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The role of peripheral immunity in Parkinson's Disease

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Abstract

In recent years the contraposition between inflammatory and neurodegenerative processes has been increasingly challenged. Inflammation has been emphasized as a key player in the onset and progression of Parkinson's disease (PD) and other neurodegenerative disorders. Evidence of microglial activation, profound imbalance in phenotype and composition of peripheral immune cells, and impaired adaptive and innate immune responses, seem to contribute to the pathogenesis of the disease. Furthermore, peripheral inflammatory mechanisms and immunogenetic factors are likely to be implicated. Even though several lines of preclinical and clinical studies are supporting and defining the complex relationship between the immune system and PD, the exact mechanisms are currently unknown. The temporal and causal connections between innate and adaptive immune responses and neurodegeneration are unsettled as well, thus challenging our ambition to define an integrated and holistic model of the disease. Despite these difficulties, current evidence is providing the unique opportunity to develop immune-targeted approaches for PD, thus enriching our therapeutic armamentarium. This thesis provides an extensive overview of past and present studies that explored the implication of the immune system in neurodegeneration, thus paving the road for the concept of disease modification in PD.

Abstract

Negli ultimi anni la contrapposizione tra processi infiammatori e neurodegenerativi è stata sempre più messa in discussione. L'infiammazione è stata recentemente valorizzata come un fattore chiave per l'insorgenza e la progressione della malattia di Parkinson (MdP) così come di altre malattie neurodegenerative. Diversi studi hanno evidenziato il contributo del sistema immunitario nella MdP, suggerendo ad esempio l'attivazione microgliale, l'alterazione nella composizione e funzionalità delle cellule immunitarie periferiche e la compromissione della risposta innata e adattativa come elementi cruciali nella patogenesi della malattia. Inoltre, è probabile che fattori d'infiammazione periferica e immunogenetici siano coinvolti. Sebbene diversi studi preclinici e clinici stiano supportando e definendo la complessa relazione tra il sistema immunitario e la MdP, i meccanismi precisi sono attualmente sconosciuti. Anche le connessioni temporali e causali tra le risposte immunitarie innate e adattative e la neurodegenerazione sono in corso di definizione, il che rappresenta una sfida nell'elaborazione di un modello integrato e olistico della malattia. Nonostante queste difficoltà, diverse linee di ricerca stanno attualmente facendo da base per diverse strategie di targeting del sistema immunitario per la MdP, nella speranza così di arricchire il nostro armamentario terapeutico. Questa tesi fornisce un'ampia panoramica degli studi passati e presenti che hanno esplorato il ruolo dell'immunità nella neurodegenerazione della MdP, aprendo così la strada al concetto di "terapie modificanti il decorso di malattia" nell'ambito di questa disabilitante patologia.

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Chapter 1

1.1 Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, with more than 6 million people affected worldwide. The clinical picture is characterized by motor symptoms, including bradykinesia, rigidity, tremor, postural instability, and a wide array of non-motor symptoms [1]. The pathological hallmark of the disease is represented by intraneuronal α-synuclein-positive inclusions called Lewy bodies and loss of dopaminergic neurons in the substantia nigra pars compact (SNc), the dorsal motor nucleus of the vagal nerve, the locus coeruleus, the pedunculopontine nucleus, and the nucleus basalis of Meynert [2]. Several factors have been involved in the pathogenesis, and the impairment of the immune system is being increasingly recognized as a key factor as well. The involvement of immune mechanisms in PD has been supported by several lines of research. Firstly, several single-nucleotide polymorphisms (SNPs) significantly associated with PD are linked to the function of the immune system [3, 4]. Furthermore, previous studies have found a common background between PD and other autoimmune and inflammatory diseases [5, 6], further suggesting the influence of the peripheral immune system in the pathogenesis of PD. Even though age is a key factor in the development of several neurodegenerative diseases, immunosenescence has been an underappreciated factor in the neurodegeneration field. Immunosenescence is characterized by age-dependent immunodeficiency and the production of inflammatory mediators from chronically stimulated immune cells [7, 8].

Both innate and adaptive immunity may present defective competence with aging and are notably altered in PD and other neurodegenerative disorders. Central and peripheral neuroinflammatory mechanisms will be discussed.

1.2 Central neuroinflammation

1.2.1 The role of microglia

Microglial cells are one of the most crucial central nervous system (CNS) components: they protect the CNS by maintaining environmental homeostasis and clearing cellular debris [9]. Nevertheless, senescence and pathologic overactivation of microglia can lead the brain to exaggerated inflammatory responses and neuronal damage. McGeer et al. provided crucial evidence linking neuroinflammation to the pathogenesis of PD [10]: the authors found HLA-DR+ microglia in postmortem tissue from PD patients. Extensive analysis of PD brain tissue showed that microgliosis may occur before cell death and even in its absence [11] and prodromal disease stages [12]. A relationship between microglial activation and dopamine terminal loss in early PD was observed as well [13], thus supporting the hypothesis that neuroinflammatory responses by intrinsic microglia contribute significantly to the progressive degeneration process. In this scenario, the *in vivo* evaluation of microglial activity is being increasingly performed using positron emission tomography (PET) ligands to measure neuroinflammation in PD patients. Nonetheless, the correlation between the levels of microglial activation and clinical severity has yielded conflicting results so far [14, 15].

In response to inflammatory challenges, microglia polarize towards the proinflammatory M1 phenotype or the immunosuppressive M2 phenotype [16].

Activation of microglia can occur in response to early signs of neuronal dysfunction and distress, i.e. Damage-Associated Molecular Patterns (DAMPs) released by dying neurons [17] or changes in the structure of endogenous proteins like α-synuclein. The association between α-synuclein and microglial activation has been extensively investigated: it seems that α-synuclein alone is sufficient to trigger neuroinflammation through both the stimulation of adaptive immunity [18] and monocyte recruitment [19]. Further studies showed that the ability of α-synuclein to start a pro-inflammatory response depends on its specific aggregation state [20] and the inflammatory properties of αsynuclein fibrils are linked to their intrinsic structure [21]. Converging evidence suggested that α-synuclein can also activate microglia through the Toll-like receptor (TLR) 4 or TLR2 pathways [22, 23]. Several other mechanisms, i.e. conversion of astrocytes into neurotoxic phenotypes, antigen presentation via major histocompatibility complex (MHC), and production of pro-inflammatory cytokines have been proposed to explain how microglial cells exert their detrimental effects on dopaminergic neurons [24].

When astrocytes are exposed to interleukin (IL)-1, tumor necrosis factor (TNF), and complement C1q, they lose their neuroprotective phenotype and become neurotoxic [25]. In a PD mouse model, blocking the conversion of astrocytes with a glucagon-like peptide 1 receptor (GLP1R) agonist (NLY01) preserved the neuroprotective properties of astrocytes [26].

As previously mentioned, the activation of both innate and adaptive immune responses is strongly influenced by MHC class II: in a study by Harms et al. expression of full-length

human α-synuclein determined the induction of MHCII expression by microglia, whereas knock-out of MHCII prevented microglial activation, antigen presentation, immunoglobulin (Ig)G deposition, and the degeneration of dopaminergic neurons [27]. Previous evidence [28] confirmed in the 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) mouse model lymphocytic infiltration of CD4+ and CD8+ T cells in the SN and striatum, with an enhanced MHC class I and II antigens expression on microglia. Intriguingly, treatment with dexamethasone could inhibit T-cell infiltration and MHC class II expression, diminishing glial reaction and neuronal impairment [28, 29]. Furthermore, targeting the MHCII through RNA silencing of the class II transactivator was effective in an α-synuclein mouse model [30].

1.2.2 Cytokines and inflammasomes

Production of pro-inflammatory cytokines is another key mechanism in dopaminergic neuron damage: one of the most relevant signal transducers of the immune system, TNF-α, is significantly higher in the brain and cerebrospinal fluid (CSF) from parkinsonian patients than in controls [31]. Another study confirmed in 22 *de novo* PD patients significantly increased CSF levels of IL-6, correlating with the severity of motor impairment and especially bradykinesia [32]. A complex dysregulation in 25 mRNAs including members of the complement system, colony-stimulating factors, Toll family, cytokines IL-8, IL-6, IL-6 Signal Transducer (IL6ST), IL-1β, TNF-α family, IL-10, transforming growth factor (TGF)β family, cathepsins, and integrin family, was proved to be region dependent in PD brain areas such as SNc, putamen, frontal cortex, and angular gyrus [33]. Interestingly, immune dysregulation in both the periphery and the

brain may determine the upregulation of inflammatory cytokines ultimately leading to a pro-inflammatory cascade. In a similar manner to what happens in the brain, levels of pro-inflammatory TNF, interferon (IFN)-γ, IL-1β, IL-6, IL-2, CXC-chemokine ligand 8 (CXCL-8), and C-C Motif Chemokine Ligand 2 (CCL2) are elevated in the serum of PD patients and a correlation with disease severity and disability has been found [34, 35]. Even though conflicting results about plasma levels (either increased or decreased) of cytokines have been reported [36–39] several studies suggested their usefulness as prognostic biomarkers of motor and non-motor symptom progression. Hofmann et al. reported a negative correlation between serum IL-6 levels and activities of the daily living scale [40], whereas Williams-Gray et al. reported an association between an antiinflammatory cytokine profile and slower motor and cognitive progression [41].

Activation of inflammasomes, which are multiprotein complexes, can be involved in the release of cytokines [42]. Inflammasomes act as sensors of environmental and cellular stress. The NLR family pyrin domain-containing 3 (NLRP3) inflammasome is composed of the NLRP3 sensor, the signaling adapter apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the caspase-1 protease. The assembly of this complex triggers caspase-1–mediated release of IL-1β and IL-18, thus initiating inflammatory responses [43]. Cleaved caspase-1 and the ASC were elevated in the SN of PD patients and NLRP3 inhibition abolished fibrillar α-synuclein–mediated inflammasome activation in mouse microglial cells [44]. Furthermore, the use of an inflammasome inhibitor ameliorated motor deficits, nigrostriatal dopaminergic degeneration, and accumulation of □-synuclein aggregates in rodent PD models [44].

1.2.3 Crosstalk between brain and periphery: the role of the blood-brain barrier

In this scenario, brain-resident mast cells may also be relevant: their degranulation and the interaction with glia and neurons can modulate blood-brain barrier (BBB) permeability through vasodilation [45]. The BBB is a key regulator of the crosstalk between peripheral circulation and the CNS. Even though many CNS diseases are associated with compromised BBB function, initial evidence in PD seemed to suggest an intact BBB [28, 46]. Nevertheless, pro-inflammatory cytokines released by activated microglia can likely alter cell junctions and BBB permeability. For example, increased levels of TNF-α, IL-1β, and IL-6 reduce ZO-1 and occluding proteins [47]. Immune cell migration could be mediated by the intercellular adhesion molecule-1 (ICAM-1) and the lymphocyte function-associated antigen 1 (LFA-1), which are involved in inflammatory processes and T-cell mediated host defense system [48]. Large numbers of ICAM-1 positive reactive astrocytes and LFA-1 positive lymphocytes have been detected in both the SN of PD patients and MPTP-exposed monkeys [49]. Additionally, either blocking LFA-1 in Th17 cells or blocking ICAM-1 in mesencephalic neurons with neutralizing antibodies abolished Th17-induced neuronal death, thus suggesting that Th17 cells infiltrate into the brain parenchyma depending on LFA-1/ICAM-1 interaction [50].

1.3 Peripheral inflammation

The peripheral immune system is a complex network consisting of two arms: the innate immune system, which represents the first line of defense against invading pathogens; and the adaptive immune system, which involves antigen-specific responses highly adapted to the specific pathogen. An altered balance in cellular and humoral responses

has been observed in PD, and the crosstalk between brain and periphery may influence the progression of neurodegeneration.

1.3.1 Innate immunity

In addition to the role of microglia in the SNC, monocytes may contribute to disease pathogenesis in the periphery as well. Blood monocytes are usually classified based on CD16 and CD14 expression [51]: more in detail, classical monocytes (CD14+/CD16-) are critical for the initial inflammatory response and they release upon activation IL-10, IL-6, RANTES, and CCL2 [52]. Non-classical monocytes (CD14-/CD16+) have been described as an anti-inflammatory subpopulation, as they maintain endothelial cell integrity [53, 54]. Intermediate monocytes (CD14+/CD16+) were proposed as a transitional population and have a high expression of MHCII [52]. Frequencies of classical monocytes are elevated in PD patients, and these cells display an altered transcriptome [55]. Funk et al. [56] observed a strong upregulation in the percentage of peripheral monocyte precursors and enhanced expression of the CCR2 receptor on the surface of classical monocytes. Moreover, monocytes from PD patients are hyperactive in response to both free and extracellular vesicle-associated α-synuclein [57]. Lastly, leucine-rich repeat kinase 2 (LRRK2) levels are elevated in monocyte populations from PD patients and contribute to monocyte dysregulation [58, 59].

Other key components of the innate immune system are represented by natural killer (NK) cells and the complement system. NK involvement in antibody-dependent cell-mediated cytotoxicity (ADCC) was explored in a study by Bokor et al.: the authors found a significant correlation between NK activity and the severity of disease staging [60].

Another study found that the expression of the inhibitory NKG2A receptors was significantly lower in PD compared with healthy controls, thus increasing the susceptibility to target-cell-dependent NK activation [61]. Moreover, an increased percentage of peripheral NK cells, directly correlating with the Unified Parkinson's Disease Rating Scale (UPDRS) scores, was also found [62]. In contrast with these findings, a significant reduction of cell frequencies and absolute numbers of invariant natural killer (iNKT) cells and γδ T cells was reported in peripheral blood samples of PD patients [63].

The complement system recruits a cascade of protease enzymes and substrates to neutralize the pathogen. This is accomplished by the activation and recruitment of phagocytes, opsonization, and the formation of the membrane attack complex (MAC). The presence of C3d, C4d, C7, and C9 complement proteins in the Lewy bodies of PD patients suggests the possibility of complement-mediated neuronal destruction [64]. Furthermore, modulation of complement receptor 3 (CR3) through genetic deletion or the blockade using a CD11b antibody determined the amelioration of dopaminergic neurodegeneration in a PD mouse model [65].

1.3.2 Adaptive immunity

The adaptive immune system, comprising B and T lymphocytes, forms the second arm of the immune system and provides specific responses against foreign antigens, generation of immunologic memory, and regulation of host immune homeostasis.

1.3.2.1 T lymphocytes

Notably, T cells are essential mediators of humoral and cellular adaptive immune responses: highly specific receptor-mediated clonal selection and expansion of T cells allow both antigen-specific immunity and immunological memory against known pathogens. Precursors of T cells migrate to the thymus and develop into two distinct subsets, CD4 + and CD8 +, based on their peculiar surface markers. Previous studies have shown that T cells play a key role both in the CNS and the periphery, leading to a profound imbalance in the immune network of PD patients. Brochard et al. found CD8+ and CD4+ T cells but not B cells in postmortem human PD brains, and T cell-mediated dopaminergic toxicity was almost exclusively arbitrated by CD4+ T cells [66]. Other studies supported the prominent role of CD4+ in T cell-mediated dopaminergic toxicity [67–69]. A seminal research by Sulzer et al. explored whether T cells recognize epitopes derived from α-synuclein and found that the Y39 and S129 regions act as epitopes [70], thus triggering an immune response mostly driven by IL-5-secreting CD4 + T cells and IFN-γ CD8 + cytotoxic T cells. Another research also reported that α-synuclein-specific T cell activation was predominant in early-stage PD [71].

Concerning the involvement of CD8+ T cells, recent neuropathological evidence [72] observed T cell infiltration throughout different PD stages, and nigral cytotoxic CD8 + T cell infiltration was the strongest in the early stage of the disease when no α-synuclein aggregation and dopaminergic neuronal death were present yet.

It is conceivable that the alteration of T cells in the CNS is mirrored in the periphery, likely as a consequence of BBB disruption in PD patients [73], thus determining an imbalance of

different subpopulations. Concerning the CD8+ subset, several studies observed increased levels of IFN-*γ*–producing CD8 + T cells [74–76], even though conflicting evidence detecting no significant differences compared with healthy controls was reported as well [77–79]. Reduced levels of circulating CD3 + and CD4 + T cells were also found [36, 75, 76, 80] as further confirmed in a meta-analysis [81]. In contrast with these results, other researchers reported in PD patients an increase in the percentage of CD3 + and CD4 + [82], or no significant difference in the percentage of both CD4 + and CD8 + compared with healthy controls [37, 77, 78].

