UNIVERSITY OF TLEMCEN – ABOU-BEKR BELKAID

SNV-STU FACULTY – BIOLOGY DEPARTMENT

LABORATORY OF APPLIED MOLECULAR BIOLOGY AND IMMUNOLOGY - BIOMOLIM

TLEMCEN

N° of Order

Thesis

Presented for obtaining the degree of

Master in Biological Sciences

Immunology specialty

By:

Soumeya BENAMAR

Thesis defended on JUNE 29, 2022

Title:

Exploration of neutrophil respiratory burst and NETosis in patients with Parkinson's disease

Under the supervision of Professor Mourad ARIBI

Dr. El MEZOUAR Chahrazed	**MAA**	University of Tlemcen, Algeria	President
Pr. ARIBI Mourad	**Professor**	University of Tlemcen, Algeria	Dir. of Thesis
Dr. DJALTI Farah	**MCB**	University of Tlemcen, Algeria	Examiner

Juin29, 2022

Abstract

Introduction :
Parkinson disease (PD) is a progressive neurodegenerative disease that affects peripheral organs as well as the central nervous system and is widely associated with neuroinflammation process, Neutrophils are myeloidinnate immune cells that are qualified as professional phagocytes and play a pivotal role in inflammatory responses.

Materiels et methodes :oxydative burst biomarkers (NO, H2O2), Netosis Formation based on MPO activity, and phagocytosis were determinated on serum and cell culture

Results:we have shown a remarkable downregulation of NETosis mediated MPO activity, as well as oxidative burst activity mediated NO production and H2O2, nevertheless, we observerd an upregulation of phagocytosis.

Conclusions:
Parkinson disease (PD) is an inflammatory disorder which has shown a remarkable downregylation of both Netosis and oxidative burst activity, while induced phagocytosis.

Key words: NETosis, Phagocytosis, RespiratoryBurst, Neutrophile, inflammation desoerder,Parkinson disease.

Résumé

Introduction :
La maladie de Parkinson (PD) est une maladie neurodégénérative progressive qui affecte les organes périphériques ainsi que le système nerveux central et est largement associée au processus de neuroinflammation. Les neutrophiles sont des cellules immunitaires myéloïdes qualifiées de phagocytes professionnels et jouent un rôle central dans les réponses inflammatoires.

Matériels et méthodes : des biomarqueurs de burst oxydatif (NO, H2O2), la formation de Netosis basée sur l'activité MPO et la phagocytose ont été déterminés sur sérum et culture cellulaire

Résultats : nous avons montré une régulation à la baisse remarquable de l'activité MPO médiée par NETosis, ainsi qu'une activité de rafale oxydative médiée par la production de NO et de H2O2, néanmoins, nous avons observé une régulation à la hausse de la phagocytose.

Conclusion :
La maladie de Parkinson (MP) est un trouble inflammatoire qui a montré une remarquable régulation à la baisse de l'activité de la nitose et de la rafale oxydative, tout en provoquant une phagocytose.

Mots clés :NETosis, Phagocytose, RespiratoryBurst, Neutrophile, inflammation desoerder,Parkinson disease.

Acknowledgements

First of all I thank my god Allah for everything he gave me in this life, and I deeply wish he accept it as I did it for his sack only.

This work is a result of a Master training for the years 2020/2022 in the Laboratory of Applied Molecular Biology and Immunology (BIOMOLIM W0414100, University of Tlemcen), under the direction and the supervision of Professor ARIBI Mourad. Warmly I thank him, for the professional quality of the training I received and for accompanying and helping me tirelessly throughout this period, with constant availability and endless benevolence despite the responsibilities entrusted to him.

I would like to thank so much the members of the defense: Dr. Chahrazed EL MEZOUAR ,Dr.Farah DJALTI and Pr Mourad ARIBI for accepting to evaluate the work of this master thesis. Thank you for your interest in it, and for your time.

At the same time, I would like to express my warm thanks to all members of the laboratory of BIOMOLIM, especially Sara Dahou, RabiaMessali ,Aida ,Nour el houda , Zakaria, And without forgetting the trainees Lamis, Ramadan , Rania, Ikram, Ghizelane , Nadir,Houda,Zoukha and Amina You gave me a lot, and each of you left an imprint on me.

Many thanks to all my teachers at different levels, especially those who have left positive marks on me. I especially mention my teacher DJAFOUR Fouzia because she taught me how to be me.

Thanks to the promotion of immunology 2020/2022, thanks to our team Houria, Djihane ,Farah, Amine, Sara , Riyad, Iram,Zineb,Ahlem,Amina

Thanks to professors, doctors and health coworkers especially to Pr.Bouchnak who give us the access to the neurology department in the hospital of Tlemcen. And thanks to Wahiba and Abdelnour.

Special thanks to my online friends Leoned, Mark, Rick, Atika, Aslania, Biyanie and Arif.

DEDICATIONS

This work and all my future works are the fruit of my dear beloved parents. This is for their love, support and Encouraged in the most difficult times.

To my grandmother “mima” may Allah rest her soul in peace, I’ll achieve what you always wanted me to achieve one day.

To those who gave me everything with love Mohamed el amine, Djaoued, Sid ahmed, Oussama and Ziyade my dearest brothers.

To my sisters in law, my nephews and nieces that spread extra joy in my life.

To all my family aunties, anculs and cousins.

To my soul mates Houia and Abir .

This work is dedicated to all Parkinson’s patients.

Finally I would like to thank myself for being strong and I would say this is just the beginning.

Table of contents

List of Figures

List of Abbreviations

B

Biomolim *Applied Molecular Biology and Immunology*
BM *bone marrow*

C

Ca^{2+} *L'ion de Calcium*
CFU *Colony Forming Units*
CMP *common myeloid progenitor lineage*
CRP *C reactive protein*

D

D1 *direct pathway*
D2 *indirect pathway*
DA Dopaminergic
DCs *Dendritic cells*
DNA *acide désoxyribonucléique*

G

G-CSF *granulocyte-colony stimulating factor*
GM-CSF *granulocyte-monocyte colony-stimulating factor*
GMP *common granulocyte-monocyte progenitor*

H

H2O2 *Hydrogen peroxide*
HOCl *hypochlorous acid*

I

IL-8 *Interleukin 8*
iNOS *inductibleNOS*

L

LB Lymphocytes B
LRRK2 Leucinerich repeat kinase-2

M

MPO *myeloperoxidase*
MSA *Mannitol Salt Agar*

N

NaBr *sodium bromide*
NADPH *Nicotinamide adénine dinucléotide phosphate*
$NaNO2^{-}$*sodium nitrite*
NBT *Nitro-blue tetrazolium*

NCF1 *neutrophil cytosolic factor 1*
NETs *Neutrophil extracellular traps*
NLRs *intracellular receptors such as NOD-like receptors*
NO *Nitric Oxide*
NOx *nitrite and nitrate*

