki, K. (2017). Diagnostic accuracy of fecal calprotectin in assessing the severity of inflammatory bowel disease: From laboratory to clinic. Caspian J Intern Med *8*, 178-182.

Morkl, S., Lackner, S., Meinitzer, A., Mangge, H., Lehofer, M., Halwachs, B., *et al.* (2018). Gut microbiota, dietary intakes and intestinal permeability reflected by serum zonulin in women. Eur J Nutr *57*, 2985-2997.

Mortensen, O.H., Nielsen, A.R., Erikstrup, C., Plomgaard, P., Fischer, C.P., Krogh-Madsen, R., *et al.* (2009). Calprotectin--a novel marker of obesity. PLoS One *4*, e7419.

Mulak, A., Koszewicz, M., Panek-Jeziorna, M., Koziorowska-Gawron, E., and Budrewicz, S. (2019). Fecal Calprotectin as a Marker of the Gut Immune System Activation Is Elevated in Parkinson's Disease. Front Neurosci *13*, 992.

Niehaus, I., and Lange, J.H. (2003). Endotoxin: is it an environmental factor in the cause of Parkinson's disease? Occup Environ Med *60*, 378.

Nishiwaki, H., Ito, M., Ishida, T., Hamaguchi, T., Maeda, T., Kashihara, K., *et al.* (2020). Meta-Analysis of Gut Dysbiosis in Parkinson's Disease. Mov Disord *35*, 1626-1635.

Nuzum, N.D., Loughman, A., Szymlek-Gay, E.A., Hendy, A., Teo, W.P., and Macpherson, H. (2020). Gut microbiota differences between healthy older adults and

individuals with Parkinson's disease: A systematic review. Neurosci Biobehav Rev *112*, 227-241.

Ohlsson, B., Orho-Melander, M., and Nilsson, P.M. (2017a). Higher Levels of Serum Zonulin May Rather Be Associated with Increased Risk of Obesity and Hyperlipidemia, Than with Gastrointestinal Symptoms or Disease Manifestations. Int J Mol Sci *18*.

Ohlsson, B., Roth, B., Larsson, E., and Hoglund, P. (2017b). Calprotectin in serum and zonulin in serum and feces are elevated after introduction of a diet with lower carbohydrate content and higher fiber, fat and protein contents. Biomed Rep *6*, 411-422.

Olsen, I., Kell, D.B., and Pretorius, E. (2020). Is Porphyromonas gingivalis involved in Parkinson's disease? Eur J Clin Microbiol Infect Dis *39*, 2013-2018.

Pagano, G., Taylor, K.I., Anzures-Cabrera, J., Marchesi, M., Simuni, T., Marek, K., *et al.* (2022). Trial of Prasinezumab in Early-Stage Parkinson's Disease. N Engl J Med *387*, 421-432.

Pal, G.D., Shaikh, M., Forsyth, C.B., Ouyang, B., Keshavarzian, A., and Shannon, K.M. (2015). Abnormal lipopolysaccharide binding protein as marker of gastrointestinal inflammation in Parkinson disease. Front Neurosci *9*, 306.

Perez-Pardo, P., Dodiya, H.B., Engen, P.A., Forsyth, C.B., Huschens, A.M., Shaikh, M., *et al.* (2019). Role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice. Gut *68*, 829-843.

Postuma, R.B., Berg, D., Stern, M., Poewe, W., Olanow, C.W., Oertel, W., *et al.* (2015). MDS clinical diagnostic criteria for Parkinson's disease. Mov Disord *30*, 1591-1601.

Raetz, C.R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. Annu Rev Biochem *71*, 635-700.

Sampson, T.R., Debelius, J.W., Thron, T., Janssen, S., Shastri, G.G., Ilhan, Z.E., *et al.* (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell *167*, 1469-1480 e1412.

Sapone, A., de Magistris, L., Pietzak, M., Clemente, M.G., Tripathi, A., Cucca, F., *et al.* (2006). Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes *55*, 1443-1449.

Scheperjans, F. (2016). Gut microbiota, 1013 new pieces in the Parkinson's disease puzzle. Curr Opin Neurol *29*, 773-780.

Scheperjans, F., Aho, V., Pereira, P.A., Koskinen, K., Paulin, L., Pekkonen, E., *et al.* (2015). Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord *30*, 350-358.

Schwiertz, A., Spiegel, J., Dillmann, U., Grundmann, D., Burmann, J., Fassbender, K., *et al.* (2018). Fecal markers of intestinal inflammation and intestinal permeability are elevated in Parkinson's disease. Parkinsonism Relat Disord *50*, 104-107.

Schumann, R.R. (2011). Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. Biochem Soc Trans *39*, 989-993.

Seethaler, B., Basrai, M., Neyrinck, A.M., Nazare, J.A., Walter, J., Delzenne, N.M., *et al.* (2021). Biomarkers for assessment of intestinal permeability in clinical practice. Am J Physiol Gastrointest Liver Physiol.

Shannon, K.M. (2022). Gut-Derived Sterile Inflammation and Parkinson's Disease. Front Neurol *13*, 831090.

Talley, N.J., Holtmann, G.J., Jones, M., Koloski, N.A., Walker, M.M., Burns, G., *et al.* (2020). Zonulin in serum as a biomarker fails to identify the IBS, functional dyspepsia and non-coeliac wheat sensitivity. Gut *69*, 1-3.

Vatanen, T., Kostic, A.D., d'Hennezel, E., Siljander, H., Franzosa, E.A., Yassour, M., *et al.* (2016). Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. Cell *165*, 1551.

Walsham, N.E., and Sherwood, R.A. (2016). Fecal calprotectin in inflammatory bowel disease. Clin Exp Gastroenterol *9*, 21-29.

Weis, S., Schwiertz, A., Unger, M.M., Becker, A., Fassbender, K., Ratering, S., *et al.* (2019). Effect of Parkinson's disease and related medications on the composition of the fecal bacterial microbiota. NPJ Parkinsons Dis *5*, 28.

WHO (2023). World Health Organisation. Parkinson disease. https://www.who.int/news-room/fact-sheets/detail/parkinson-disease. Last update 13.06.2022. Access Date: 19.02.2023.

Wijeyekoon, R.S., Kronenberg-Versteeg, D., Scott, K.M., Hayat, S., Kuan, W.L., Evans, J.R., *et al.* (2020). Peripheral innate immune and bacterial signals relate to clinical heterogeneity in Parkinson's disease. Brain Behav Immun *87*, 473-488.

Yan, Z., Yang, F., Cao, J., Ding, W., Yan, S., Shi, W., *et al.* (2021). Alterations of gut microbiota and metabolome with Parkinson's disease. Microb Pathog *160*, 105187.

Yao, Z., Mates, J.M., Cheplowitz, A.M., Hammer, L.P., Maiseyeu, A., Phillips, G.S., *et al.* (2016). Blood-Borne Lipopolysaccharide Is Rapidly Eliminated by Liver Sinusoidal Endothelial Cells via High-Density Lipoprotein. J Immunol *197*, 2390-2399.

Zhao, Y., Walker, D.I., Lill, C.M., Bloem, B.R., Darweesh, S.K.L., Pinto-Pacheco, B., *et al.* (2023). Lipopolysaccharide-binding protein and future Parkinson's disease risk: a European prospective cohort. J Neuroinflammation *20*, 170.