Concerning CD4 + T cells, specific subsets are known to orchestrate different immune functions [83]: T helper (Th)1 and Th17 target bacterial and viral pathogens mainly through the release of IFN-*γ*, IL-17A, IL-21, and other pro-inflammatory cytokines. Th2 activity is focused on parasitic and allergic responses, in particular through IL-4, IL-5, and IL-13, which act as anti-inflammatory cytokines. Regulatory T cells (Tregs) modulate T cell activation and inflammation.

An imbalance of CD4+ T cell composition and function has been reported in PD. Chen et al. [80] observed in the peripheral blood of PD patients increased levels of circulating Th1 and Th17 cells and a decreased number of Th2 and Tregs. Compared with the control group, the Th1/Th2 and Th17/Treg ratios were significantly increased with a shift towards Th1 and Th17 subsets, and a significant association with motor function scores (assessed through the UPDRS-III) was found. The shift towards Th1 lineage was further confirmed by Kustrimovic et al. [36] in both drug-naïve and drug-treated patients, with profound modifications of transcription factor genes expression and increased

production of IFN-γ and TNF-α. The imbalance in CD4 + T cells transcription factors could be of great interest since it represents a peculiar molecular signature shared by idiopathic REM sleep behavior disorder and PD patients [84] as well as potential biomarkers of motor complications [85].

Another key component in the pro-inflammatory bias is represented by Th17 cells. This subset is involved in host defense against extracellular pathogens and plays a central role in the pathophysiology of several autoimmune diseases through the production of IL-17, IL-17F, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GMCSF). Increased levels of Th17 in early-stage PD were reported in several studies [74, 80, 86], as well as significant correlations with subscales I and II of the Movement Disorder Society (MDS)-UPDRS [74]. Nonetheless, conflicting results observing no differences or reduced levels of Th17 cells were also described [36, 87].

The prevalence of a pro-inflammatory phenotype in PD may also be favored by an altered anti-inflammatory response promoted by Th2 and Treg cells. Several studies have observed lower absolute numbers and frequency of Th2 cells in PD compared with healthy controls [36, 75], and increased mRNA levels of both GATA3 and STAT6 [36]. Furthermore, increased levels of STAT6 were reported in PD patients with motor fluctuations [85], thus suggesting the suitable involvement of Th2 cells in the complex stage of the disease. On the other side, Alvarez-Luquin et al. demonstrated no significant difference in Th2 cell counts in PD patients compared with controls, even though a significant increase in IL-13 levels was observed [87], and significantly increased levels of IL-4-producing Th2 have been reported as well [74].

Tregs represent another T cell subset likely involved in the disruption of immune balance. Tregs are responsible for the preservation of immune tolerance and inhibition of autoimmunity, acting as negative regulators of inflammation. It was previously reported in PD patients an impaired ability to suppress effector T cell function [88] and reduced absolute numbers of Tregs have been found as well [36, 75, 87]. Interestingly, dysregulation of the Treg compartment was also associated in PD patients with crucial non-motor symptoms, such as cognitive impairment [89] and constipation [90].

Reynolds et al. demonstrated a potential neuroprotective role for Tregs in the MPTP mouse model of PD: the adoptive transfer of CD3-activated Tregs to MPTP-intoxicated mice protected the nigrostriatal system in a dose-dependent manner [91], probably by attenuating Th17-mediated neurodegeneration [67]. Also in the MPTP mouse model examined by Li et al., Treg transfer along with anti-TNFα antibody administration increased Tregs and reduced Th1 cells leading to an amelioration of PD severity [92]. However, the necessity of a timely modulation of Treg expansion was highlighted in recent research: early treatment with superagonistic anti-CD28 monoclonal antibodies attenuated dopaminergic neurodegeneration in the SN, whereas later treatment failed to exert this neuroprotective effect [93].

1.3.2.2 B lymphocytes

B lymphocytes, which are the key players in humoral immunity, depend on CD4+ T helper cells for their antibody-secreting function. A decrease in the number of peripheral blood B cells in PD patients has been reported [94, 95], but the role of B cells in PD is less well understood and is being recently explored. IgG deposits and the IgG

receptor FcγRI have been found on dopaminergic neurons and activated microglia, suggesting the role of humoral immunity in neuroinflammation and neurodegeneration [96]. Furthermore, antibodies against α-synuclein, dopamine, and melanin have been detected in the sera and CSF of PD patients [97, 98], and correlations with disease progression and the severity of clinical symptoms were also found [99, 100]. Even though the role of anti-α-synuclein antibodies is not clear, *in vitro* [101] and *in vivo* [102] studies supported the notion of a neuroprotective effect, thus suggesting immunization strategies aimed at raising their levels [103].

1.4 Genetic PD and immune involvement

Mutations in several genes cause autosomal dominant and autosomal recessive monogenic forms of PD, and some genetic variants may modulate the risk of idiopathic PD as well. Intriguingly, growing research is linking PD-associated genes to the immune network function.

The first identified mutation to cause PD was in the *SNCA* gene, which encodes αsynuclein. *SNCA* is expressed in several immune cells including T cells, B cells, NK cells, microglia, and monocytes [104]. Microglia from knock-out α-synuclein mice displayed a basally increased reactive phenotype compared with the wild-type cells [105], thus suggesting the homeostatic role of α-synuclein. Due to the more rapid disease progression observed in patients with *SNCA* gene triplications than those with duplications, a dosage effect of the gene is hypothesized. Overexpression of α-synuclein increases neuroinflammation both in the CNS [106, 107] and the periphery. Indeed, α-

synuclein peptides can stimulate the secretion of TNF in lymphocytes from PD patients but not in controls [108] and trigger the activation of helper and cytotoxic T cells.

Mutations in *LRRK2* are the most common cause of autosomal dominant PD [109]. *LRRK2* expression is increased in response to pathogens in human B cells, T cells, macrophages, and non-classical monocytes [58, 110–112]. Implications in both cell differentiation and function have been hypothesized as well. *LRRK2* mRNA levels are lower in pre-B compared to mature B cells, where it is more expressed in the B2 subtype, and its inhibition determined an impairment of myeloid progenitors and myeloid cell maturation [113]. Furthermore, peripheral pro-inflammatory cytokines are higher in asymptomatic individuals carrying the G2019S mutation [114], and *LRRK2* levels are increased in the immune cells of PD patients [58]. Besides G2019S, the R1441G mutation is the second most frequent *LRRK2* mutation. Gillardon et colleagues showed that micederived microglia cells carrying this mutation displayed significantly increased mRNA levels of pro-inflammatory cytokines, i.e. IL-1, IL-12, CCL4, CXCL1, and CCL3L1, thus expanding the immune regulation properties exerted by this gene [115].

Another PD-related gene involved in immune pathways is *Parkin (PRKN)*, which encodes a multidomain protein involved in the regulation of proteasomal degradation and ubiquitin-mediated signaling [109]. Parkin was proved to repress mitochondrial antigen presentation [116]. Mutations in this gene may therefore block these inhibitory effects and increase immune responses. Chronic intraperitoneal lipopolysaccharide (LPS) administration in *Parkin* knock-out mice triggers subtle motor deficits and loss of dopaminergic neurons in the SN [117], suggesting that loss of Parkin function increases

the vulnerability of nigral dopaminergic neurons to inflammation-related degeneration. Tightly connected to *PRKN*, *PINK1* encodes a ubiquitin kinase accumulating on the outer mitochondrial membrane when mitochondria are damaged. Therefore, the loss of either of these genes dysregulates mitophagy and increases mitochondrial stress. Mitoinflammation has been recognized as a key component in PD mouse models deficient in *PRKN* or *PINK1* that undergo acute or chronic mitochondrial stress [118, 119]. More in detail, mitochondrial stress can induce dopaminergic neurodegeneration in SNc when either *PRKN* or *PINK1* are absent, mostly through STING signaling [120].

Mutations in the *GBA* gene (which encodes the glucocerebrosidase enzyme) are also linked to the involvement of inflammatory pathways. For example, the deletion of *GBA* in neurons, oligodendrocytes, and astrocytes increases in mice the production of inflammatory cytokines and oxidative stress [121]. Furthermore, mice expressing the L444P mutation display multisystem inflammation and B cell hyperproliferation [122]. Evidence supporting the interaction between *GBA* and immunity derives also from human studies: patients with GBA-PD have higher plasma levels of CXCL8, CCL2, and CCL3 than patients with idiopathic PD [123]. Furthermore, enzymatic GBA activity is significantly reduced in monocytes from patients with PD, both GBA-associated and idiopathic [124].

DJ1, which is encoded by *PARK7*, is involved in both innate and adaptive immunity. In mouse models, it participates in the regulation of TLR signaling in primary astrocytes [125]. Additionally, DJ1-deficient mice exhibit decreased Treg and generate increased levels of reactive oxygen species (ROS) [126].

1.5 Inflammation and the gut-brain axis

Intestinal immune activation and dysbiosis could represent one potential peripheral driver in PD inflammatory state. Colonic dysfunction is a common non-motor symptom in PD and several lines of evidence support the spreading of α-synuclein pathology from the gastrointestinal tract to the brainstem and eventually the SN [127]. Furthermore, growing evidence is supporting the notion that inflammatory processes in the gut may play a pathogenic role in PD [128, 129]. For example, significant co-occurrence of irritable bowel syndrome and PD has been reported [130, 131]. Among patients with inflammatory bowel disease who were treated with anti-TNF therapy, the incidence of PD was reduced by 78% compared with not exposed patients [132]. Moreover, gastrointestinal infections are associated with a higher risk of developing PD later in life [133]. According to several studies, *Helicobacter pylori* (HP) infection could be associated with the worsening of motor symptoms and decreased levodopa efficacy [134].

In animal models, chronic mild focal intestinal inflammation accelerated brain neuropathology and motor dysfunction in α-synuclein mutant mice [135]. Furthermore, colonization of α-synuclein-overexpressing mice with microbiota from PD patients enhanced physical impairment compared with microbiota transplants from healthy human donors [136]. Among mediators of gut-derived inflammation, TLR4 has been identified as one of the most relevant: for example, in rotenone-treated mice, intestinal and brain inflammation was less extensive in TLR4-knocked-out mice compared with wild-type ones [137].

Further supporting T-cell-driven inflammation of gut mucosa, Houser and colleagues examined stool samples from 156 PD patients and 110 controls and found increased levels of IL-1α, IL-1β, CXCL8, and CRP [138]. Another study found an increase in mRNA transcripts encoding TNF, IFN-γ, IL-6, and IL-1β and glial markers (glial fibrillary acidic protein and Sox-10) in colonic biopsies of PD patients compared with age-matched healthy controls [139]. Moreover, quantitative analysis of fecal biomarkers revealed in PD higher levels of fecal calprotectin as a marker of the gut immune system activation; nevertheless, no correlation with PD duration has been observed [140].

In summary, several lines of evidence point to the hypothesis that PD development is triggered in the intestine, and gut microbiota produces inflammatory mediators in the gut mucosa [129]. Based on this premise, *in vitro* evidence [141] suggested that probiotics decrease pro-inflammatory cytokines, oxidative stress, and potentially pathogenic bacterial overgrowth. However, to further confirm the immunomodulatory effect of probiotics, *in vivo* longitudinal data will be necessary.

1.6 Aim of the project

The constant growth of this exciting and promising field is not only providing meaningful insights into the pathophysiology of the disease but will also hopefully allow the identification of novel therapeutic targets able to slow or even reverse neurodegenerative processes [142]. The immune system may be involved in neurodegeneration through the release of pro-inflammatory cytokines and the conversion of several cell lines towards a damaging phenotype both in the CNS and the

periphery. Nonetheless, whether immune activation represents an epiphenomenon of the neurodegenerative process or is a cause itself, is still not fully understood.

In this context, our project aims to further elucidate the association between the peripheral immune system and several motor and non-motor manifestations of PD, discussing the role of this impaired immune network in the pathophysiology of the disease.

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Chapter 2: Published and submitted works

2.1 Relationship between circulating CD4+ T lymphocytes and cognitive impairment in patients with Parkinson's disease

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Abstract

Introduction: Parkinson's disease (PD) is characterized by loss of dopaminergic neurons. Neuroinflammation may represent an important factor in the pathophysiology of PD and recent findings indicate that PD patients present a pro-inflammatory peripheral

profile of CD4+ T lymphocytes, which may correlate with motor disability. However, no data are currently available on the relationship between CD4+ T lymphocytes and cognitive

function in PD. The aim of our study is to evaluate the relationship between cognitive profile and circulating CD4+ T lymphocyte subsets in PD patients.

Methods: PD patients underwent blood withdrawal and CD4+ T lymphocyte subpopulations, including CD4+ T naïve and memory cells, Th1, Th2, Th17, Th1/17 and T regulatory (Treg) cells were evaluated by flow cytometry. Cognitive evaluation was performed using Addenbrooke Cognitive Examination (ACE-R).

Results: 43 consecutive PD patients (31 males; age [mean ± SD]: 68.9 ± 8.4 years) were enrolled. 14/43 (32.6%) were drug naïve. Based on the ACE-R score, patients were divided in two groups using defined cutoff values. In comparison to patients with normal cognitive profile, patients with cognitive impairment had a higher number of circulating lymphocytes. Moreover, drug naïve patients with a worse cognitive outcome had a lower number of resting Treg and higher number of activated Treg. Furthermore, we found a correlation between proinflammatory peripheral immune phenotype and worse cognitive outcome in the ACE-R total and sub-items scores.

Conclusions: In our cohort of PD patients, cognitive impairment was associated with higher number of circulating lymphocytes, and – at least in drug naïve patients – with dysregulation of the Treg compartment. Further studies are needed to assess whether and to what extent peripheral immunity mechanistically contributes tocognitive decline in PD.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, affecting 10 million people worldwide (Wirdefeldt et al., 2011). PD is clinically defined by a motor syndrome with bradykinesia, rest tremor and rigidity (Kalia and Lang, 2015). Nonetheless, patients often complain of so-called "non motor symptoms" such as anxiety, depression, hyposmia, constipation, impulsive-compulsive disorder, REM sleep behavior disorders and autonomic disturbances (Chaudhuri et al., 2011). Moreover, about 30–40% of PD patients will present over disease course a cognitive decline ranging from mild cognitive impairment (PD-MCI) to dementia (PDD) (Cammisuli et al., 2019; Corrado et al., 2018). Loss of dopaminergic neurons in the pars compacta of the substantia nigra with concomitant accumulation of Lewy Bodies (LB) in the surviving neurons are the pathological hallmarks of PD. The main component of LB is alpha-synuclein (α-syn), whose aggregation and accumulation perpetuates the degenerative process (Tofaris and Spillantini, 2005). Noteworthy, α-syn accumulates not only in the central but also in the peripheral nervous system, and such widespread distribution probably accounts for the variety of non motor symptoms (Comi et al., 2014). The exact mechanisms leading to neuronal loss in PD are not yet fully understood, but a growing number of studies highlight the key role of inflammation and peripheral immune system (Cappellano et al., 2013). Immune mechanisms may be involved in both the central nervous system (CNS) and the periphery. McGeer et al. (1988) demonstrated the presence of CD3+ cells, a marker for T cells, within CNS of PD patients. More recently, Brochard et al. (2009) showed infiltration of CD4+ and CD8+ T cells in the substantia nigra of PD patients

suggesting that CD4+ T cells are responsible for T cell-induced death of dopaminergic neurons. In the periphery, peptides derived from α-syn may activate lymphocytes

(particularly cytotoxic and T helper) responses in PD patients but not in healthy controls (Sulzer et al., 2017) and a proinflammatory profile of circulating CD4+ T cells has been recently described in patients with PD (Kustrimovic et al., 2018). The contribution of peripheral immunity in the development of cognitive decline has been investigated in different neurodegenerative diseases, such as Alzheimer's disease (AD) and multiple sclerosis (MS). Findings obtained in APS/PS1 murine models of AD showed that transfer of Th1 cells increased amyloid deposition, while Th2 decreased levels of circulating proinflammatory cytokines such as IFN-γ and TNF-α (Cao et al., 2009). In addition, Th17 cells may contribute to cognitive decline in AD. Accordingly, AD patients displayed higher serum levels of IL17 compared to controls (Chen et al., 2014), and administration of anti

IL17 antibodies led to improvement of cognitive function in murine models of AD (Cristiano et al., 2019). In MS, the percentage of IFNpositive CD4+ and CD8+ T lymphocytes showed a significant correlation with cognitive decline (Heesen et al., 2010). Few data are available on the relationship between peripheral immunity and cognitive function in PD and most studies were limited to the assessment of serum levels of cytokines. Of note, two studies found a correlation between a pro-inflammatory cytokine profile and worse cognitive outcome, evaluated with the Mini Mental State Examination (MMSE) (Menza et al., 2010; Williams-Gray et al., 2016). The aim of our study is to describe whether, in PD patients, progression of cognitive impairment may

correlate with circulating lymphocytes and in particular with CD4+ T cell subsets. To this end, we compared PD patients with and without cognitive impairment assessed by the Addenbooke Cognitive Examination.