O

OXPHOS *oxidative phosporylation*

P

PAB *buffer for peroxide*
PAD4 *peptidyl arginine deaminase 4*
PD *Parkinson's disease*
PMN *Polymorphonuclear Neutrophils*
PMNs *Polymorphonuclear neutrophils*
PRRs *pattern recognition receptors*
PRS *Phenol Red Buffered Solution*

R

RNS *nitrogen species*
ROS reactive oxygen species

S

SN *Neurvous System*
SNc substantia nigra pars compacta
SNC *Central Nervous System*

T

TCA *trichloroacetic acid*
TLRs *Toll-like receptors*
TNF Tumor Necrosis Factor

Introduction

Parkinson's disease (PD) is an idiopathic disease of the nervous system characterized by both motor and non-motor system manifestations, which affect elderly people(Beitz, 2014). It is the second most common neurodegenerative disease (Sherer et al., 2012). Resulting from progressive loss of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and the presence of alpha-synuclein positive cytoplasmic inclusions, termed Lewy bodies, in surviving neurons(Heller et al., 2014). Recent studies shown that aging itself and environmental stress may promote the development of Parkinson's disease(Beitz, 2014). Although the majority of the diagnosed PD does not arise from genetic mutations, there is growing appreciation that specific genetic variants in various genes expressed in immune cells can modify risk for the development of PD. In particular, mutations in the gene encoding Leucinerich repeat kinase-2 (LRRK2) are the most common cause of familial PD. (Hernandez et al., 2016).

Parkinson's disease like other disease induce activation of innate and adaptative immune system responses, indeed the process that link the immune system with the PD remain unclear (Tan et al., 2020). Many studies shown that Tumor Necrosis Factor (TNF) expression in SN from patients and the presence of elevated pro-inflammatory cytokines in brain and cerebrospinal fluid prompted the "inflammation hypothesis of neurodegeneration" in PD(Tansey and Romero-Ramos, 2019). And the excess accumulation of reactive oxygen species (ROS) in neurons takes part in the process and might even initiate it(Vitte et al., 2004). As we know the major source of ROS in the organism are the electron transport chain of oxidative phosporylation (OXPHOS) in mitochondria and the NADPH oxidase of phagocytic cells including monocytes, macrophages and polymorphonuclear neutrophils (Vitte et al., 2004).

Polymorphonuclear Neutrophils (PMN) are a component of innate immune response, that are the first barrier of defense and its sole purpose was to release powerful oxidants and proteases in an attempt to kill pathogens(Kubes, 2018). In addition the polymorphonuclear neutrophils has a new effector function that utilize to kill pathogens which is the production of Neutrophil extracellular traps (NETs)(Brinkmann et al., 2004). However, it has been proposed that as a last gasp effort to try to kill pathogens, the neutrophil mixes its inner proteolytic contents together with its DNA, makes a home-made bomb and explodes, releasing a chromatin NET decorated with proteases. Of note polymorphonuclear neutrophils express many receptors involved in achieving their function, including expression of FPR1/2, FCgR, nAChR, CR3, C5aR (when IFN-β is used as a priming cytokine) in NETosis(Jorch and Kubes, 2017).

Literature review

1. Parkinson's disease (PD)

1.1. Definition

Parkinson disease is a chronic and progressive neurodegeneration, occur because of the death of dopaminergic neurons in the basal ganglia, a brain area that controls movement. Resulting a unintended movements. (Lindqvist et al., 2012)

1.2. History

Parkinson's was discovered decades ago, in AD 175 in western medicine it was described by the physician Galen as "Shaking palsy" (Obeso et al., 2017).This timeline regroups the most important events in the history of Parkinson's disease.

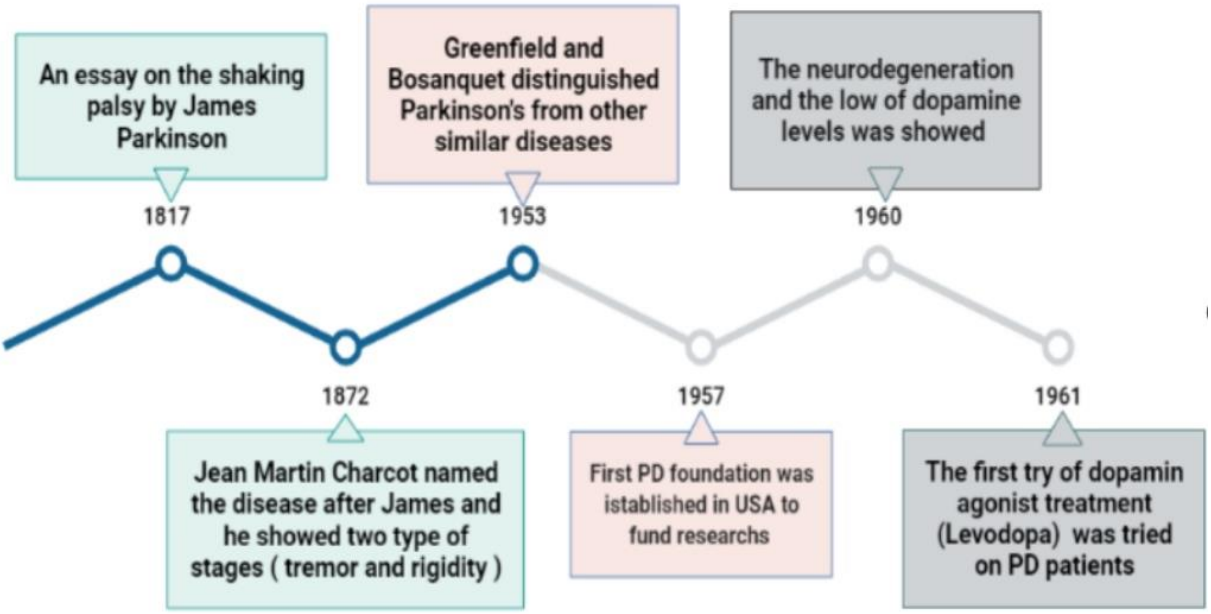

Figure 1 Time line of the most important events in the history of PD (Goetz, 2011)

1.3. Epidemiology

Parkinson's disease is one of the most common neurological disorders; it has an overall prevalence thought to be increasing, based on epidemiological studies in 2020 about 10 million people worldwide were living with PD (Prasad and Hung, 2021).

The onset of Parkinson's disease usually peaks between the ages of 55-65 years and generally has a slowly progressive onset.(Ou et al., 2021) and men are more susceptible than women with a prevalence ratio of approximately 3:2 (Tolosa et al., 2021)

1.4. Etiology

The etiology of PD is not completely clear, although there are some genetic causes like mutations in the PINK1, PARKIN or Alpha Synuclein Genes.