2. Patients and methods

2.1. Study participants

Patients with PD were consecutively enrolled at the Movement Disorders Center of Maggiore Hospital, Novara, Italy and PD diagnosis was established according to the Movement Disorders Society Criteria (Postuma et al., 2015). For each patient, the following clinical parameters were considered: age, gender, disease duration, Levodopa Equivalent doses (LED) (Tomlinson et al., 2010); Hohen and Yahr stage (Goetz et al., 2004) and UPDRS part III score (Goetz et al., 2008). Patients with a past or concomitant autoimmune disease and with a previous or ongoing immune modulating or suppressive therapy were excluded. Furthermore, patients with diffuse cortical brain atrophy or with a marked subcortical vascular involvement were excluded (assessed with a brain CT scan or MRI). Patients' cognitive profile was evaluated using the Addenbrooke Cognitive Examination (ACE-R) (McColgan et al., 2012). This study was approved by the local Ethics Committee (CE 65/16) and patients were included after having read and signed an informed consent form for research purpose. The study was performed according to the Declaration of Helsinki and to the relevant ethical guidelines for research on humans.

2.2. Sample collection

All patients underwent a peripheral venous blood withdrawal in EDTA-coated tubes (BD Vacutainer). Tubes were subsequently coded and stored at room temperature for 24 h

until processing, to ensure homogeneous treatment of all the samples. Complete blood cell count with differential analysis was conducted on separate blood samples collected in EDTA-coated tubes (BD Vacutainer). Moreover, for each patient C-reactive protein and erythrocytes sedimentation rate were evaluated in order to detect deficits or activation of immune system.

2.3. Cell population labeling for flow cytometry analysis

Analysis of circulating CD4+ T cells was performed by flow cytometry as previously described (Kustrimovic et al., 2016, 2018) without DR staining. Briefly, three aliquots of 100 µl whole blood were prepared form each subject. The first one was used for CD4+ T helper (Th) subsets staining by incubating sample with a cocktail of anti-human CD4, CXCR3 (CD183), CCR4 (CD194), and CCR6 (CD196) antibodies (ab) for the identification of CD4+ T lymphocytes and the following Th subsets: Th1 cells (CD4+ CXCR3+ CCR4CCR6−), Th2 cells (CD4+ CXCR3-CCR4+ CCR6−), Th17 cells (CD4+ CXCR3-CCR4+ CCR6+) and Th1/17 cells (CD4+ CXCR3+ CCR4-CCR6+). The second aliquot of whole blood was used for the analysis of CD4+ Treg according to Miyara et al. (2009). Sample was incubated with a cocktail of antihuman CD4, CD25, CD127 and CD45RA ab for the identification of CD4+ T lymphocytes, of total Treg (CD4+ CD25highCD127low), and of naïve Treg (CD4+ CD25highCD127lowCD45RA+) and activated Treg (CD4+ CD25highCD127lowCD45RA−). After 20 min at room temperature (RT) in the dark erythrocytes were removed by means of a lysis buffer ((g/L) NH4Cl 8.248, KHCO3 1.0, EDTA 0.0368). Samples were then centrifuged, supernatants were removed and cells were resuspended in 350 µl PBS ((g/L) NaCl 8.0, KCl 0.2, Na2HPO4 1.42, KH2PO4 0.24,

pH7.4) and left on ice until acquisition. Analysis of CD4+ T naïve and memory subsets was performed according to previously established method (Kustrimovic et al.,2016). Briefly, the third aliquot of whole blood was before lysed with lysis buffer to remove erythrocytes, then washed with PBS and incubated with a cocktail of anti-human CD3, CD4, CD8, CD45RA and CCR7 (CD197) ab for the identification of T lymphocytes (CD3+), T helper (CD3+ CD4+ CD8−) and T cytotoxic (CD3+ CD4-CD8+) lymphocytes and the following CD4+ T cell subsets: naïve (CD3+ CD4+ CD45RA+ CCR7+), central memory (TCM, CD3+ CD4+ CD45RACCR7+), and effector memory (TEM CD3+ CD4+ CD45RACCR7−). After 20 min at RT in the dark, sample was washed, centrifuged to remove supernatant, resuspended in 350 µl PBS and left on ice until acquisition. Acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson, Milan, Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by means of their classical forward scatter (FSC) and side scatter (SSC) signals, and a minimum of 20,000 lymphocytes from each sample was collected in the gate. Data were analyzed with the FlowJo software (version 8.3.2). The results were finally expressed as absolute numbers (106/L) as well as percentage of positive cells (%).

2.4. Statistical analysis

Statistical significance of the differences between groups were analyzed by MannWhitney test or t test according to the distribution of the values assessed by the D'Agostino & Pearson normality test. Correlations were assessed by Pearson or Spearman correlation analysis, as appropriate. Calculations were performed using a

commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results

3.1. Clinical characteristics of patients

Forty-three consecutive PD patients were enrolled (31 men and 12 women, age [mean ± SD]: 68.9 ± 8.4 years). The characteristics of the population are summarized in Table 1.

Table 1: Demographic characteristics of the whole population. Data are means±SD unless otherwise indicated. a. 6 were taking Levodopa alone, 1 Levodopa + DA (rotigotine), 5 Levodopa + IMAO-B (2 rasagiline, 2 selegiline and 1 safinamide), 6 Levodopa + DA + IMAO-B. b. 2 were taking DA alone (1 pramipexole and 1 ropinirole), 8 DA + IMAO-B. Abbreviations: BMI, body mass index DA, dopamine agonist; IMAO-B, Monoamine Oxidase B inhibitor; LED, levodopa equivalent dose.

Variables	PD patients (N=43)
Age (years)	68.9±8.4
Gender (M/F)	31/12
Disease duration (years)	3.3±1.9
LED (mg/die)	450.4±258.7
Drugs	
Levodopa (n) [a]	18
DA agonists (n) [b]	17
IMAO-B (n)	19

H&Y stage	1.3±0.4
1 (n)	29
1.5-2 (n)	14
UPDRS III	15.7±7.2
1-10 (n)	13
11-20 (n)	23
>20 (n)	7
BMI (Kg/m^2)	25±2.7

PD patients were defined as drug-naïve if, at the time of enrollment, they had never been treated with antiparkinsonian drugs, or as drug treated if they had been already put on dopaminergic substitution treatments. Fourteen patients (32.6%) were drug naïve (DN). Mean disease duration was 3.8 ± 2.4. Mean UPDRS part III was 1.6 ± 3.6 while

H&Y stage was 1.3 ± 0.4. We found a statistically significant inverse correlation between ACE-R total score and age of patients at evaluation ($r = -0.47$; $p = 0.001$). Patients were then stratified according to their ACE-R total score: group 1 with a total score ≥83 and group 2 with a total score < 83 (Bruno and Schirmann Vignaga, 2019). Consistently, group 1 were significantly younger than group 2 patients (66.4 ± 8.7 vs 73.3 ± 5.9; $p = 0.009$), but the two groups did not differ for LED, disease duration, H&Y stage or UPDRS part III score (see Table 2). Age difference between group 1 and 2 patients was confirmed in drug treated (DT) (29/43) (67.3 ± 8.9 vs 75 ± 4.6; $p = 0.01$) but not in DN patients (64.7 ± 8,6 vs 69.4 ± 7.2; $p = 0.319$).

Table 2: Demographic data of the study population according to ACE-R . Data are means±SD unless otherwise indicated. Notes: ACE-R total score: group 1 ≥83 group 2< 83. Abbreviations: BMI, body mass index, LED, levodopa equivalent dose

	PD patients			**Drug naïve patients**			**Drug treated patients**		
	Group 1	**Group 2**	**P**	**Group 1**	**Group 2**	**P**	**Group 1**	**Group 2**	**P**
Age (years)	66.4±8.7	73.3±5.9	**0.009**	64.7±8.6	69.4±7.2	0.319	67.3±8.9	75±4.6	**0.014**
Gender (M/F)	19/8	11/5		4/5	3/2		15/3	8/3	
Disease duration (years)	3.4±2.0	3.2±1.7	0.720	2.3±2.2	1.6±1.5	0.520	3.9±1.8	3.9±1.3	0.955
LED (mg/die)	498.1± 310.1	377± 125.3	0.230	-	-	-	498.1± 310.1	377.0± 125.3	0.230
H&Y stage	1.2±0.4	1.3±0.5	0.358	1.2±0.4	1.5±0.5	0.302	1.2±0.4	1.3±0.5	0.743
UPDRS III	14.7±5.2	17.4±9.6	0.242	12.6±6.0	19.2±5.1	0.058	15.8±4.5	16.5±11.2	0.797
BMI (kg/m^2)	24.8±2.7	25.4±2.8	0.478	23.5±1.6	24.2±2.2	0.505	25.4±2.9	25.9±3.0	0.649
Addenbrooke's Cognitive Examination									
Total	90.2±5.1	72.9±7.8	**<0.001**	89.9±5.2	72.2±8.8	**<0.001**	90.3±5.1	73.3±7.7	**<0.001**
Attention/orientation	17.4±0.9	16.8±1.3	0.074	17.1±1.2	15.8±1.5	0.091	17.5±0.7	17.2±1.1	0.344
Memory	22.1±2.6	14.1±4.4	**<0.001**	21.2±3.5	14.2±3.1	**0.003**	22.6±2.1	14.0±5.0	**<0.001**
Fluency	9.9±1.9	6.6±2.7	**<0.001**	10.4±1.7	5.8±2.8	**0.002**	9.7±2	7.0±2.7	**0.005**
Language	25.1±1.0	22.6±2.8	**<0.001**	25.2±0.8	23.0±0.7	0.099	25.1±1.1	22.5±2.5	**0.001**
Visuospatial	15.7±2.3	12.9±2.1	**<0.001**	15.9±0.3	13.4±2.1	**0.003**	15.6±2.9	12.6±2.2	**0.007**

3.2. Blood count and CD4+ T cell profile

Cognitively impaired patients (group 2, total score < 83) had a significantly higher number of total lymphocytes compared to group 1 patients ($2.1 \pm 0.8 \times 10^9$/L vs $1.6 \pm 0.4 \times 10^9$/L; p = 0.025; Table 3).

Table 3: Complete blood count in drug treated patients according to the ACE-R. Data are means±SD unless otherwise indicated. Notes: Group 1: ACE-R total score: group 1 ≥83 group 2< 83. Abbreviations: RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells.

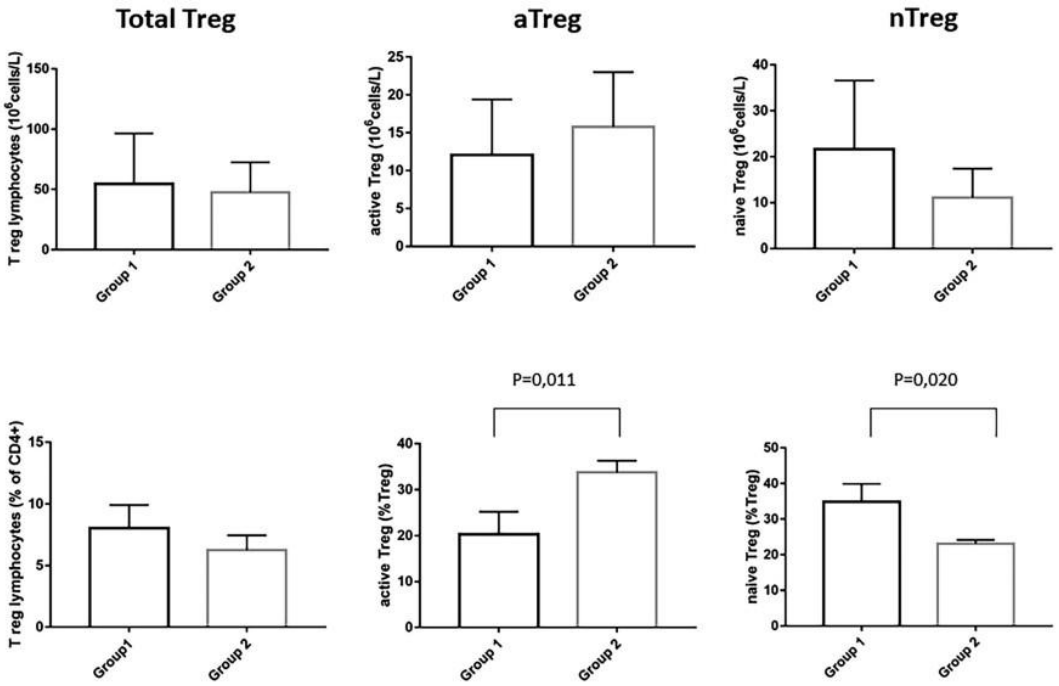
			Addenbrooke								
			PD patients			**Drug naïve patients**			**Drug treated patients**		
	Units	**range**	**Group 1**	**Group 2**	**P**	**Group 1**	**Group 2**	**p**	**Group 1**	**Group 2**	**P**
RBC	10^{12}cells /L	4.7-6.1	4.8±0.4	4.8±0.6	0.872	4.8±0.2	4.9±0.3	0.329	4.8±0.5	4.7±0.7	0.870
Hemoglobin	g/dL	14-18	14.1±1.2	14.2±1.6	0.907	14.1±0.6	14.6±0.8	0.132	14.1±1.5	13.9±1.8	0.758
Hematocrit	%	42-52	42.5±3.4	42.9±3.7	0.716	42.4±2.4	44.0±2.2	0.244	42.5±3.9	42.4±5.5	0.965
MCV	Fl	80-94	89.2±4.7	89.5±3.1	0.849	88.6±2.8	89.3±3.0	0.703	89.5±5.5	89.6±3.3	0.974

MCH	Pg	27-31	29.7±2.1	29.5±1.3	0.802	29.4±1.2	29.7±0.7	0.647	29.8±2.4	29.4±1.5	0.671
MCHC	g/dL	32-36	33.2±1.2	33.0±1.1	0.539	33.2±1.5	33.3±1.2	0.933	33.2±1.1	32.9±1.1	0.389
Platelets	10^9 cells/L	130-400	226.0±47.6	221.1±62.2	0.774	252.6±47.6	212.0±22.6	0.101	212.7±42.9	225.3±74.4	0.567
WBC	10^9 cells/L	4.8-10.8	6.5±1.0	7.3±2.2	0.103	6.6±0.6	7.6±1.6	0.112	6.4±1.1	7.1±2.5	0.296
Lymphocytes	10^9 cells/L	1.3-2.9	1.6±0.4	2.1±0.8	**0.025**	1.6±0.4	2.4±1.3	0.079	1.7±0.4	1.9±0.5	0.203
	%	20.5-45.5	25.7±5.9	28.7±7.5	0.151	24.1±5.5	30.6±9.4	0.125	26.5±6	27.8±6.8	0.582
Monocytes	10^9 cells/L	0.3-0.8	0.5±0.2	0.6±0.2	0.098	0.5±0.1	0.5±0.1	0.213	0.6±0.2	0.7±0.2	0.180
	%	5.5-11.7	8.2±2.0	8.9±2.4	0.286	6.9±1.9	7.1±1.3	0.874	8.8±1.9	9.7±2.4	0.236
Neutrophils	10^9 cells/L	2.2-4.8	4.1±0.9	4.3±1.7	0.582	4.4±0.7	4.4±0.7	0.988	4±0.9	4.3±2.1	0.557
	%	43-65	63.4±6.8	58.6±9.0	0.056	66.8±6.7	58.9±8.4	0.078	61.7±6.3	58.5±9.6	0.289

Eosinophils	10^9 cells/L	0-0.2	0.1±0.1	0.2±0.1	0.098	0.1±0.1	0.2±0.2	0.203	0.2±0.1	0.2±0.1	0.283
	%	0.9-2.9	2.2±1.5	3.1±2.5	0.151	1.6±1.1	2.7±2.7	0.307	2.5±1.7	3.3±2.5	0.308
Basophils	10^9 cells/L	0-0.1	0.0	0.1±0.0	0.098	0.0	0.0±0.1	0.263	0.0	0.0	0.238
	%	0.2-1	0.6±0.3	0.7±0.3	0.251	0.6±0.2	0.8±0.4	0.462	0.6±0.3	0.7±0.3	0.399

Analyzing T lymphocytes subpopulations, group 2 DN showed significantly lower resting Treg (23.1 ± 1.1% vs 34.9 ± 5.0% of total Treg; p = 0.020) and higher activated Treg (33.8 ± 2.5% vs 20.3 ± 4.9% of total Treg; p = 0.011) than group 1 DN patients, expressed as % of total Treg (Fig. 1).