This genetic locus are modified environmentally (e.g., pesticides, water pollutants) or behaviorally (e.g., use of tobacco, coffee, exercise, or head trauma) have been found to have a role in the pathogenesis of Parkinson's disease in different populations. (Tansey et al., 2022)

In rare cases Parkinson symptoms might be caused by MPTP, a toxic impurity that can be found in recreational drug MPPP and DNA variants genes like LRRK2.(Rousseaux et al., 2017)

1.5. Diagnostic

Currently there are no laboratory tests to diagnose non-genetic cases of Parkinson's. Physicians diagnose the disease by a neurological examination and usually the diagnose is based on the clinical history of the patients (Tolosa et al., 2021).

1.6. Clinical aspect

Figure 2 Clinical features of PD and critical symptoms(Váradi, 2020)

1.7. Physiopathology of PD and implication of the immune system

As we already know innate and adaptive immune responses are intimately linked and studies showed the interaction of both of their compounds in the Central Nervous System (SNC) is

present in any contexts of autoimmunity, inflammation and the PD associated genes with immune cells.

The main process of all this is the cellular infiltration of (Lymphocytes B and T, Dendritic cells (DCs) among others. However the mechanism that link the immune system with the PD remain unclear (Tan et al., 2020).

- **A brief Anatomy**

The basal ganglia describe a series of cells (bodies grey matter) that are located together within the deep subcortical white matter of the brain.(Thau et al., 2021)

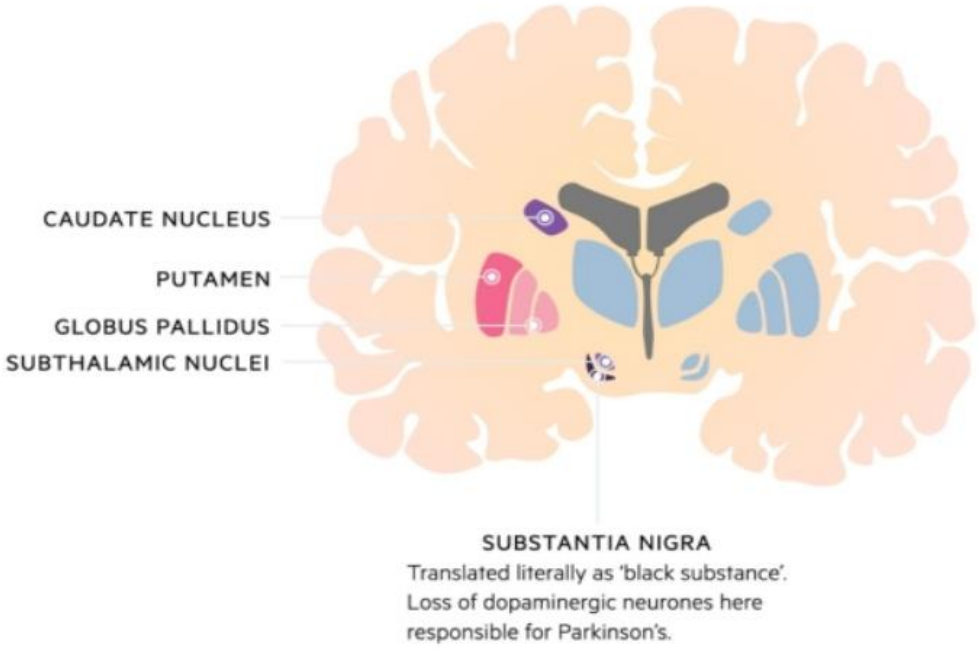

Figure 3 Abrief anatomy of the brain to show the Substantia Nigra localization

- **Two pathways must to mention**

Direct pathway

Mostly a stimulatory pathway (‘on’ pathway) that is shorter.

Activation of the direct pathway leads to a series of neural connections through the basal ganglia, which eventually leads to the initiation of movement. Dopamine that is released from the substantia nigra via dopaminergic neurons is able to activate the direct pathway via D1 receptors leading to the generation of movement.

Indirect pathway

An inhibitory pathway (‘off’ pathway) that is longer.

Activation of the indirect pathway is essential in the inhibition of muscular tone to prevent unnecessary movement. Dopamine that is released from the substantia nigra via dopaminergic neurons is able to inhibit the indirect pathway via D2 receptors therefore leading to the generation of movement. (MacMahonCopas et al., 2021)

1.8. Treatment

Medicines and surgical treatments can relieve some symptoms of PD patients by:

- Increasing the level of dopamine in the brain
- Helping control non-movement symptoms

The main therapy for Parkinson's is **Levodopa**, usually taken with **carbidopa** to reduce side effects.

An alternative therapy for Levodopa is the **Deep brain stimulation** which is a surgical procedure consisting of stimulating the brain to relieve symptoms (Prasad and Hung, 2021)

2. Polymorphonuclear neutrophils (PMNs)

2.1. Definition

Polymorphonuclear Neutrophils (PMN) also known by microphages or (neutrocytes ,heterophils) are a component of innate immune defense, that are the first barrier of defense to fight pathogens, cancer and autoimmune disease and immune deficiencies to maintain tissue homeostasis thanks to their high mobility.

They consist of the first present cells and granulocyte of the immune system at the infection site and are distinguished by their antimicrobial and antifungal potential, which is permitted by their cellular elements: antimicrobial peptides, neutrophil-specific proteolytic enzymes, in addition their production of ROS and neutrophil extracellular traps (NETs).(Quail et al., 2022)

Neutrophils are very abundant, they are the largest leukocyte type in the peripheral human blood , accounting from ~ 40 to 70% of the total white blood cells under normal conditions and ~10–20% in mice and they thrives in a neutral pH ~6.5 and 7.5.

The half-life of PMN is short, from 8 to 20 hours in circulation, although after migration into tissues, their life is notably extended to (1–4 days). It's been showed that the life span of PMN can be markedly elongated under inflammatory conditions, namely in the presence of granulocyte-colony stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), Interleukin 8 (IL-8), and C reactive protein (CRP), among other proinflammatory(Di Carlo et al., 2001).

2.2. Ontogeny-phylogeny

Neutrophils are born and differentiate within the bone marrow (BM) or continuously replenished by yielding short-lived cytotoxic cells whose decline and flow in the circulation and tissues where they meet the pathogene and die after several attacks, they are diurnally regulated.(Boes and Durham, 2017).

They are derived from a common myeloid progenitor lineage (CMP) through a common granulocyte-monocyte progenitor (GMP) . After that they assure subsistence stages which are: myeloblasts, promyelocytes, myelocytes, metamyelocytes (also called banded neutrophils or immature neutrophils) and mature neutrophils and then they get released into the blood circulation and then they move into the tissues (Lawrence et al., 2018)

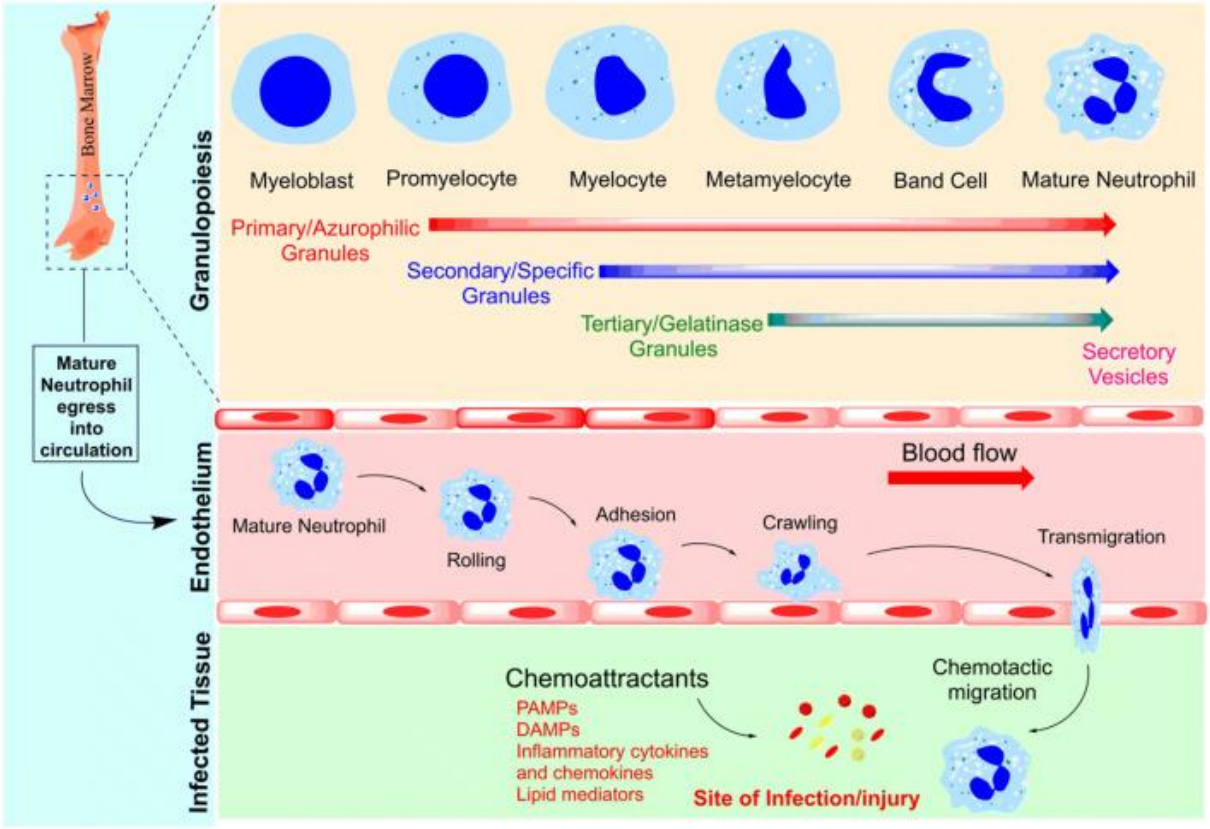

Figure 4 Ontogeny and Phylogeny of PMNs

The difference between the cellular transition stages of the maturation of PMN is the presence of granules, while The primary granules are common almost in all stages of development, thus secondary granules and secretary granules are more specific towards mature Neutrophils, without interacting with each other within the cell. (Rawat, Syeda, etShrivastava 2021)

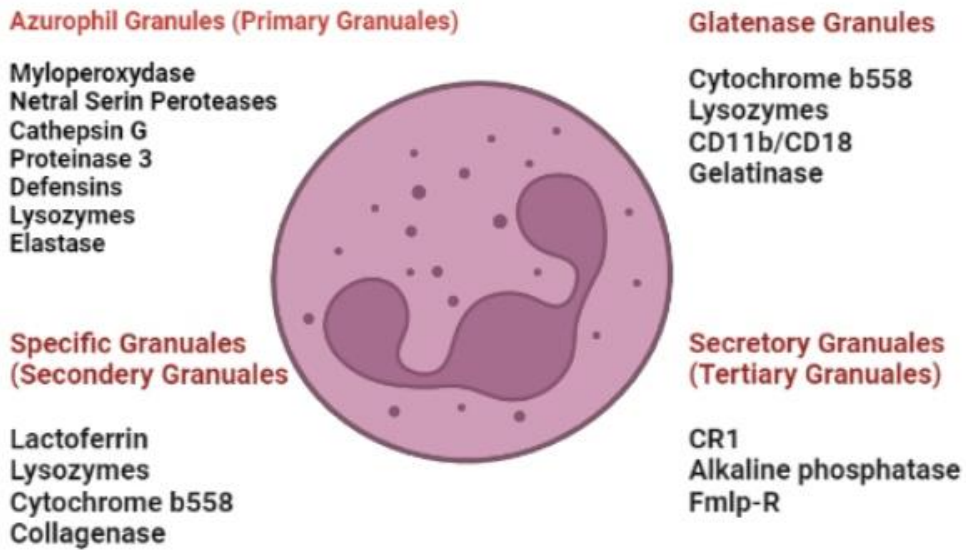

Figure 5 PMNs have at least three distinct granule subsets, (Rawat, Syeda, etShrivastava 2021)

2.3. Identification markers

PMN express a vast number of membrane receptors that allow them to recognize and eliminate infectious agents effectively and respond appropriately to microenvironmental stimuli that regulate neutrophil functions, such as activation, migration, generation of reactive oxygen species, formation of neutrophil extracellular traps, and mediator secretion, among others. We name of them receptors involved in distinct mechanisms of cell death of neutrophils (Jorch and Kubes, 2017)

- NETosis: FPR1/2 FCgRnAChR CR3 C5aR (when IFN-β is used as a priming cytokine)
- Phagocytosis: FCγR
- Apoptosis: TNFR FAS (APO-1/CD95) DR3 (APO-3/TRAMP) DR4 (TRAIL-R1) DR5 (TRAIL-R2) DR6

Including membrane receptors such as scavenger receptors, mannose receptors, Dectin-1 (, CD14, C1qR, receptors for IgG (FcγR) , C3b/C3biRs (CR1,CR3) , collectins (CD91-calreticulin complex), and Toll-like receptors (TLRs) ; they also express intracellular receptors such as NOD-like receptors (NLRs). Through these receptors , known as pattern recognition receptors (PRRs).

The identification of neutrophils in humans can be realized byCD11b+ CD15+ CD66b+ CD14−. In addition, CD10 is proving to be a key marker for the maturation and suppressive potential of neutrophils.(Di Carlo et al., 2001b)

2.4. Functions

The current knowledge related to the diverse roles of neutrophils throughout the progression; their principal innate functions include degranulation, phagocytosis, and release of neutrophil extracellular traps (NETs; expelled DNA webs decorated with microbicidal proteins such as myeloperoxidase, elastase, and defensins). (Mantovani et al., 2011; Matlung et al., 2018).