Fig. 1: Treg cells in drug naïve patients. ACE-R total score: group 1 ≥ 83, group 2 < 83.



Furthermore, group 2 among DT patients had a higher percentage of Th1 lymphocytes (14.7 ± 10.1% vs 18.0 ± 4.5%, p = 0.011 – Table 4).

Table 4: Lymphocyte subpopulation in drug treated patients according to the ACE-R. Data are means±SD unless otherwise indicated. Notes: ACE-R total score: group 1 ≥83 group 2< 83. Abbreviations: T_{CM}, T central memory; T_{EM}, T effector memory.

		Addenbrooke								
	Units	**PD patients**			**Drug naïve patient**			**Drug treated patients**		
		Group 1	**Group 2**	**P**	**Group 1**	**Group 2**	**P**	**Group 1**	**Group 2**	**P**
CD3+	10^6 cells/L	981.9 ± 349.6	1116.4 ± 393.1	0.308	1005 ± 379.8	1228 ± 411.7	0.40 4	973.8 ± 350.4	1060.6 ± 3994	0.585
	%Lym	59.2 ± 13.8	55.3 ± 14.5	0.439	59.3 ± 14.4	50.3 ± 9.2	0.20 1	59.2 ± 14	57.8 ± 16.5	0.828

CD4+	10^6 cells/L	593.6 ± 220.6	633.9 ± 187.9	0.594	649.9 ± 291.4	711.9 ± 258	0.739	573.8 ± 197	594.8 ± 147.5	0.791
	%CD3+	62.4 ± 14.5	59.3 ± 11.8	0.526	65.4 ± 14.6	57.6 ± 1.8	0.251	61.4 ± 14.8	60.2 ± 14.6	0.848
CD8+	10^6 cells/L	264.9 ± 185.1	330.6 ± 211.6	0.385	214.6 ± 151.9	356.5 ± 183.9	0.218	282.6 ± 196.6	316.8 ± 235.1	0.707
	%CD3+	25.4 ± 12.7	26.7 ± 11.9	0.768	21.1 ± 14	27.8 ± 7.0	0.411	26.9 ± 12.3	26.2 ± 14.1	0.897
CD4+/ CD8+		3.8 ± 3.2	3.8 ± 4.3	0.689	5.2 ± 4.3	2.2 ± 0.5	0.153	3.3 ± 2.7	4.6 ± 5.1	0.953
TCM	10^6 cells/L	139.1 ± 57.5	157.0 ± 70.6	0.424	153 ± 52.8	182.1 ± 95.2	0.546	134.1 ± 59.9	144.4 ± 58.4	0.690
	%CD4+	24.3 ± 7.3	24.1 ± 6.0	0.926	24.8 ± 6.3	24.7 ± 6.0	0.992	24.2 ± 7.8	23.8±6.4	0.793
T naïve	10^6 cells/L	236.1 ± 154.9	207.4 ± 79.8	0.553	308.8 ± 215.1	241 ± 115.8	0.584	210.4 ± 125.8	190.6±57.5	0.593
	%CD4+	37.3 ± 15.0	24.7 ± 14.7	0.624	43.8 ± 12.8	35.8 ± 18.4	0.433	35.1 ± 15.4	34.2±13.9	0.895
TEM	10^6 cells/L	175.6 ± 84.6	228.5 ± 105.9	0.089	151.6 ± 47.6	233.8 ± 129.5	0.185	184.0 ± 94.0	225.8±102.0	0.162

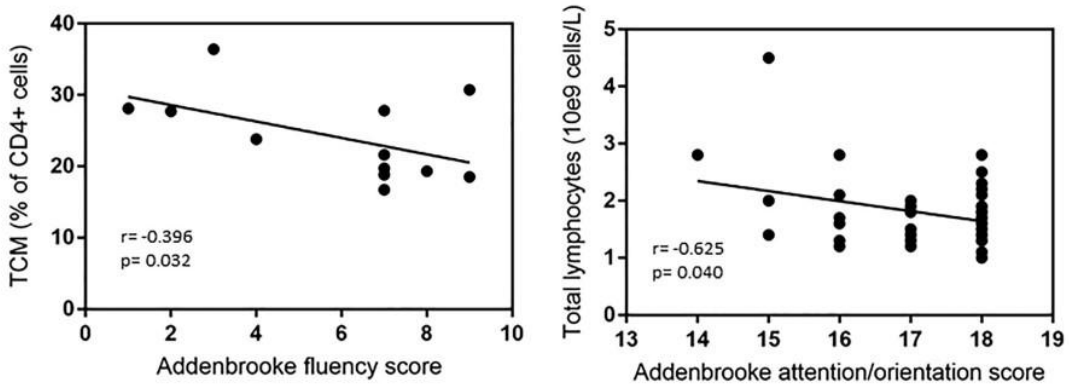
	%CD4+	31.0 ± 12.1	35.0 ± 11.6	0.237	25.2 ± 8.2	32.4 ± 12.7	0.303	33.0 ± 12.8	36.3±11.7	0.294
Th2	10^6 cells/L	62.1 ± 44.2	62.9 ± 40.8	0.747	39.3 ± 21.6	71.6 ± 24.1	0.047	71.0 ± 47.9	59.4±46.5	0.472
	%CD4+	10.1 ± 5.4	8.8 ± 3.1	0.681	7.5 ± 2.0	9.5 ± 2.7	0.201	11.2 ± 5.9	8.6±3.3	0.212
Th17	10^6 cells/L	61.1 ± 24.8	68.2 ± 40.9	0.781	51.5 ± 14.9	70.7 ± 14.3	0.067	64.9 ± 27.2	67.1±48.4	0.517
	%CD4+	11.0 ± 3.9	10.0 ± 4.0	0.453	10.4 ± 2.3	9.6 ± 2.9	0.644	11.2 ± 4.4	10.1±4.6	0.540
Th1	10^6 cells/L	89.9 ± 71.2	114.4 ± 54.2	0.050	82.1 ± 43.2	114.2 ± 63.4	0.338	92.9 ± 80.4	114.5±53.8	0.084
	%CD4+	14.9 ± 8.9	16.7 ± 5.0	0.061	15.4 ± 5.2	13.4 ± 5.1	0.545	14.7 ± 10.1	18.0±4.5	**0.011**
Th1/17	10^6 cells/L	55.2 ± 28.3	71.9 ± 46.4	0.242	45.6 ± 21.6	92.3 ± 51.1	0.058	58.9 ± 30.2	63.8±44.5	0.924
	%CD4+	9.9 ± 4.8	9.9±4.5	0.965	9.0 ± 3.7	12.1 ± 5.4	0.287	10.2 ± 5.2	9.0±4.1	0.649
CD25h CD127l	10^6 cells/L	49.4 ± 26.4	46.6 ± 18.9	0.986	54.6 ± 41.8	47.6 ± 24.9	0.833	47.2 ± 17.5	46.4±19.2	0.921
	%CD4+	7.8 ± 1.7	7.1 ± 1.6	0.221	8.0 ± 1.9	6.3 ± 1.2	0.259	7.8 ± 1.7	7.3±1.7	0.475

Active Treg	10^6 cells/L	12.8 ± 5.6	12.9 ± 6.5	0.950	12.1 ± 7.3	15.8 ± 7.2	0.557	13.1 ± 5.0	12.3±6.6	0.747
	%Treg	26.0 ± 8.0	28.9 ± 6.2	0.244	20.3 ± 4.9	33.8 ± 2.5	0.01	28.2 ± 8.0	27.8±6.3	0.898
Naïve Treg	10^6 cells/L	14.4 ± 9.8	10.9 ± 4.5	0.456	21.6 ± 15	11.1 ± 6.3	0.317	11.7 ± 5.6	10.8±4.5	0.708
	%Treg	27.4 ± 7.2	25.6 ± 6.2	0.433	34.9 ± 5.0	23.1 ± 1.1	0.020	24.6 ± 5.7	25.9±6.8	0.616

3.3. Correlations between CD4+ T cells and cognitive profile

Correlation analyses were performed in the whole population controlling for age. We detected a significant inverse correlation between total lymphocytes number and ACER attention-orientation sub-item score ($r = -0.369$, $p = 0.032$). Though not significant, a positive correlation was found between percentage of Th2 and Addenbrooke language sub-item score ($r = 0.299$; $p = 0.085$). Moreover, when considering independently patients with cognitive impairment, a significant inverse correlation was found between percentage of TCM lymphocytes and Addenbrooke fluency sub-item score ($r = -0.625$, $p = 0.040$) (Fig. 2).

Fig. 2: Correlations between CD4+ T cells and cognitive profile. Abbreviation: TCM T central memory cells.



4. Discussion

To the best of our knowledge, this is the first study investigating the relationship between peripheral CD4+ T cells subpopulations and cognitive status in PD patients. Our findings showed that patients with poor cognitive scores at ACE-R test displayed some differences in peripheral immune phenotype. Particularly, they presented a significant higher number of total lymphocytes (2.1 ± 0.8 vs 1.6 ± 0.4 × 10^9 cell/L). Different factors can be taken into account. Firstly, the two groups are different in terms of age but its real effect on total lymphocytes count is not yet clearly established (Apoil et al., 2017). Secondly, in our population this difference seems to be more evident among drug naïve than in drug treated patients, opening questions whether this may represent a compensatory increase in the first stages of the disease or be the result of a possible influence of pharmacological therapy on blood cells populations. When analyzing the different T lymphocytes subpopulations, drug treated patients with cognitive impairment had a significant higher percentage of Th1 lymphocytes (14.7 ± 10.1 vs 18.0 ± 4.5, p = 0.01) thus displaying a preferential pro-inflammatory peripheral immune

phenotype. A shift towards a pro-inflammatory peripheral immune-phenotype in PD has already been reported (Chen et al., 2015; Kustrimovic et al., 2018) but the suitable influence on motor disease progression has not yet fully understood. Particularly, Chen et al. (2015) detected an increase of Th1 and a decrease of Th2 lymphocytes, which correlated with disease severity evaluated with the UPDRS part III score. Kustrimovic et al. (2018) demonstrated that PD patients presented a lower number of Th2 lymphocytes and an increased number of Th1 as percentage of total CD4+ T cells compared to healthy controls. In vitro studies, showed also a preferential Th1 differentiation of naïve T cells confirmed by a higher amount of interferon gamma (IFN-γ) producing Th lymphocytes. Moreover, Hutter Saunders et al. (2012) evidenced an increase of effector/memory T cells in patients with worse motor symptoms (defined as UPDRS III score > 30). Dealing with cognitive decline, changes in peripheral immunity have already been reported in patients with dementia, with different patterns according to the disease etiology, but all presenting a reduction of T and B cells (Busse et al., 2017). Focusing on PD, Hu et al. (2018) showed that cognitive impaired patients displayed a significant lower number of CD4+, CD8+, CD3+, and CD4+/CD8+ lymphocytes. In our population, we detected significant correlations between ACE-R sub-items scores and CD4+ sub-populations. Particularly, we found inverse correlations between attention sub-item and total lymphocytes count and between fluency sub-item and percentage of TCM lymphocytes and attention/orientation sub-item. Furthermore, our data show that DN patients with a worse cognitive outcome presented a significantly higher percentage of active Treg and a lower percentage of naïve Treg. Treg is a T cell subpopulation that has the capacity to reduce inflammation by counterbalancing the pro-inflammatory functions of Th17

lymphocytes, as demonstrated in MPTP murine models, through the interaction with microglia (Reynolds et al., 2010). Two main subpopulations have been detected: activated and naïve Treg (respectively aTreg and nTreg). Generally, aTreg originate from nTreg which, in turn, can modulate this conversion. Physiologically, aged people present a higher number of aTreg but under pathologic conditions (i.e. autoimmune diseases) this proportion can invert with a decreased number of aTreg (Miyara et al., 2009).

Intriguingly, a neuroprotective role of Treg has already been suggested in other neurodegenerative diseases. Particularly, patients with amyotrophic lateral sclerosis presented a significantly decreased number of Treg, whose number also negatively correlated with disease progression (Rentzos et al., 2012). In APPPS1 murine models of Alzheimer's disease, depletion of Treg populations accelerated the onset of cognitive deficits while their amplification determined an increased recruitment of plaqueassociated protective microglia with consequent improvement of cognitive functions (Dansokho et al., 2016). Both animal models and in vitro studies have investigated whether Treg may be protective also in PD. Huang et al. (2017) have studied Treg in the MPP+ murine model and found that protection is achieved through a direct cell to cell interaction between Treg and dopaminergic neurons rather than a release of Treg cytokines, such as transforming growth factor (TGF)-β1 and interleukin (IL)-10. Kustrimovic et al. (2018) firstly demonstrated a significantly reduced number of Treg (both aTreg and nTreg) in patients compared to healthy controls and secondly a functional impairment of these cells consisting in a reduced ability to counteract the production of INF-γ and tumor necrosis factor alpha (TNF-α) by T CD4+ effectors. In our

cohort we detected a significant difference in percentage of aTreg and nTreg between drug naïve patients with and without cognitive impairment, thus questioning about the suitable role of therapy on the expression of T cells subpopulations. Indeed, it has been demonstrated that dopaminergic therapy, particularly after long-term treatment, may influence T cell proteome and consequently exert effects on the immune system. (Alberio et al., 2012). All these data strengthen the idea of immune system as a mechanism involved in cognitive decline in PD patients. Particularly, patients showing a higher pro-inflammatory compartment (in this case Th1 lymphocytes) and a dysregulation of Treg population may be more vulnerable in the development of cognitive dementia. Probably this unbalance in CD4+ T cells subpopulations may contribute to a reduced ability in counteracting pro-inflammatory responses and to an overproduction of pro-inflammatory cytokines that can reach the central nervous system trough a disrupted blood brain barrier (Brugger et al., 2015). This inflamed environment can determine a more rapid motor and non motor deterioration, contributing, among others, to the cognitive decline development in PD. The notion that immune mechanisms play a significant role in neurodegenerative diseases has been driving research with immunomodulatory therapies. In a randomized double-blind phase 1 clinical trial, administration of Sargramostim, a human recombinant granulocyte–macrophage colony-stimulating factor, to PD patients, was able to induce a Treg immune response, along with a modest improvement of motor symptoms (Gendelman et al., 2017). This aspect is of particular interest in this context, since PD patients with cognitive decline may present a dysregulation of Treg compartment. Moreover, in vitro data obtained on peripheral blood mononuclear cells of PD patients

suggest that peripheral immune function can be modulated by probiotics. Indeed, probiotics belonging to the *Lactobacillus* and *Bifidobacterium* genus exerted antiinflammatory effects by modulating the release of cytokines toward an antiinflammatory profile and counteracting the production of reactive oxygen species (Magistrelli et al., 2019). Our study presents also some limitations, including crosssectional design and sample size. Furthermore, lymphocytes subpopulations were identified using surface antigens and not through transcription factors expression (such as FoxP3 for Treg). This study was intended as hypothesis generating, to eventually open the way to a longitudinal study, trough which provide a better outlook on the predictive value of immune changes on the cognitive outcome of PD. On the other hand, our study population, though small, reflects the main risk factor for dementia development in PD, i.e. older age at evaluation (Nie et al., 2019). Moreover, our PD patients were well characterized both in terms of clinical and immunological parameters. All patients underwent a complete clinical evaluation for both motor and cognitive profiles, using validated clinical scales and tests. Particularly we decided to use the ACE-R test since it is a rapid test to explore different cognitive domains. Furthermore, it is valid tool for cognitive screening in PD patients with a specific cut-off value, as demonstrated by Reyes et al. (2009). At the same time, the immunological evaluation was very thorough, since it included a large panel of lymphocytes subpopulations. In conclusion, our study, though exploratory in nature, suggests that PD patients with an exaggerated proinflammatory peripheral immune phenotype and a Treg compartment dysregulation may be more vulnerable to cognitive decline development. These alterations might also represent both candidate biomarkers and therapeutic targets.