2.4.1. Phagocytosis

Neutrophils are exceptionally efficient phagocytes and are capable of gobbling up prey opsonized with IgG in <20 s, in comparison with macrophages, which need up to several minutes to ingest similar amounts of opsonized particles.

The recruitment of NADPH oxidase during phagosome maturation is significantly higher than in macrophages due to the fusion of cytoplasmic granules with the phagosome. These, and probably other mechanisms, result in the neutrophil phagosome to retain a pH close to neutral for longer periods of time, in comparison with the maturation of the phagolysosome in macrophages, in which acidification can reach pH values of 4.5–5.

Phagocytosis is an important physiological mechanism that allows the elimination of excess cells or damaged cells and their elimination in cases of infection by pathogens. Because many of the eat-me signals are displayed on necrotic cells or cells in the process of apoptosis, it was thought that phagocytes only feed on dead cells or cells condemned to die.

However, there is now evidence that phagocytosis of viable cells can cause their death, a process that has been termed primary phagocytosis or phagoptosis(Lee et al., 2003)

2.4.2. Oxidative burst

PMN contains granules with an arsenal of cytotoxic factors, such as antimicrobial compounds, serine proteases, lysozyme, defensins, metalloproteases, and enzymes that mediate oxidative burst. They also enzymatic machinery for the production of free radicals that allows a much more effective elimination of pathogens.

The high concentrations of reactive oxygen species produced by the NADPH oxidase are an important marker for triggering apoptosis or necrosis. In resting neutrophils, the NADPH is inactive and its components are distributed between the cytosol and cellular membranes. Upon neutrophil activation, the cytosolic components (p47phox, p67phox, p40phox) and the Rho family GTPase Rac-2 migrate toward the membranes of intracellular granules and the

cell membrane to participate in the assembly of the NADPH oxidase complex, which mediates the metabolic burst and ROS production (Dahlgren and Karlsson, 1999).

In the last years, it has been suggested that ROS produced by the NADPH oxidase participate as signaling molecules and it has been shown that the molecular pathways leading to apoptosis of neutrophils depend on ROS generat Since neutrophils can secrete a whole range of factors stored in their many granules as well as produce reactive oxygen and nitrogen species upon stimulation (Amulic et al., 2012)

2.4.3. NETosis

Currently, it has been shown that activated neutrophils can accomplish their effector functions and simultaneously activate mechanisms of cell death in response to different intracellular or extracellular factors

the mechanisms of cell death of neutrophils have distinctive properties, such as a high production of reactive oxygen species (ROS) and nitrogen species (RNS), that are important for their effector function in infections and pathologies such as cancer, autoimmune diseases, and immunodeficiencies, influencing their cell death mechanisms (Kumar and Sharma, 2010a)

This process is regulated, among others, by peptidyl arginine deaminase 4 (PAD4), an enzyme that contains a putative sequence of nuclear localization, but that resides principally in cytosolic structures in resting neutrophils, although it can also enter into the nucleus and citrullinated the histones and diverse transcription factors. (Zhou et al., 2018)

Therefore, it can participate in the epigenetic regulation of gene expression and cellular differentiation (94, 95). PAD4 is associated with the cytosolic subunits p47phox (also known as neutrophil cytosolic factor 1, NCF1) and p67phox (NCF2) that form part of the NADPH oxidase complex involved in the respiratory burst. Activation of the cell leading to the elevation of intracellular Ca^{2+} to physiological levels does not result in activation of PAD4 enzymatic activity. However, high levels of intracellular calcium (higher than those of physiological neutrophil activation, such as those observed by disruption of the cell membrane) lead to activation of PAD4 and rapid citrullination of p47phox/NCF1 and p67phox/NCF2, as well as their dissociation from PAD4. Citrullination of NCF1 and NCF2 prevents the assembly of NADPH oxidase complex. Originally, it was reported that the presence of NADPH oxidase and myeloperoxidase (MPO) was necessary for the correct formation of NETs; until recently, all the activators capable of inducing NETs formation were known to require in some manner the presence of ROS. However, this has been a

controversial theme in recent years due to the description of novel NETosis activation pathways that appear to be independent of NADPH oxidase .(V. Kumar et Sharma 2010)

3. Problematic and objectives

3.1. Problematic

Parkinson disease (PD) is a progressive neurodegenerative disease that affects peripheral organs as well as the central nervous system and is widely associated with neuroinflammation process. The pathophysiology of PD is characterized by an ageing immune system of both innate and adaptive responses, and immunosenescence, which is the result of a prior age-acquired immunodeficiency and inflammageing, described with an excess low-level production of circulating inflammatory mediators and cytokines from chronically stimulated innate cells, including neutrophils. Neutrophils are myeloid innate immune cells that are qualified as professional phagocytes and play a pivotal role in inflammatory responses.They achieve their host defense role by phagocytosing pathogens, secreting their granules full of cytotoxic enzymes, or expelling neutrophil extracellular traps (NETs) during the process of NETosis.The neutrophil phagosome is a niche of redox signaling and metabolic reactions referred to asthe respiratory burst, which generates superoxide to form H2O2 after dismutation. H2O2 and Cl- ions may be catalyzed by the myeloperoxidase to generate hypochlorous acid (HOCl).Furthermore, neutrophil may produce termed neutrophil extracellular traps (NETs) throughNETosis, which is considered as an armdouble-edged sword, it contributes to the activation of the immune system during inflammation, nevertheless, its overexpression may leads toexacerbation of inflammatory disease. In this contexte, we have investigated the implication of phagocytosis, oxidative burst and Netosis exerted by neutrophil in the pathogenesis of PD.

3.2. Objectives

- ✓ Determination of respiratory burst biomarkers, including, H2O2, NO in patients with PD.
- ✓ Determination of MPO activity and HOCL production in patients with PD.
- ✓ Evaluation of phagocytosis in patients with PD.

3.3. Purpose

Our goal is to explore the implication of phagocytosis, oxidative burst and Netosis exerted by neutrophil in the inflammatory process during PD.

2. Materiel and methods

2.1. Study design

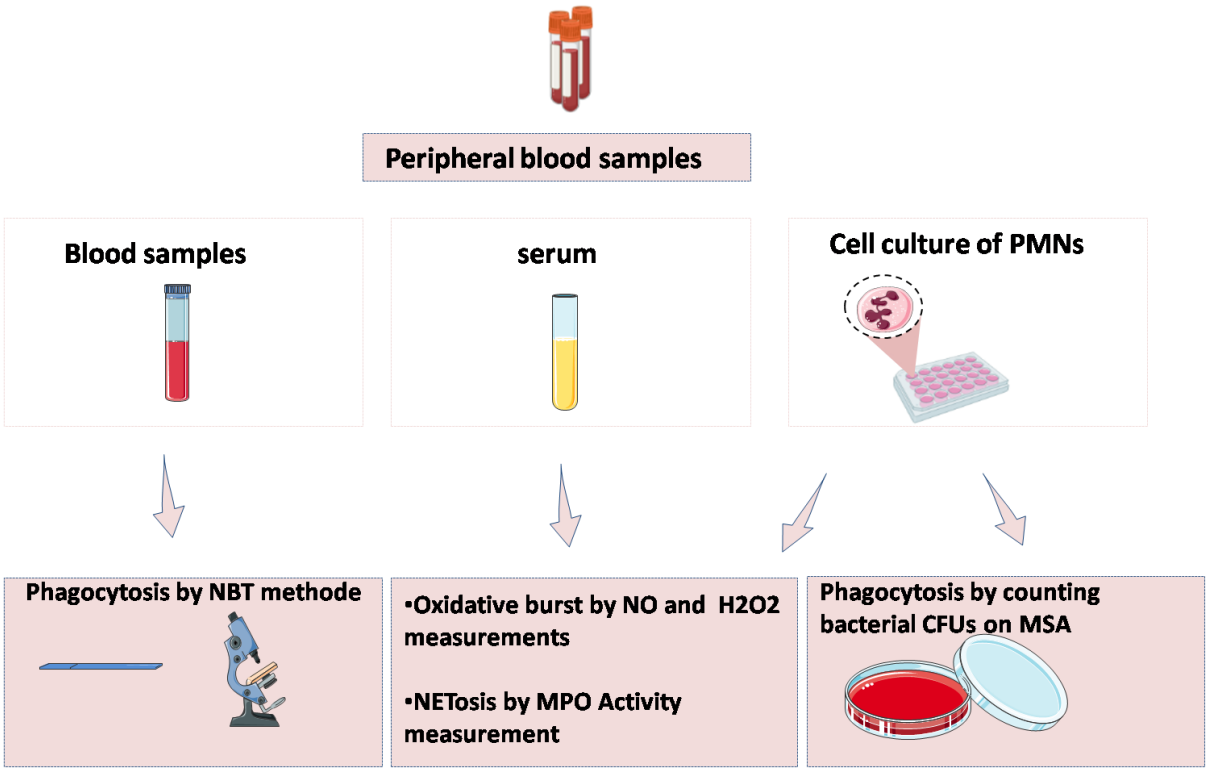

Figure 6 Study design

CFU: Colony **F**orming **U**nits

MSA: Mannitol **S**alt **A**gar

2.2. Specimen recolte

Peripheral blood was collected from twenty PD patients and age- and sex-matched neurologically healthy controls (n=20) , whose were enrolled in the study between May and June in 2022. Patients were recruited from neurological department in Tlemcen's Hospital, and were invited there for enrollment in the study. All study participants gave written consent for participation in the study.

Blood samples were taken on an empty stomach, at 9 a.m., on Vacutainers tubes via the median cubital vein. The tubes were transported within 20 minutes, in a cooler to **Laboratory of Biomolim W0414100.**

2.3. Phagocytosis in blood samples"Nitro-blue tetrazolium assay"

The measurement of the phagocyte activity of PMNs was carried out using the NBT method (nitroblue tetrazolium, Sigma-Aldrich, Germany). This method consists on preparing two Eppendorf tubes each containing 100 µL of NBT and 0.5 mL of blood, with or without addition of a dose of 10 µM of ASX in the final volume. In the first tube, 100 µL of latex particles were added; in the second, 100 µL of physiological water. Then, both tubes were

vortexed at 20 revolutions per minute, then incubated at 37°C for 20 minutes, followed by incubation for 15 minutes at room temperature. The number of PMNs was calculated on smears after double staining with May-Grünwald Giemsa.

The demonstration of phagocytosis was based on the measurement of the level of reduction of NBT in formazan in black-blue color in each leukocyte cell, in the presence or in the absence of internalized latex particles, using Technologies ImageJ (NIH, USA) and Optika microscopic Analysis.

2.4. Phagocytosis in cell culture of PMNs

The phagocytosis assay was performed as described(Aribi, 2018).

2.5. Oxidative burst

The oxidative burst or respiratory burst was evaluated by measuring the rate of NO production and H2O2 either in the serum and cell culture (Aribi, 2018).

2.5.1. Measurement of Nitric Oxide (NO)

The rate of NO produced in the serum has been determined after 30 minutes of incubation by measuring the accumulation of oxidative metabolites (NOx, nitrite and nitrate), by a colorimetric Griess reaction, using trichloroacetic acid (TCA), Vanadium (III) chloride and Griess's reagent, as previously described (Aribi et al., 2015; Guevara et al., 1998). Absorbance was read at 540 nm and NO concentration was determined from a calibration curve having concentrations ranging from 0 to 150 µmol/L of sodium nitrite ($NaNO_2^-$).(Aribi, 2018).

2.5.2. Measurement of Hydrogen peroxide (H2O2)

H2O2 production was quantified in both cell culture and serum at 610 nm by the colorimetric method of Pick and Keisari (Pick and Keisari, 1980) later modified (Aribi, 2018). The test is based on the oxidation of phenol red by H2O2 via peroxidase.Brievely, Phenol Red Buffered Solution (PRS) was used, which contains a PAB buffer for peroxide assay buffer (5.0 mM K2HPO4, 1.0 mM KH2PO4, 140 mMNaCl, 0.5 mM glucose adjusted to pH 7.4), 0.28 mM (0.1 g/L) phenol red and 8.5 U/L (50 µg/mL) horseradish peroxidase (HRPO, EC 1.11.1.7). The PRS solution was prepared immediately before performing the test by adding phenol red and HRPO to 2.1 mL of PAB at a final concentration of 0.46 mM and 0.046 U/mL, respectively. Cell lysates were added to the prepared mix with a ratio of 1-4, and then incubated for 30 min at 37°C (Pericone et al., 2000). To stop the reaction, 10 µL of NaOH with a normality of 1 have been added. The amount of H2O2 produced was quantified from a standard curve prepared using 30% dilutions of H2O2 (Aribi, 2018).

2.6. Netosis

2.6.1 Measurement of Myeloperoxidase (MPO) activity

Extracellular MPO activity gives an estimate of the oxidative stress in inflammatory diseases, while intracellular MPO activity correlates well with tissue neutrophil content. We measure the activity by dividing the concentration of HOCL on the concentration of Total proteins.(Pulli et al., 2013).

2.6.2 Measurement of Hypochlorous acid (HOCL)

Etosis's assessment was based on the indirect measurement of MPO activity after its action on hydrogen peroxidase (H2O2) .(Harwood et al., 2006)(Pulli et al., 2013) "modified by Aribi; laboratory W0414100. University of Tlemcen, 13000 Tlemcen, Algeria".