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2.2 Expression of Transcription Factors in CD4 + T Cells as Potential Biomarkers of Motor Complications in Parkinson's Disease

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Abstract

Background: Management of motor complications (MC) represents a major challenge in the long-term treatment of Parkinson's disease (PD) patients. In this context, the role of peripheral adaptive immunity may provide new insights, since neuroinflammatory mechanisms have been proved crucial in the disease.

Objective: The aim of this study was to analyze the transcription factors genes involved in CD4 + T cells development to uncover specific molecular signatures in patients with

(PMC) and without (WMC) motor complications.

Methods: mRNA levels of CD4 + T lymphocytes transcription factor genes TBX21, STAT1, STAT3, STAT4, STAT6, RORC, GATA3, FOXP3, and NR4A2 were measured from 40 PD patients, divided into two groups according to motor complications. Also, 40 age- and sex-matched healthy controls were enrolled.

Results: WMC patients had higher levels of STAT1 and NR4A2 ($p = 0.004$; $p = 0.003$), whereas in PMC we found higher levels of STAT6 ($p = 0.04$). Also, a ROC curve analysis confirmed STAT1 and NR4A2 as feasible biomarkers to discriminate WMC (AUC = 0.76, 95% CI 0.59–0.92, $p = 0.005$; AUC = 0.75, 95% CI 0.58–0.90, $p = 0.007$). Similarly, STAT6 detected PMC patients (AUC = 0.69, 95% CI 0.52–0.86, $p = 0.037$).

Conclusion: These results provide evidence of different molecular signatures in CD 4 + T cells of PD patients with and without MC, thus suggesting their potential as biomarkers of MC development.

INTRODUCTION

Levodopa represents the main symptomatic treatment for Parkinson's disease (PD). Nonetheless, after a variable time, its administration can lead to the development of complications (MC) such as motor fluctuations (MF) and levodopa-induced dyskinesias (LIDs). MF, defined as the worsening or reappearance of motor symptoms (often in parallel with low levodopa plasma concentration), include wearing-off, delayed-ON, noON, random ON-OFF, and early morning or nocturnal akinesia [1]. LIDs comprise different hyperkinetic involuntary movements (most commonly chorea and dystonia),

which can be peak dose-related or diphasic. Both MF and LIDs have a great impact and affect patients' quality of life [1, 2]. Known predictors for MC development are the presence of nonmotor features as well as medications and demographic factors (e.g., younger age at PD onset and lower body mass index) [3]. The pathological background of MC is complex and not yet fully understood. Interestingly, peripheral adaptive immunity is known to be a key regulator of neuroinflammation both in the central nervous system and in the periphery: accordingly, on one side T cells can be found in the substantia nigra of PD brains and on the other CD4 + T lymphocytes subsets from peripheral blood may have a crucial role as well [4, 5]. We recently reported reduced CD4 + T cells in PD patients [6] with relatively increased Th1 resulting in a putative proinflammatory Th1 bias. Remarkably, the peripheral immune imbalance observed in PD patients was recapitulated by a peculiar transcription factor (TF) gene expression profile in CD4 + T cells [6]. More specifically, in comparison with control subjects, PD patients had lower levels of TBX21, STAT3, STAT4, RORC, and NR4A2, and higher levels of STAT6, GATA3, and FOXP3. A similar peculiar molecular signature in CD4 + T cells, strongly resembling cells from PD patients, was reported in idiopathic REM sleep behavior disorder (iRBD): in both iRBD subjects and PD patients lower levels of TBX21, STAT3, and STAT4, and higher levels of FOXP3 were reported. iRBD represents the strongest risk factor for prodromal PD, and these findings suggested early involvement of peripheral immunity in the disease [7]. Whether neuroinflammation may be involved in disease progression, and in particular in the development of MC, is however still unclear. Boi and colleagues showed in an animal model that thalidomide and 3,6'-dithiothalidomide significantly attenuate the severity of L-dopa induced dyskinesias by

reducing tumor necrosis factor (TNF)-α levels in the striatum and substantia nigra pars reticulata and increasing the levels of interleukin (IL)-10 [8]. Moreover, amantadine (a noncompetitive antagonist of the N-methyl-D-aspartate glutamate receptor commonly used to treat LIDs) can act on microglia by inhibiting its inflammatory activation [9]. Based on available evidence, it has been recently suggested that in PD abnormally activated microglia and astrocytes lead to altered neuronal-glial communication, thus affecting synaptic activity and neuroplasticity and contributing to the development of LIDs [10]. Similarly, a study from Teema et al. [11] showed in a rat model that treatment with ibuprofen or piroxicam in combination with l-dopa ameliorated wearing-off at the end of week 10, delayed the development of dyskinesia, and decreased striatal cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) levels. Taken together, these results point to a potential role of the immune system in the development of MC by affecting both presynaptic and postsynaptic mechanisms. Although neuroinflammation is increasingly regarded as a key mechanism in MC occurring during PD progression, no information exists so far on the possible involvement of peripheral immunity. In the present study we, therefore, decided to explore in CD4 + T cells of PD patients with and without MC, the TF gene expression profile which we previously reported as dysregulated in both established PD as well as in iRBD [6, 7]. The identification of suitable molecular signatures could indeed be crucial for the early identification of subjects at risk of MC development, as well as to find new therapeutic targets and eventually delay MC onset in PD.

MATERIALS AND METHODS

Subjects

This is a retrospective case-control study: patients with an established diagnosis of idiopathic PD [12] were selected from an electronic database established in the context of a research program aimed at the study of peripheral immunity in PD [6, 7, 13]. The database currently includes 205 PD patients and 94 healthy controls (HC). At the time of enrollment, all patients underwent neurological assessment, performed by experienced neurologists in Movement Disorders. Motor symptoms and disease staging were assessed in "ON" condition using respectively the Unified Parkinson's Disease Rating Scale (UPDRS) part III and the Hoehn and Yahr scale [14, 15]. Other relevant data were collected, including duration and type of antiparkinsonian treatment, age at PD onset, disease duration, interval between PD diagnosis and MC onset, interval between MC onset and blood withdrawal, and levodopa equivalent daily dose or LEDD [16]. For the aforementioned research program protocol, established exclusion criteria were: a history of chronic autoimmune diseases or cancer and administration of immunomodulatory treatment. A peripheral blood venous sample was obtained at enrollment and used to assess complete blood count and peripheral CD4 + T lymphocytes TF gene expression. For the present study, we carefully revised the records of the 205 PD patients: drug-naïve patients (n = 69) and subjects with a later diagnosis of atypical parkinsonism (n = 4) were excluded from the analysis. Only drug-treated patients with comprehensive clinical and CD4 + T cells gene expression data available were included (n = 132). We subsequently selected those with motor complications (PMC), defined as those subjects who reported one or more of the following

(wearingoff, delayed-ON, no-ON, random ON-OFF, early morning/nocturnal akinesia, peak-dose or diphasic dyskinesias). Of the 25 PMC identified, 5 patients were excluded because of moderate/severe dementia (Mini-Mental State Examination < 19 [17]). For each PMC, we selected a PD patient without motor complications (WMC), nearly perfectly matched with respect to sex, UPDRS part III score (±2 points), and duration of antiparkinsonian and levodopa treatment (±2 years). Finally, HC were also selected and matched to each

PMC and WMC patient, according to sex and age (±2 years). The institutional Ethics Committee approved the study protocol (CE 65/16) and all subjects gave their informed written consent. The study was conducted according to the Declaration of Helsinki and following the international research ethical principles involving human subjects. Isolation of CD4 + T cells and real-time PCR Detailed procedures for isolation of peripheral blood mononuclear cells (PBMC) from whole blood, immunomagnetic isolation of CD4 + T cells, and total RNA extraction and reverse transcription, are described elsewhere [6, 7]. Expression of the TF genes TBX21, STAT1, STAT3, STAT4, STAT6, RORC, GATA3, FOXP3, and NR4A2 was measured by Real-Time PCR. To start RealTime PCR reactions 2 µl aliquots were obtained (cDNA final concentration: 1 µg/µl) and the following thermal protocol was used: 20 s at 95◦C (x 1, hot start); 2-step cycles as follows: 1 s at 95◦C, 20 s at 60◦C (x 40). Assays were performed in triplicate for each sample, and levels of mRNA were finally expressed as 2-DCt where DCt = [Ct (sample) - Ct (housekeeping gene)]. Relative expression was determined by normalization to the expression of RPS18, which is the gene for 18S cDNA. Statistical analysis Variables were expressed as counts and percentages when categorical and as mean ± standard

deviation when continuous. Normal distribution of data was assessed using the ShapiroWilk test. Differences between groups were analyzed through ANOVA analysis of variance, after testing for the independence of observations, normality of residuals, homogeneity of variances (Levene statistics) and checking for outliers, or with nonparametric Mann-Whitney U test if these assumptions were not met. Comparisons between categorical variables were assessed using Fisher's exact test. When adjusting for covariates in the two-way ANCOVA model, assumptions for homogeneity of regression slopes and absence of interaction between each covariate and factors were tested. A receiver operating characteristic (ROC) curve analysis was carried out to establish the biomarkers' discriminatory power. Area under the curve (AUC) and significance values were obtained and AUC values interpretation was determined according to Mandrekar [18]. Optimal cut-offs were chosen by coordinate tracing of the ROC curve according to Youden's index analysis. Sensitivity, specificity, positive, and negative likelihood ratios (LR+, LR-), positive and negative predictive values (PPV, NPV) were computed. Biomarkers' combination was explored by estimating predicted probabilities from a logistic regression model and using the values obtained as the test variable in the ROC analysis. The significance level was set to $p < 0.05$. All analyses were performed using SPSS Version 25 (IBM Corporation, Armonk, USA) and Graphpad Prism version 8 (GraphPad Software Inc., San Diego, USA).

RESULTS

For this cross-sectional study we identified 20 PMC, 20 matched WMC, and 40 age- and sex-matched HC. Complete clinical and demographic data are shown in Table 1.

Table 1: Clinical and demographic characteristics of PD patients and healthy controls

Number of patients **(N=40)**	**PMC** **(N=20)**	**WMC** **(N=20)**	**HC** **(N=40)**	***p* value** **PMC *VS* WMC**	***p* value** **PD *VS* HC**
Age (years), mean (SD)	65.75 (10.3)	70 (8.6)	69.2 (8.6)	0.13	0.52
Sex (M/F)	12/8	14/6	26/14	0.74	1.0
Age at PD onset (years), mean (SD)	57.15 (9.5)	62.65 (7.8)		0.07	
Disease duration, mean (SD)	8.60 (4.9)	7.60 (3.6)		0.62	
UPDRS part III, mean (SD)	15 (7.5)	16.3 (6)		0.36	
Hoehn and Yahr stage stage 1, n (%) stage 2, n (%) stage 3-4, n (%)	 8 (40%) 10 (50%) 2 (10%)	 13 (65%) 6 (30%) 1 (5%)		 0.20 0.33 0.50	
Antiparkinsonian therapy Levodopa, n (%) Levodopa + COMTI, n (%) DA, n (%) MAOIs, n (%)	 19 (95%) 6 (30%) 12 (60%) 12 (60%)	 17 (85%) 2 (10%) 14 (70%) 12 (60%)		 0.60 0.23 0.74 1.0	
Duration of antiparkinsonian treatment (years), mean (SD)	7.25 (4.5)	6.2 (3.99)		0.47	
Duration of levodopa treatment (years), mean (SD)	4.28 (2.7)	4.85 (2.8)		0.51	

LEDD (mg/die), mean (SD)	745.5 (353)	625.5 (218.8)	0.18
Interval PD diagnosis-MC onset (years), mean (SD)	5.1 (2.7)		
Interval MC onset-blood withdrawal (years), mean (SD)	2.31 (1)		
Motor complications, n (%)	Wearing off, 20 (100%); morning/nocturnal akinesia, 3 (15%); delayed ON/no ON/random ON-OFF, 4 (20%); peak dose dyskinesias, 9 (45%)		

COMTI, Catechol-O-methyltransferase inhibitors; *DA*, Dopamine Agonists; *HC*, healthy controls; *LEDD*, levodopa equivalent daily dose; *MAOIs*, Monoamine oxidase inhibitors; *MC*, motor complications; *PD*, Parkinson's Disease; *PMC*, patients with motor complications; *WMC*, patients without motor complications; *UPDRS*, Unified Parkinson's Disease Rating Scale

PMC reported wearing off (100%), morning and nocturnal akinesia (15%), delayed or noON/random ON-OFF (20%), and peak-dose dyskinesias (45%). Fisher's exact test and Mann-Whitney U test didn't reveal any statistically significant difference between groups for any variable. Complete blood count showed slightly increased values of mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in PMC compared to WMC (data not shown). Detailed data of TF mRNA levels in PMC, WMC and HC are included in Supplementary Table S1.