The rate of decomposition of H2O2 by MPO is determined by measuring the rate of hypochlorous acid (HOCl), which is generated from H2O2 and chloride at extracellular medium as follows:

$H2O2 + Cl \rightarrow HOCl + OH$(Tvrdá and Benko, 2020)

HOCl produced by activated neutrophils can oxidize bromide (Br) in vitro.

Briefly, 10 µl extracellular medium in PBS (PH 7.4) was added to 10 µl 22mM sodium bromide (NaBr). The HOBr concentration was determined by measuring its absorbance at 330 nm (PH 12, E330=332M^-1 cm^-1) immediately without incubation , as reported (Kumar and Margerum, 1987).The HOCl level was estimated using a standard prepared under the same conditions by adding 10 µl 22mM sodium bromide to 10 µl 20mM HOCl, 5.25% in PBS (PH 7.4). The results of Etosis were validated by fluorescence microscopy using Floid Cell Imaging Station.

2.7. Measurement of total proteins

Total protein concentration was measured from serum. In using a commercial kit (SPINREACT Ctra.Santa Coloma, SPAIN), the proteins present in serum interact with the Biuret reagent to form a colored complex quantifiable by spectrophotometry at 540 nm.

3. Results


CONFIDENTIAL

4. Discussion

Parkinson's disease (PD) is a well-known progressive neurodegenerative disease, which is characterized by neuroinflammation, associated with ageing of the immune system(Tansey et al., 2022). Age-associated alterations in the immune system include immunosenescence and inflammageing as well asan impaired adaptive immune system is thought to be implicated to pathogenesis of PD. Inflammageing induce uncontrolled downregulation of circulating inflammatory mediators and cytokines from chronically stimulated innate and adaptive cells, including neutrophils. furthermore, many studies were reported that PD is strongly associated with systemic inflammation(Adams et al., 2019).During PD, necrosis of neurons is observed following acute brain injury and neurotropic infections or as a result of the release of damaging chemicals such as glutamate, nitric oxide (NO), and reactive oxygen species (ROS)(Chao et al., 2014).

4.1. Oxydative burst activity in PD patients

4.1.1. NO production in PD patients

Nitric oxide (NO)is an intra- and extracellular messenger and a key signaling molecule that play wide biological role in many physiological processes including neuronal signaling, immune response, inflammatory response, modulation of ion channels, phagocytic defense mechanism, and cardiovascular homeostasis(Tuteja et al., 2004).NO is secreted by Nitric oxide synthase, which distinguish 3 different isoforms, iNOS: inductible NOS, eNOS and nNOS. NO, synthesized in neurons of the central nervous system, acts as a neuromediator with many physiological functions, including the formation of memory, coordination between neuronal activities. In peripheral nervous system, NO have a central role in neurotransmission(Tuteja et al., 2004).in our study, we have shown a very remarkable downregulation of NO production in PD patients compared to controls.

4.1.2. H2O2 production in PD patients compared to control

H2O2, is Our study shows difference between H2O2 production in PD patients when compared to control but the difference was not significant.

4.2. Phagocytosis activity in PD patients

Phagocytosis assay was performed using imaging latex-based Phagocytosis (CTCF) as mentiened the phagocytosis activity was significantly upregulated in patients with PD compared to control, respiratory burst was shown by NBT reduction assay,

4.3. NETosis formation in PD patients

NETosis related to MPO activity and HOCL production is crucial process, where neutrophile expelling neutrophil extracellular traps (NETs) in response to different stimuli. its over expression may leads to exacerbation of inflammatory disease.

Conclusion

Parkinson disease (PD) is a progressive neurodegenerative disease that affects peripheral organs as well as the central nervous system and is widely associated with neuroinflammation process. which induce immune cells infiltration in injured tissu, PD induce no reversible disorder in innate and adaptive immune system including age-acquired immunodeficiency and inflammageing, leading to excess deficiency in inflammatory mediators and cytokines. Neutrophils as professional phagocytes may contribute to the physiopathology of PD. Our study has shown very promising results which need to becomplemented by cell culture analysis of Netosis, phagocytosis and Oxidativeburst.

Bibliography

REFRENCES

Amulic, B., Cazalet, C., Hayes, G.L., Metzler, K.D., and Zychlinsky, A. (2012). Neutrophil Function: From Mechanisms to Disease. Annu. Rev. Immunol. 30, 459–489. https://doi.org/10.1146/annurev-immunol-020711-074942.

Aribi, M. (2018). Macrophage Bactericidal Assays. Methods Mol. Biol. Clifton NJ 1784, 135–149. https://doi.org/10.1007/978-1-4939-7837-3_14.

Boes, K.M., and Durham, A.C. (2017). Bone Marrow, Blood Cells, and the Lymphoid/Lymphatic System. In Pathologic Basis of Veterinary Disease, (Elsevier), pp. 724-804.e2.

Dahlgren, C., and Karlsson, A. (1999). Respiratory burst in human neutrophils. J. Immunol. Methods 232, 3–14. https://doi.org/10.1016/s0022-1759(99)00146-5.

Di Carlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., and Musiani, P. (2001a). The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97, 339–345. https://doi.org/10.1182/blood.V97.2.339.

Di Carlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., and Musiani, P. (2001b). The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97, 339–345. https://doi.org/10.1182/blood.V97.2.339.

Harwood, D.T., Kettle, A.J., and Winterbourn, C.C. (2006). Production of glutathione sulfonamide and dehydroglutathione from GSH by myeloperoxidase-derived oxidants and detection using a novel LC–MS/MS method. Biochem. J. 399, 161–168. https://doi.org/10.1042/BJ20060978.

Jorch, S.K., and Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. Nat. Med. 23, 279–287. https://doi.org/10.1038/nm.4294.

Kumar, K., and Margerum, D.W. (1987). Kinetics and mechanism of general-acid-assisted oxidation of bromide by hypochlorite and hypochlorous acid. Inorg. Chem. 26, 2706–2711. https://doi.org/10.1021/ic00263a030.

Kumar, V., and Sharma, A. (2010a). Neutrophils: Cinderella of innate immune system. Int. Immunopharmacol. 10, 1325–1334. https://doi.org/10.1016/j.intimp.2010.08.012.

Kumar, V., and Sharma, A. (2010b). Neutrophils: Cinderella of innate immune system. Int. Immunopharmacol. 10, 1325–1334. https://doi.org/10.1016/j.intimp.2010.08.012.

Lawrence, S.M., Corriden, R., and Nizet, V. (2018). The Ontogeny of a Neutrophil: Mechanisms of Granulopoiesis and Homeostasis. Microbiol. Mol. Biol. Rev. MMBR 82, e00057-17. https://doi.org/10.1128/MMBR.00057-17.

Lee, W.L., Harrison, R.E., and Grinstein, S. (2003). Phagocytosis by neutrophils. Microbes Infect. 5, 1299–1306. https://doi.org/10.1016/j.micinf.2003.09.014.