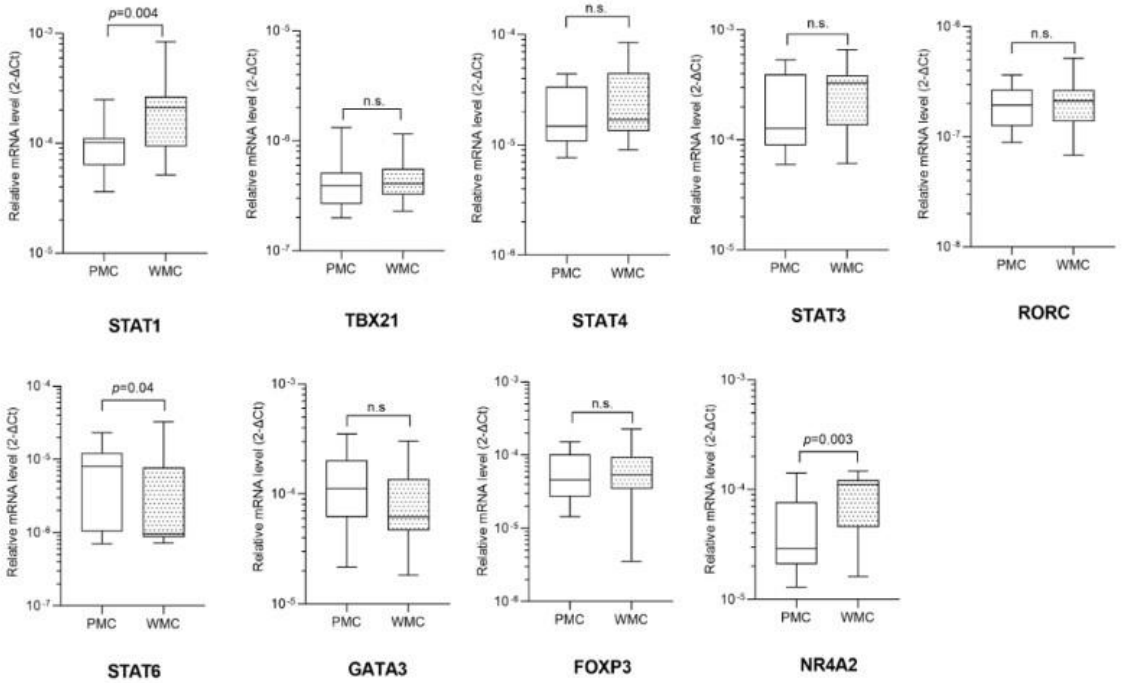
Supplementary Table S1: Comparison of CD4+ T lymphocytes transcription factors mRNA levels between patients with and without motor complications and healthy controls

Genes ($2^{-\Delta Ct}$)	PMC (N=20)	WMC (N=20)	HC (N=40)	*p* value PMC *VS* WMC	*p* value PD *VS* HC
TBX21	3.9E-007 (2.7E-007-5.17E-007)	4.1E-007 (3.2E-0075.5E-007)	2.4E-006 (7.3E-0071.2E-005)	0.465	*<0.001*
STAT1	1.0E-004 (6.4E-0051.1E-004)	2.1E-004 (9.5E-0042.6E-004)	6.5E-005 (5.5E-0051.5E-004)	*0.004*	*0.005*
STAT3	1.3E-004 (9E-0053.9E-004)	3.3E-004 (1.4E-0043.9E-004)	5E-004 (2E-004-7E-004)	0.304	*0.001*
STAT4	1.5E-005 (1.1E-0053.4E-005)	1.7E-005 (1.3E-0054.5E-005)	4.7E-005 (1.8E-0051.5E-004)	0.130	*<0.001*
RORC	1.9E-007 (1.3E-0072.7E-007)	2.1E-007 (1.4E-0072.6E-007)	1.6E-007 (9.1E-008-3E-007)	0.607	0.635
GATA3	1.1E-004 (6.2E-005-2E-004)	6.2E-005 (4.7E-0051.4E-004)	5.7E-005 (3.1E-0056.8E-005)	0.07	*<0.001*
STAT6	8.1E-006 (1.1E-0061.2E-005)	9.7E-007 (8.8E-0077.7E-006)	2E-006 (4.7E-0073.8E-006)	*0.04*	*0.046*
FOXP3	4.6E-005 (2.8E-005-1E-004)	5.4E-005 (3.5E-0059.4E-005)	4.5E-006 (1.8E-006-6E-005)	0.665	*<0.001*

NR4A2	2.9E-005 (2.1E-005 7.6E-005)	1.1E-004 (4.6E-005 1.2E-004)	1.2E-004 (8.4E-005 1.5E-004)	*0.003*	*<0.001*

Compared to HC, PD patients displayed significantly higher levels of STAT1, GATA3, STAT6, and FOXP3. When considering TF mRNA levels between PD subgroups, we found significantly higher levels of STAT1 and NR4A2 in WMC (respectively p = 0.004, p = 0.003), whereas PMC had higher levels of STAT6 (p = 0.04). Complete Real-Time PCR data, including TBX21/STAT4 (pro-Th1), STAT3/RORC (pro-Th17), and GATA3 (pro-Th2), can be seen in Fig. 1.

Figure 1: Transcription factors mRNA levels in CD4+ T lymphocytes in PD patients with (PMC) and without (WMC) motor complications. Data are plotted as medians with 25°-75° percentiles (boxes) and min–max values (whiskers)



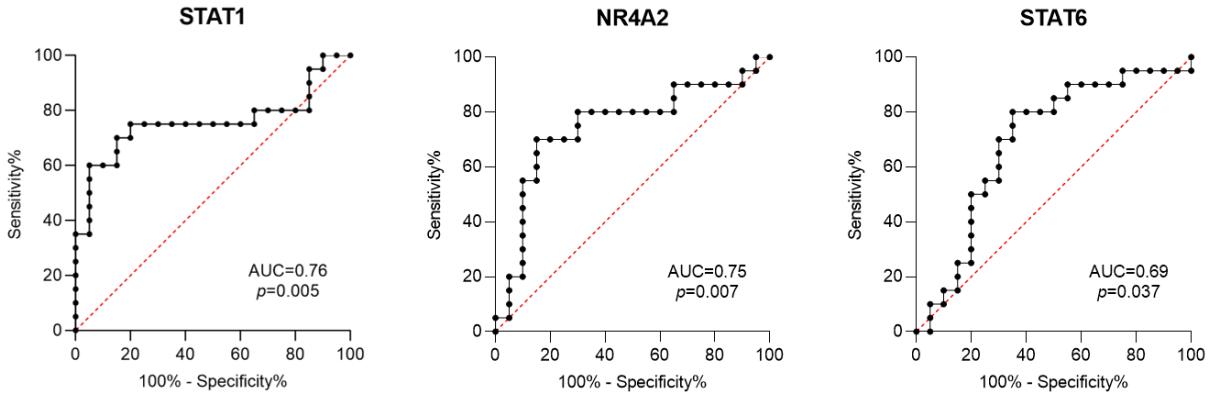
When controlling in a two-way ANCOVA model for remaining potential confounders such as age, sex [19, 20], and disease duration, between-group differences were statistically significant for STAT1 and NR4A2 (see Table 2).

Table 2: Two-way ANCOVA for *STAT1*, *NR4A2*, and *STAT6* adding age, disease duration, and sex as covariates in the model

	***F* value**	***p* value**	**Partial eta squared**
		STAT1	
Age	F(1,34)=0.051	0.823	0.001
Disease duration	F(1,34)=1.683	0.203	0.047
Sex	F(1,34)=0.037	0.848	0.001
Groups	F(1,34)=5.844	*0.021*	0.147
		NR4A2	
Age	F(1,34)=0.616	0.438	0.018
Disease duration	F(1,34)=4.948	*0.033*	0.127
Sex	F(1,34)=0.856	0.361	0.025
Groups	F(1,34)=6.243	*0.017*	0.155
		STAT6	
Age	F(1,34)=0.182	0.672	0.050
Disease duration	F(1,34)=2.174	0.150	0.060
Sex	F(1,34)=0.234	0.631	0.007
Groups	F(1,34)=3.091	0.088	0.083

We, therefore, evaluated the ability of TF mRNA levels in CD4 + T cells to discriminate between PD subgroups using ROC curve analysis: STAT1 and NR4A2 showed good AUC values (respectively 0.76, 95% CI 0.59–0.92, p = 0.005; 0.75, 95% CI 0.58–0.90, p = 0.007) thus efficiently identifying WMC. The combination of STAT1 and NR4A2 did not increase significantly the discrimination of WMC (AUC = 0.77, p = 0.003). On the other hand, STAT6 allowed MC identification (AUC = 0.69, 95% CI 0.52–0.86, p = 0.037), see Fig. 2.

Figure 2: ROC curves of transcription factors mRNA levels as candidate biomarkers to discriminate between patients with and without motor complications. Also AUC and *p* values are shown



Choosing for STAT1 an optimal cut-off value of 1.99E–004, the LR+and LR- were respectively 12 and 0.4 (sensitivity = 60%, 95% CI 38.6%–78.1%; specificity = 95%, 95% CI 76.4%–99.7%; PPV = 92.3%, NPV = 70.4%). Considering for NR4A2 a cut-off value of 1.09E–004, LR+was 5.5 and the LR– 0.5 (sensitivity = 55%, 95% CI 34.2%–74.2%;

specificity = 90%, 95% CI 69.9%–98.2%; PPV = 84.6%, NPV = 66.7%). The optimal cut-off value for STAT6 was found at 9.157E–006, with a LR+value of 2.5 and a LR- value of 1.6 (sensitivity = 50%, 95% CI 29.93%–70.07%; specificity = 80%, 95% CI 58.40%–91.93%; PPV = 71%, NPV = 61%). Cut-off values for each TF with sensitivity, specificity, LR+, LR-, PPV and NPV are summarized in Table 3.

Table 3: Sensitivity, specificity, positive likelihood ratio (LR+), negative likelihood ratio (LR-), positive predictive value (PPV), and negative predictive value (NPV) for established cut-offs

Genes	**Cut-off value**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**LR+**	**LR-**	**PPV**	**NPV**
STAT1	1.99E-004	60% (38.6-78.1)	95% (76.4-99.7)	12	0.4	92.3%	70.4%
NR4A2	1.09E-004	55% (34.2-74.2)	90% (69.9-98.2)	5.5	0.5	84.6%	66.7%
STAT6	9.157E006	50% (29.9370)	80% (58.40-91.9)	2.5	1.6	71%	61%

DISCUSSION

This study provides evidence that PMC and WMC exhibit striking differences in lymphocytes TF. Distinct molecular patterns have previously been observed in drugnaïve/drug-treated PD patients and healthy controls using the same TF gene panel [6] but no significant data were found concerning disease progression. It is well recognized that STAT1, together with TBX21 and STAT4, drives Th1 differentiation whereas STAT3/RORC are involved in Th17, STAT6/GATA3 in Th2, and FOXP3/NR4A2 in

Treg development [21–26]. More in detail, STAT1 and STAT3, in concert with Jmjd3 gene, activate microglia thus driving the production of neurotoxic molecules such as proinflammatory cytokines, chemokines, and nitric oxide [27]. The role of NR4A2 is fairly complex since it is crucial for the development and specification of midbrain dopamine neurons [28] and Le et al. found that its reduced expression increased the vulnerability of mesencephalic dopaminergic neurons to N-methyl–4-phenyl-1,2,3,6 tetrahydropyridine (MPTP)-induced injury [29]. Moreover, Montarolo et al. observed that in peripheral blood mononuclear cells (PBMC) from PD patients there is a marked down-regulated expression of the whole NR4A family subsets (NR4A1, NRA4A2, NRA4A3) [30]. The present study, on one side confirms the role of CD4 + T lymphocytes in discriminating PD patients from HC, while on the other points out novel and intriguing findings. We demonstrated that remarkable differences in STAT1, NR4A2, and STAT6 expression can be detected in PD patients with and without MC. In our previous study [6] these TF genes were similar in drug-treated and drug-naïve patients, thus opening the question of whether therapy or disease progression may influence their expression. Accordingly, the confounding effect of longer disease duration in our PD group (8.10 ± 4.3 years) has been carefully addressed in the ANCOVA model, confirming the prominent role of STAT1 and NR4A2 in identifying WMC subgroup. The role of neuroinflammation in MC has been explored in various preclinical studies: Barnum et al. tested the anti-dyskinetic effect of corticosterone in 6-hydroxydopamine (6-OHDA)lesioned rats (made dyskinetic by l-DOPA chronic treatment) and observed that l-DOPAtreated animals showed increased striatal expression of pro-inflammatory cytokines, particularly interleukin-1beta (IL-1β), which was prevented by corticosterone

administration [31]. Another study found that there are strong correlations between LIDs occurrence and expression of a pro-inflammatory microglia phenotype: in 6-OHDAlesioned rats, pulsatile l-DOPA administration but not continuous subcutaneous l-DOPA infusion determined progressive development of abnormal involuntary movements [32]. Nevertheless, evidence from human studies is lacking and the association between peripheral immunity and long-term treatment complications is still elusive. In this scenario, the present study provides for the first time distinctive molecular patterns that discriminate PMC and WMC. Among limitations, the retrospective case-control design, the small sample size which limited subgroup analysis in the PMC group, and missing information related to circulating CD4 + T lymphocytes should be mentioned. However, the limited number of patients recruited was due to the strict matching procedure performed to control for epidemiological and clinical variables: for each PMC we selected a paired patient WMC with similar features. In addition, even though gene expression does not necessarily mirror gene transcript activity and should be interpreted just as a general indication of CD4 + T cells involvement, in previous studies we observed that lower TBX21, STAT3, and STAT4 and higher FOXP3 were associated with reduced Th2, Th17, and Treg, with a relative increase of Th1 and increased production of interferon- γ and tumor necrosis factor (TNF)-α [6]. Based on this scenario, we therefore suggest that the present findings about TF mRNA levels in PD-associated MC likely underlie specific phenotypic and functional immune profiles possibly peculiar of MC, which deserve careful assessment in future investigations. The present study addressed only molecular patterns in CD4 + T cells from PD patients, providing evidence that STAT1 and NR4A2 show respectively excellent

and good positive likelihood ratios and better specificity than sensitivity in identifying the lack of motor complications: a thorough analysis of circulating CD4 + T cells subsets and longitudinal prospective studies are now warranted to elucidate the potential immunological background of long-term treatment complications in PD.

CONCLUSIONS

To the best of our knowledge, this is the first study providing distinctive molecular signatures of PD patients with and without motor complications. Though exploratory, these results shed light on the suitable involvement of the peripheral immune system, thus opening new landscapes in the therapeutic management of PD patients.

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2.3 Lymphocyte Count and Neutrophil-to-lymphocyte Ratio are Associated with Mild Cognitive Impairment in Parkinson's Disease: a Single-center Longitudinal Study

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Abstract

Lymphocyte count and neutrophil-to-lymphocyte ratio (NLR) may represent useful biomarkers of Parkinson's disease (PD), but their role in PD-related mild cognitive impairment (MCI) has not been fully elucidated. The present study aimed to confirm whether these immunological measures can discriminate PD patients from healthy controls (HC) and establish their feasibility as prognostic biomarkers of MCI in PD.

Immunological data at baseline were analyzed in 58 drug-naïve PD patients and 58 HC matched 1:1 for age, sex, and cardiovascular comorbidities. We selected a subgroup of 51 patients from this initial cohort who underwent longitudinal neuropsychological assessments through the Addenbrooke's Cognitive Examination Revised (ACE-R) test. We considered the last examination available to analyze the relationship between ACER test scores and immunological measures. We found that lymphocyte count was lower and NLR higher in PD than HC (p=0.006, p=0.044), with AUC=0.649 and 0.608, respectively. Secondly, in PD-MCI there were significantly higher levels of circulating lymphocytes (p=0.002) and lower NLR (p=0.020) than PD with normal cognitive status (PD-NC). Correlations between lymphocyte count and ACE-R total score and memory subitem (r=-0.382, p=0.006; r=-0.362, p=0.01) as well as between NLR and ACE-R total score and memory subitem (r=0.325, p=0.02; r=0.374, p=0.007), were also found. ROC curve analysis showed that lymphocyte count and NLR displayed acceptable discrimination power of PD-MCI with AUC=0.759 and 0.691, respectively. In conclusion, we suggest that an altered peripheral immune phenotype could foster cognitive decline development in PD, thus opening the possibility of immune-targeting strategies to tackle this disabling non-motor feature.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases. The clinical picture is characterized by motor symptoms, including bradykinesia, rigidity, tremor and postural instability, and a wide array of non-motor features [1]. The pathological hallmark of PD is represented by intraneuronal α-synuclein-positive inclusions called Lewy bodies and loss of dopaminergic neurons in the substantia nigra pars compact (SNc), the dorsal motor nucleus of the vagal nerve, the locus coeruleus, the pedunculopontine nucleus, and the nucleus basalis of Meynert [2]. Many pathogenic pathways, including endolysosomal and mitochondrial dysfunction, have been considered key factors. Moreover, a deeper understanding of the immune mechanisms involved in PD is being considered as well: recent evidence suggested that lower lymphocyte count was associated with an increased risk of subsequent PD diagnosis [3] and could predict ApoE ε4-related cognitive decline in PD [4]. In particular, T lymphocytes can be found in the brain of both postmortem human PD subjects and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD [5] whereas reduced CD4+ T cells with increased levels of T helper (Th) 1 were observed in the peripheral blood of PD patients compared with healthy controls (HC) [6]. In the context of a disrupted immune network largely involving lymphocytes, a promptly available indicator of peripheral inflammation is represented by the neutrophil-to-lymphocyte ratio (NLR). NLR is based on two distinct but complementary leukocyte subpopulations and alterations of this index can be found in a wide variety of medical conditions such as cancer, inflammatory and cardiovascular diseases [7–9]. Even though controversial

results have been reported and it is unclear whether the NLR can adequately reflect peripheral inflammation, several studies observed high NLR values in PD patients [10].

Furthermore, a connectometry analysis by Haghshomar and colleagues revealed in early PD significant negative correlations between NLR and white matter quantitative anisotropy in bilateral cingulum, body and left crus of fornix, body, and splenium of corpus callosum, bilateral corticospinal tract, and superior cerebellar peduncle [11]. Some of these structures, in particular the fornix, the corpus callosum, and the superior cerebellar peduncle, have been implicated in short and long-term memory impairment in PD patients [12], but longitudinal evaluations assessing the contribution of peripheral immune mechanisms to cognitive impairment are still lacking.