Lindqvist, D., Kaufman, E., Brundin, L., Hall, S., Surova, Y., and Hansson, O. (2012). Non-Motor Symptoms in Patients with Parkinson's Disease – Correlations with Inflammatory Cytokines in Serum. PLoS ONE 7, e47387. https://doi.org/10.1371/journal.pone.0047387.

MacMahonCopas, A.N., McComish, S.F., Fletcher, J.M., and Caldwell, M.A. (2021). The Pathogenesis of Parkinson's Disease: A Complex Interplay Between Astrocytes, Microglia, and T Lymphocytes? Front. Neurol. 12. .

Obeso, J.A., Stamelou, M., Goetz, C.G., Poewe, W., Lang, A.E., Weintraub, D., Burn, D., Halliday, G.M., Bezard, E., Przedborski, S., et al. (2017). Past, present, and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy: The Shaking Palsy: Past, Present and Future. Mov. Disord. 32, 1264–1310. https://doi.org/10.1002/mds.27115.

Ou, Z., Pan, J., Tang, S., Duan, D., Yu, D., Nong, H., and Wang, Z. (2021). Global Trends in the Incidence, Prevalence, and Years Lived With Disability of Parkinson's Disease in 204 Countries/Territories From 1990 to 2019. Front. Public Health 9, 776847. https://doi.org/10.3389/fpubh.2021.776847.

Prasad, E.M., and Hung, S.-Y. (2021). Current Therapies in Clinical Trials of Parkinson's Disease: A 2021 Update. Pharm. Basel Switz. 14, 717. https://doi.org/10.3390/ph14080717.

Pulli, B., Ali, M., Forghani, R., Schob, S., Hsieh, K.L.C., Wojtkiewicz, G., Linnoila, J.J., and Chen, J.W. (2013a). Measuring Myeloperoxidase Activity in Biological Samples. PLOS ONE 8, e67976. https://doi.org/10.1371/journal.pone.0067976.

Pulli, B., Ali, M., Forghani, R., Schob, S., Hsieh, K.L.C., Wojtkiewicz, G., Linnoila, J.J., and Chen, J.W. (2013b). Measuring Myeloperoxidase Activity in Biological Samples. PLoS ONE 8, e67976. https://doi.org/10.1371/journal.pone.0067976.

Quail, D.F., Amulic, B., Aziz, M., Barnes, B.J., Eruslanov, E., Fridlender, Z.G., Goodridge, H.S., Granot, Z., Hidalgo, A., Huttenlocher, A., et al. (2022). Neutrophil phenotypes and functions in cancer: A consensus statement. J. Exp. Med. 219, e20220011. https://doi.org/10.1084/jem.20220011.

Rousseaux, M.W.C., Shulman, J.M., and Jankovic, J. (2017). Progress toward an integrated understanding of Parkinson's disease. F1000Research 6, 1121. https://doi.org/10.12688/f1000research.11820.1.

Tan, E.-K., Chao, Y.-X., West, A., Chan, L.-L., Poewe, W., and Jankovic, J. (2020). Parkinson disease and the immune system — associations, mechanisms and therapeutics. Nat. Rev. Neurol. 16, 303–318. https://doi.org/10.1038/s41582-020-0344-4.

Tansey, M.G., Wallings, R.L., Houser, M.C., Herrick, M.K., Keating, C.E., and Joers, V. (2022). Inflammation and immune dysfunction in Parkinson disease. Nat. Rev. Immunol. 1–17. https://doi.org/10.1038/s41577-022-00684-6.

Thau, L., Reddy, V., and Singh, P. (2021). Anatomy, Central Nervous System (StatPearls Publishing).

Tolosa, E., Garrido, A., Scholz, S.W., and Poewe, W. (2021). Challenges in the diagnosis of Parkinson's disease. Lancet Neurol. 20, 385–397. https://doi.org/10.1016/S1474-4422(21)00030-2.

Tvrdá, E., and Benko, F. (2020). Free radicals: what they are and what they do. In Pathology, (Elsevier), pp. 3–13.

Váradi, C. (2020). Clinical Features of Parkinson's Disease: The Evolution of Critical Symptoms. Biology 9, 103. https://doi.org/10.3390/biology9050103.

Zhou, Y., An, L.-L., Chaerkady, R., Mittereder, N., Clarke, L., Cohen, T.S., Chen, B., Hess, S., Sims, G.P., and Mustelin, T. (2018). Evidence for a direct link between PAD4-mediated citrullination and the oxidative burst in human neutrophils. Sci. Rep. 8, 15228. https://doi.org/10.1038/s41598-018-33385-z.

Váradi, Csaba. 2020. « Clinical Features of Parkinson's Disease: The Evolution of Critical Symptoms ». Biology 9 (5): 103. https://doi.org/10.3390/biology9050103.

Beitz, J.M. (2014). Parkinson's disease: a review. Front Biosci (Schol Ed) 6, 65–74. https://doi.org/10.2741/s415.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. Science 303, 1532–1535. https://doi.org/10.1126/science.1092385.

Heller, J., Dogan, I., Schulz, J.B., and Reetz, K. (2014). Evidence for gender differences in cognition, emotion and quality of life in Parkinson's disease? Aging Dis 5, 63–75. https://doi.org/10.14366/AD.2014.050063.

Hernandez, D.G., Reed, X., and Singleton, A.B. (2016). Genetics in Parkinson disease: Mendelian versus non-Mendelian inheritance. J. Neurochem. 139, 59–74. https://doi.org/10.1111/jnc.13593.

Jorch, S.K., and Kubes, P. (2017). An emerging role for neutrophil extracellular traps in noninfectious disease. Nat Med 23, 279–287. https://doi.org/10.1038/nm.4294.

Kubes, P. (2018). The enigmatic neutrophil: what we do not know. Cell Tissue Res 371, 399–406. https://doi.org/10.1007/s00441-018-2790-5.

Sherer, T.B., Chowdhury, S., Peabody, K., and Brooks, D.W. (2012). Overcoming obstacles in Parkinson's disease. MovDisord 27, 1606–1611. https://doi.org/10.1002/mds.25260.

Tan, E.-K., Chao, Y.-X., West, A., Chan, L.-L., Poewe, W., and Jankovic, J. (2020). Parkinson disease and the immune system — associations, mechanisms and therapeutics. Nat Rev Neurol 16, 303–318. https://doi.org/10.1038/s41582-020-0344-4.

Tansey, M.G., and Romero-Ramos, M. (2019). Immune system responses in Parkinson's disease: Early and dynamic. Eur J Neurosci 49, 364–383. https://doi.org/10.1111/ejn.14290.

Vitte, J., Michel, B.F., Bongrand, P., and Gastaut, J.-L. (2004). Oxidative stress level in circulating neutrophils is linked to neurodegenerative diseases. J ClinImmunol 24, 683–692. https://doi.org/10.1007/s10875-004-6243-4.