Therefore, the present study aimed to elucidate whether i) lymphocyte count and NLR can discriminate PD patients from HC; ii) they may represent feasible prognostic biomarkers of mild cognitive impairment (MCI) in PD.

2. Materials and Methods

This study was carried out following the ethical guidelines of the local Ethics Committee and all patients gave their written informed consent (CE 65/16). Patients were recruited in the context of a study aiming to define the role of the peripheral immune system in PD progression conducted at the Movement Disorders Center of University Hospital Maggiore della Carità, Novara, Italy [6,13]. The database currently includes 70 drugnaïve PD patients enrolled in a longitudinal study and 94 HC. We considered as inclusion criteria subjects with an established clinical diagnosis of PD [14], aged between 45 and 80 years old, speaking Italian as their first language, and with adequate abilities to

perform neuropsychological tests. Exclusion criteria for all subjects were brain abnormalities on magnetic resonance imaging, a history of chronic autoimmune diseases or cancer, and administration of immunomodulatory treatment. For the specific purpose of the present study, we excluded patients with dementia or severe depression at baseline and incomplete immunological data. Complete medical records and total leukocyte count with subpopulations (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) measured in peripheral blood at baseline were analyzed. The NLR was calculated as absolute neutrophil count divided by absolute lymphocyte count. All patients were in the drug-naïve condition to exclude potential effects of antiparkinsonian treatment on the immunological profile [15,16]. At the time of enrollment, clinical examination was performed by neurologists with experience in movement disorders, and motor symptoms were assessed using the Unified Parkinson's Disease Rating Scale (UPDRS) part III and the Hoehn and Yahr (HY) scale [17,18]. To explore the usefulness of the lymphocyte count and NLR in identifying PD patients, available immunological data of HC matched 1:1 for age (± 1 year), sex, and cardiovascular disease status, were examined. Thereafter, a subgroup of patients with longitudinal neuropsychological evaluation was selected from the initial PD cohort. Based on the last examination available, PD patients were divided into two groups based on cognitive scores assessed through Addenbrooke's Cognitive Examination-Revised (ACE-R) test [19]. According to previous literature [20], a cut-off score of 89 was used to discriminate between PD-normal cognition (PD-NC) and PD with mild cognitive impairment (PD-MCI). Raw total and subitem scores were adjusted for age, sex, and education according to established correction grids [21].

Concerning statistical analysis, variables were expressed as counts and percentages when categorical and as mean ± standard deviation when continuous. The distribution and normality of data were assessed using the Shapiro-Wilk test. Accordingly, differences between groups were analyzed through the t-test for independent samples after testing for homogeneity of variances (Levene statistics) or non-parametric MannWhitney U test/Kruskal-Wallis test. Comparisons between categorical variables were assessed using Fisher's exact test or Chi-square test as appropriate. A receiver operating characteristic (ROC) curve analysis was carried out to establish the discriminatory power of lymphocyte count and NLR between PD versus HC and PD-NC versus PD-MCI. The area under the curve (AUC) and significance values were obtained, and AUC values interpretation was determined according to previous literature [22]. Optimal cut-offs were chosen by coordinate tracing of the ROC curve according to Youden's index

analysis. Sensitivity, specificity, positive and negative likelihood ratios (LR+, LR-), positive and negative predictive values (PPV, NPV) were computed. Spearman correlation analysis was carried out to find the relationship between ACE-R total and subitem scores and immunological data. The significance level was set to $p < 0.05$. All analyses were conducted using SPSS Version 25 (IBM Corporation, Armonk, USA).

3. Results

3.1. Lymphocyte count and neutrophil-to-lymphocyte ratio in Parkinson's disease versus healthy subjects

58 PD patients and 58 matched HC were recruited. The demographic, clinical, and immunological characteristics of both groups are reported in Table 1.

Table 1: Demographic, clinical, and immunological characteristics of PD patients and healthy controls

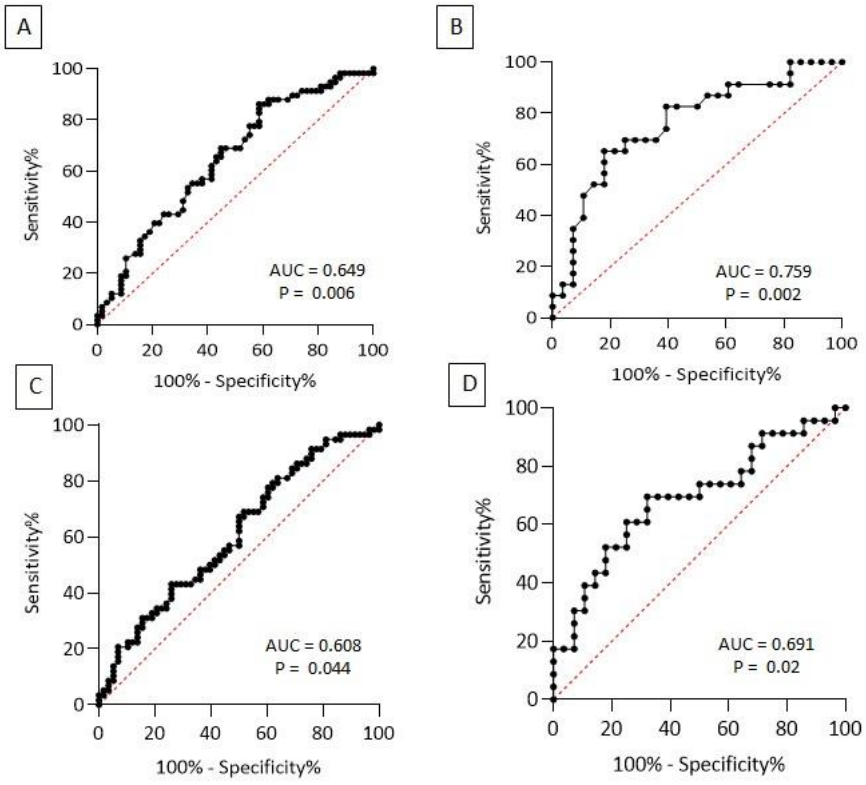
Variables	PD (n = 58)	HC (n = 58)	p-value
Age, years	69.28 (8.13)	69.31 (8.18)	0.969
Sex, M/F	42/16	42/16	1.0
History of cardiovascular diseases	34 (58.6)	31 (53.4)	0.575
Disease duration, years	1.38 (0.91)	-	-
UPDRS-III "OFF", score	13.62 (6.79)	-	-
H&Y stage			
- stage 1	41 (70.1)	-	-
- stage 2	17 (29.3)		
Tremor dominant phenotype	39 (67.2)	-	-
ACE-R total score (baseline)	93.20 (2.92)	-	-
ACE-R attention and orientation (baseline)	18 (2.24)	-	-
ACE-R memory (baseline)	25.86 (4.87)	-	-
ACE-R fluency (baseline)	11.71 (2.80)	-	-
ACE-R language (baseline)	27.37 (2.86)	-	-
ACE-R visuospatial (baseline)	16.08 (2.49)	-	-
WBC (10^3/microL)	6.47 (1.34)	6.71 (1.54)	0.600
RBC (10^6/microL)	4.69 (0.50)	4.91 (0.78)	0.251
Hemoglobin (g/dL)	14.12 (1.40)	14.24 (1.13)	0.519
Hematocrit (%)	42.39 (4.02)	42.90 (3.38)	0.467
MCV (fL)	90.62 (3.30)	91.47 (4.93)	0.588
MCH (pg)	30.15 (1.10)	30.47 (1.73)	0.118
MCHC (g/dL)	33.87 (4.44)	33.52 (2.28)	0.601

Platelets (10^3/microL)	218.75 (51.48)	222.63 (52.80)	0.612
Monocytes (10^3/microL)	0.55 (0.17)	0.56 (0.15)	0.712
Eosinophils (10^3/microL)	0.15 (0.09)	0.22 (0.21)	0.108
Basophils (10^3/microL)	0.04 (0.03)	0.04 (0.02)	0.601
Neutrophils (10^3/microL)	4.20 (1.22)	4.19 (1.16)	0.943
Lymphocytes (10^3/microL)	1.73 (0.56)	2.02 (0.65)	*0.006*
NLR	2.63 (1.15)	2.23 (0.78)	*0.044*
ESR (mm/h)	12.14 (10.98)	-	-
CRP (mg/dL)	0.37 (0.97)	-	-

Abbreviations: *ACE-R*, Addenbrooke's Cognitive Examination Revised; *H&Y*, Hoehn and Yahr; *UPDRS*, Unified Parkinson's Disease Rating Scale; *WBC*, white blood cells; *RBC*, red blood cells; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *NLR*, neutrophil-to-lymphocyte ratio; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein. Variables are expressed as mean (SD) when continuous and counts (percentage) when categorical. Significant p-values are highlighted in italics.

We found that the total number of lymphocytes was lower (p=0.006) and the NLR higher (p=0.044) in PD subjects, whereas no other statistically significant differences were found between groups. ROC curve analysis was therefore employed to detect the utility of these immunological measures in discriminating PD from HC, finding for lymphocyte count an AUC value=0.649 (95% CI 0.0549-0.748, p=0.006) and for NLR an AUC value=0.608 (95% CI 0.506-0.711, p=0.044), see Figure 1 panel A and C.

Figure 1: ROC curve of lymphocyte count to discriminate PD patients from HC (panel A) and PD-MCI from PD-NC (panel B); ROC curve of NLR values to discriminate PD patients from HC (panel C) and PD-MCI from PD-NC (panel D)



Regarding lymphocyte count, an optimal cut-off value ≤ 1.915x10^3/microL had 69% sensitivity (95% CI 56.20%-79.38%) and 55.2% specificity (95% CI 42.45%-67.25%), whereas an optimal cut-off for NLR ≥ 2.065 showed a sensitivity of 69% (95% CI 56.20%-79.38%) and a specificity of 48.3% (95% CI 35.93%-60.84%), see Table 2.

Table 2: Cut-offs of lymphocyte count and NLR to discriminate between PD versus HC and PD-NC versus PD-MCI, with sensitivity, specificity, positive and negative likelihood ratios, and positive and negative predictive values

Lymphocyte count cut-off values	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**LR+**	**LR-**	**PPV**	**NPV**
		PD VS HC				
≤ 1.915 (10^3/microL)	69% (56.20%-79.38%)	55.2% (42.45%-67.25%)	1.54	0.56	60.6%	64%
		PD-MCI VS PD-NC				
≥ 1.790 (10^3/microL)	65.2% (44.89%-81.19%)	82.1% (64.41%-92.12%)	3.64	0.42	75%	74.2%
NLR cut-off values	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**LR+**	**LR-**	**PPV**	**NPV**
		PD VS HC				
≥ 2.065	69% (56.20%-79.38%)	48.3% (35.93%-60.84%)	1.33	0.64	57.1%	60.9%
		PD-MCI VS PD-NC				
≤ 2.295	69.6% (49.13%-84.4%)	67.8% (49.34%-82.07%)	2.16	0.45	64%	73.1%

Abbreviations: *NLR*, neutrophil-to-lymphocyte ratio; *LR+*, positive likelihood ratio; *LR-*, negative likelihood ratio; *PD-MCI*, PD with cognitive impairment; *PD-NC*, PD with normal cognition; *PPV*, positive predictive value; *NPV*, negative predictive value.

3.2. Lymphocyte count and neutrophil-to-lymphocyte ratio in Parkinson's disease-related mild cognitive impairment

From the initial PD cohort, 51 subjects with longitudinal neuropsychological evaluation were then selected and divided into two groups according to cognitive status.

Demographic, clinical, and immunological characteristics of PD-NC and PD-MCI are reported in Table 3.

Table 3: Demographic, clinical, and baseline immunological characteristics of PD-NC and PD-MCI subgroups

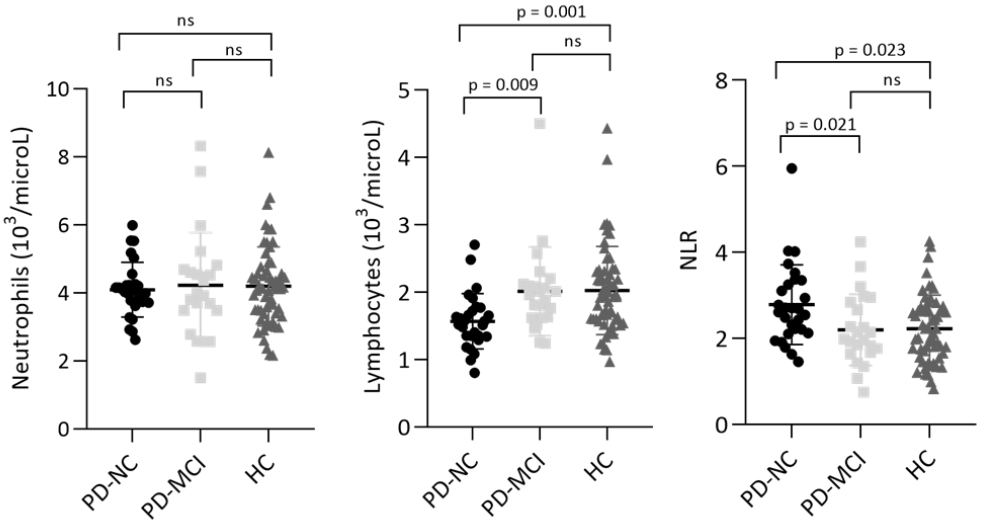
Variables	PD-NC (n = 28)	PD-MCI (n = 23)	p-value
Age at baseline, years	67.39 (9.09)	68.80 (8.48)	0.185
Sex, M/F	18/10	18/5	0.360
Scholarity, years	10.86 (4.02)	9.17 (4.72)	0.116
History of cardiovascular diseases	17 (60.71)	13 (56.52)	0.783
Disease duration, years	2.86 (1.48)	3.48 (1.65)	0.162
UPDRS-III "ON", score	13 (5.48)	13.65 (7.15)	0.753
LEDD, mg/day	360.84 (179.36)	409.65 (258.24)	0.463
H&Y stage			
- stage 1	20 (71.43)	18 (78.26)	0.749
- stage 2	8 (28.57)	5 (21.73)	
Tremor dominant phenotype	19 (67.85)	15 (65.22)	1.0
ACE-R total score	92.67 (3.40)	80.15 (7.84)	*< 0.0001*
ACE-R attention and orientation	18.13 (0.54)	20.06 (15.04)	*0.001*
ACE-R memory	26.40 (2.08)	21.57 (3.25)	*< 0.0001*
ACE-R fluency	11.63 (2.22)	9.34 (2.42)	*< 0.0001*
ACE-R language	27.35 (1.26)	26.04 (3.05)	0.117
ACE-R visuospatial	16.04 (1.24)	14.17 (1.91)	*< 0.0001*
WBC (10^3/microL)	5.95 (1.01)	7.07 (1.47)	*0.011*
RBC (10^6/microL)	4.68 (0.44)	4.67 (0.59)	0.541
Hemoglobin (g/dL)	14.12 (1.30)	13.98 (1.58)	0.40
Hematocrit (%)	42.34 (3.54)	42.27 (4.76)	0.483

MCV (fL)	90.77 (2.84)	90.66 (3.73)	0.960
MCH (pg)	30.26 (1.02)	29.97 (1.23)	0.416
MCHC (g/dL)	34.48 (6.17)	33.09 (1.02)	0.405
Platelets (10^3/microL)	214.15 (41.74)	223.35 (62.82)	0.841
Monocytes (10^3/microL)	0.54 (0.18)	0.57 (0.16)	0.351
Eosinophils (10^3/microL)	0.16 (0.09)	0.15 (0.09)	0.673
Basophils (10^3/microL)	0.04 (0.02)	0.05 (0.03)	0.150
Neutrophils (10^3/microL)	4.09 (0.80)	4.22 (1.55)	1.0
Lymphocytes (10^3/microL)	1.57 (0.41)	2.01 (0.66)	*0.002*
NLR	2.78 (0.93)	2.20 (0.83)	*0.020*
ESR (mm/h)	10.50 (7.53)	10.65 (7.22)	0.857
CRP (mg/dL)	0.19 (0.22)	0.47 (1.28)	0.595

Abbreviations: *ACE-R*, Addenbrooke's Cognitive Examination Revised; H*&Y*, Hoehn and Yahr; *UPDRS*, Unified Parkinson's Disease Rating Scale; *LEDD*, levodopa equivalent daily dose; *WBC*, white blood cells; *RBC*, red blood cells; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *NLR*, neutrophil-to-lymphocyte ratio; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein. Variables are expressed as mean (SD) when continuous and counts (percentage) when categorical. Significant p-values are highlighted in italics.

PD-MCI reported significantly lower scores in ACE-R total, attention and orientation, memory, fluency, and visuospatial subitems. Regarding immunological parameters, in PD-MCI there were significantly higher levels of circulating lymphocytes (p=0.002) and lower NLR values (p=0.020). The comparison between PD-MCI, PD-NC, and HC using Kruskal-Wallis test after Bonferroni correction is reported in Figure 2.

Figure 2: Kruskal-Wallis test with Bonferroni correction for levels of circulating neutrophils, lymphocytes, and NLR in PD and HC. Data are plotted as means and standard deviations.



ROC curve analysis was performed to establish whether lymphocyte count and NLR could discriminate between PD-MCI and PD-NC. PD-MCI were detected with acceptable AUC values by both lymphocyte count (0.759, 95% CI 0.625-0.894, p=0.002) and NLR (0.691, 95% CI 0.542-0.840, p=0.02), see Figure 1 panel B and D. An optimal cut-off value for lymphocyte count ≥ 1.790x10^3/microL had 65.2% sensitivity (95% CI 44.89%-81.19%) and 82.1% specificity (95% CI 64.41%-92.12%), whereas a cut-off value for NLR ≤ 2.295 showed a sensitivity of 69.6% (95% CI 49.13%-84.4%) and a specificity of 67.8% (95% CI 49.34%-82.07%), see Table 2.

Correlations between lymphocyte count and ACE-R total score and memory subitem (r = - 0.382, p=0.006; r = - 0.362, p=0.01) as well as between NLR and ACE-R total score and memory subitem (r = 0.325, p=0.02; r = 0.374, p=0.007), were also found, whereas no statistically significant correlations were observed between immunological measures

and age at onset, sex, disease duration, levodopa equivalent daily dose (LEDD), and UPDRS-III score.

4. Discussion

The results of the present study highlight different profiles of peripheral immune cells in PD patients compared with HC and in relation to cognitive status. More in detail, we firstly found that PD subjects display significantly lower levels of circulating lymphocytes and higher NLR than HC. Secondly, we reported in PD-MCI higher levels of circulating lymphocytes and lower NLR than PD-NC.

Decreased levels of circulating lymphocytes in PD patients have been reported in several studies [6,10,23], and the feasibility of NLR as a biomarker has been explored as well. Akıl and colleagues [24] found that NLR values ≥ 2.25 resulted in 73% sensitivity and 74% specificity in identifying PD patients, determining higher predictive power than carcinoembryonic antigen (CEA). Similarly, another study [25] established a cut-off value of 2.39 with 65% sensitivity and 75% specificity (AUC=0.714). Compared to these results, the present research showed for NLR lower AUC values and poorer prediction of PD diagnosis: however, it should be borne in mind that in our case-control design strict matching criteria, including cardiovascular comorbidities, were applied. Regarding the relationship between NLR and PD-related symptoms, another work [26] failed to find statistically significant differences in NLR between 13 akinetic-rigid and 33 tremordominant patients. Furthermore, no association with disease severity [10,27] and controversial results concerning the relationship with disease duration and LEDD [10,25,26] have also been described.

The disruption of peripheral immune mechanisms is involved in cognitive impairment as well. For example, in previous studies [28,29], lymphocyte levels were significantly decreased in Alzheimer's disease (AD) and MCI patients compared with HC.

Furthermore, altered levels of interleukin (IL)-10, IL-1β, IL-4, and IL-2 were reported in the MCI stage of dementia with Lewy bodies (DLB) and MCI-AD, thus supporting the role of the peripheral immune system early in the disease process [30]. In this context, a proinflammatory shift leading to higher NLR values was reported as an independent risk factor for MCI [28,31]. Intriguingly, high preoperative NLR values were also associated with cognitive dysfunction in patients undergoing carotid endarterectomy [32] and after acute ischemic stroke [33].

On the other hand, our results showed that PD-MCI display significantly lower NLR and higher levels of circulating lymphocytes than PD-NC, whereas no differences were found regarding other indexes of peripheral inflammation such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Moreover, ROC curve analysis showed that both lymphocyte count and NLR displayed acceptable discrimination power of PD-MCI with AUC=0.759 and 0.691, respectively. Since the neutrophil count was only mildly elevated in PD-MCI, it is unequivocal that the strongest contribution in NLR values derives from the average number of total circulating lymphocytes ($2.01x10^3$/microL in PD-MCI versus $1.57x10^3$/microL in PD-NC). It should also be noticed that both levels of circulating lymphocytes and NLR are almost identical in PD-MCI and HC ($2.01x10^3$/microL versus $2.02\ x10^3$/microL; 2.20 versus 2.23. See figure 2). The trend toward normal immunological values in PD-MCI certainly lowers the reliability of these biomarkers in

discriminating PD-MCI from HC, but at the same time rises several intriguing observations. One possible explanation for this unexpected finding is that PD patients with more severe trajectories of cognitive deterioration display higher levels of circulating lymphocytes as a result of profound adjustments leading to altered lymphocyte subpopulations. Indeed, altered levels of peripheral CD4+, CD8+, CD3+, and CD4+/CD8+ have been previously reported in cognitively impaired PD patients [34], whereas another study [35] reported in patients with worse cognitive scores higher levels of activated T regulatory cells (Treg) and Th1 and lower resting Treg. As suggested by the latter study, it is conceivable that the dysregulation of the Treg and Th1 compartments may significantly increase the vulnerability to the development of cognitive impairment. Interestingly, we also found significant correlations between lymphocyte count/NLR and the ACE-R memory subscore: Berankova and colleagues [36] demonstrated that this subscore has 90% sensitivity and 46% specificity in predicting PD dementia (PDD). Therefore, it can be speculated that an imbalance of peripheral immune cells may be involved in memory deficits and associated with an increased risk of dementia. It should also be highlighted that even though α-synuclein pathology is the main substrate of PDD, coexistent tau and amyloid-β pathologies are common and independently contribute to the development of cognitive decline in PD [37]. Indeed, several lines of evidence reported that neutrophil-related markers in peripheral blood could predict a decline in executive function in mild AD patients [38], and neutrophil extracellular traps (NETs) inside the cortical vessels and parenchyma of AD patients were also observed [39]. The discovery of NETs in AD brains suggests their role in the

exacerbation of neuroinflammatory mechanisms through vascular and parenchymal damage, but their involvement in synucleinopathies has not yet been fully understood.

In this context, the advantage of NLR comes from integrating the information of two leukocyte subtypes, as altered lymphocyte levels express the impairment of regulatory pathways whereas elevated neutrophils have been associated with increased oxidative stress and peripheral cytokine release [40]. Moreover, it overcomes the limits of absolute values of a single leukocyte subtype (which can be influenced by several factors), resulting in higher clinical significance compared with other inflammatory biomarkers [41].

To the best of our knowledge, this study demonstrates for the first time the relationship between established and readily available measures of peripheral inflammation (lymphocyte count and NLR) and the impairment of specific cognitive domains in PDMCI. One strength of this research is represented by the careful selection of patients who were drug-naïve concerning antiparkinsonian treatment at baseline immunological assessment, thus excluding the potential interference of dopaminergic therapy. Moreover, when evaluating the discrimination power for lymphocyte count and NLR of PD and PD-MCI, we thoroughly considered medical conditions potentially affecting immunological measures. Some limitations should be mentioned as well, such as the small sample size and the relatively limited time of longitudinal analysis (3.14 ± 1.56 years). Furthermore, the diagnosis of PD-MCI was established through an abbreviated assessment, thus providing less diagnostic certainty than extensive neuropsychological test batteries [42]. Nonetheless, we suggest that future studies with prospective designs

and larger cohorts should analyze the association between levels of circulating lymphocytes/NLR and MCI in early PD and explore their accuracy as biomarkers of PD progression.

5. Conclusions

Our study, though exploratory in nature, suggests that PD-MCI patients display an altered peripheral immune phenotype characterized by increased levels of circulating lymphocytes and reduced NLR. Whether this peculiar immunological profile could make patients more susceptible to cognitive decline development has yet to be fully clarified. The answer to this question could be of great interest, especially in the emerging scene of immune-targeting strategies in PD.

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2.4 Relationship between [123I]FP-CIT SPECT data and peripheral CD4+ T cell profile in newly-diagnosed drug-naïve Parkinson's disease patients

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Under review

Abstract

Background: Dysregulation of the CD4+ T cell compartment occurs in Parkinson's Disease (PD). Nonetheless, the exact relationship with dopamine transporter (DAT) SPECT denervation patterns is currently unknown.

Methods: Expression of transcription factors and levels of circulating CD4+ T cell subsets were assessed in peripheral blood mononuclear cells (PBMC) from 23 newly diagnosed drug-naïve PD patients. Semi-quantitative [^{123}I]-FP-CIT SPECT data, i.e. uptake in the

most and least affected putamen (maP, laP) and caudate (maC, laC), total striatal binding ratio (tSBR), and total putamen-to-caudate ratio (tP/C) were obtained.

Results: *FOXP3* mRNA levels correlated with the uptake in maC (r = - 0.542, *P* = 0.011), laP (r = - 0.467, *P* = 0.033), and tSBR (r = - 0.483, *P* = 0.027). Concerning flow cytometry analysis of circulating CD4+ T cell subsets, a significant relationship between tP/C, caudate uptake, and the levels of both T helper (Th)1 and 2, was detected. Furthermore, we found significant correlations between the uptake in maP and the total count of naïve and activated T regulatory cells (Treg) (r = - 0.717, *P* = 0.001; r = - 0.691, *P* = 0.002), which were confirmed after the Benjamini-Hochberg correction for multiple comparisons using a false discovery rate at level *q* = 0.10. Levels of circulating naïve Treg were higher (*P* = 0.014) in patients with more extensive dopaminergic denervation, suggesting a compensatory phenomenon.

Conclusions: Peripheral CD4+ T cell immunity is involved in early-stage PD and novel correlations with striatal DAT loss were observed.

Introduction

Many mechanisms have been implied in the pathogenesis of Parkinson's disease (PD), and the immune system is recently emerging as a potential disease-modifying target. Whether a pro-inflammatory phenotype could promote neuronal dysfunction and neurodegeneration has been extensively investigated through *in vivo* imaging. Increased microglial activation using a positron emission tomography (PET) tracer (^{11}C-PK11195) has been observed in both idiopathic PD [1] and patients with idiopathic rapid eye movement sleep behavior disorder (iRBD) [2]. Another work [3] found in leucine-rich repeat kinase 2 gene (LRRK2) non-manifesting carriers subclinical reductions of putaminal ^{18}F-DOPA uptake and raised nigral ^{11}C-PK11195 binding, thus suggesting the role of neuroinflammation in the pathophysiology of early phases of LRRK2-PD. In the context of an altered immune phenotype, previous studies have highlighted the role of adaptive immunity. T lymphocytes, in particular, are present in the substantia nigra of parkinsonian brains [4, 5], and reduced levels of circulating CD4+ T cells with a relative increase of T helper (Th) 1 subset [6] have been reported. Intriguingly, this imbalance in CD4+ T lymphocyte subpopulations is identifiable at the molecular level with a distinctive transcription factor (TF) gene expression profile. This peculiar molecular signature was also found in patients with iRBD [7] and the complex phase of PD [8]. Nonetheless, the association between peripheral inflammation and dopaminergic nigrostriatal degeneration is still elusive and has yielded conflicting results. Previous research observed significant correlations between the neutrophil-to-lymphocyte ratio as a marker of peripheral inflammation and the striatal binding ratios (SBRs) evaluated

by Dopamine Transporter Single Photon Emission Computed Tomography (DAT-SPECT) with [^{123}I]N-ω-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl) nortropane ([^{123}I]FPCIT) [9]. On the other hand, a recent study [10] confirmed the association between microglial activation and dopaminergic presynaptic disruption in a cohort of PD patients with an average disease duration of 2.5 years and ongoing dopamine replacement therapy but failed to find any significant correlation with the levels of total T cells and their subpopulations.

Therefore, in the present investigation, we aimed to thoroughly investigate in drugnaïve newly-diagnosed PD patients the relationship between CD4+ T cell TF gene expression, levels of peripheral CD4+ T lymphocyte subsets, and [^{123}I]FP-CIT SPECT data.

Materials and Methods

Study Participants

This study was carried out in accordance with the ethical guidelines of the local Ethics Committee and all patients gave their written informed consent (CE 65/16). We analyzed clinical, immunological, and DAT-SPECT data of consecutive drug-naïve PD patients referring to our Movement Disorders outpatient clinic between January 1st, 2014, and January 1st, 2021. Patients included in the database were recruited in the context of a study aiming to define the role of the peripheral immune system in parkinsonian syndromes [6, 11]. The database currently includes 70 drug-naïve parkinsonian patients enrolled in a longitudinal study and 94 healthy controls (HC). For the specific purposes of this study, we considered a diagnosis of PD as inclusion criteria according to the UK Parkinson's Disease Society Brain Bank Diagnostic Criteria [12], and

no history of current or previous therapy with antiparkinsonian agents. Exclusion criteria were brain abnormalities on magnetic resonance imaging, the occurrence of atypical signs or symptoms in conflict with a diagnosis of idiopathic PD during subsequent clinical re-assessments, a history of autoimmune diseases or cancer, concurrent (< 3 months) infection or use of anti-inflammatory drugs, the administration of chronic immunomodulatory or immunosuppressive treatment, and the use of central nervous system-acting drugs potentially interfering with DAT-SPECT analysis [11]. At the time of enrollment, neurological examination was performed by neurologists with experience in movement disorders, and motor symptoms were assessed using the Unified Parkinson's Disease Rating Scale (UPDRS) part III and the Hoehn and Yahr (HY) scale [13, 14]. Other relevant demographic and clinical data were collected.

Semi-quantitative DAT-SPECT analysis

Brain SPECTs were acquired at baseline using standard procedures [15]. Briefly, 140-180 MBq of [^{123}I]FP-CIT (DaTSCAN®, GE Healthcare Ltd, Little Chalfont, UK) were injected intravenously 40-60 minutes after administration of KClO4 400 mg to block free iodine uptake into the thyroid. Patients were imaged 4 hours post-injection using a dual-head gamma camera (Philips Axis) equipped with low-energy high-resolution parallel hole collimators. To optimize the spatial resolution of reconstructed images, patients were positioned to reduce as much as possible the rotation radius. One hundred twenty views were acquired using a step-and-shoot protocol at 3° interval and images were all inspected by experienced nuclear physicians. To meet the Basal Ganglia (BasGan) Matching Tool software requirements [15], all sets were reconstructed using a

Butterworth filter (order = 7.0, cut-off = 0.45) and corrected for attenuation using the Chang algorithm (μ = 0.10 cm−1), and reoriented images were finally analyzed. The semiquantitative analysis was performed automatically by positioning a 3D Region of Interest

(ROI) template, including an occipital ROI for background evaluation. To obtain specific SBRs, occipital background binding was subtracted from the putamen and caudate nucleus uptake from the most and least affected hemisphere in relation to motor symptoms according to the formula: SBR = (caudate or putamen ROI count density – occipital ROI count density)/occipital ROI count density. Uptake values in the most and least affected putamen (maP, laP) and caudate (maC, laC) were obtained and then compared to a reference database [16] for age adjustment. The total putamen-tocaudate ratio (tP/C) and total striatal binding ratio (tSBR) were respectively calculated by averaging the putamen-to-caudate ratio from each hemisphere and the uptake measures in the four analyzed striatal areas.

Immunological assessment

Within four weeks from clinical evaluation and DAT-SPECT analysis, patients underwent a peripheral venous blood withdrawal in EDTA-coated tubes (BD Vacutainer) for CD4+ T cell assessment and complete blood count. Tubes were stored at room temperature for 24 hours before processing. Detailed procedures are extensively described in previous works [6, 11]. Briefly, RT-PCR reactions were started using 2 μl aliquots (cDNA final concentration: 1 μg/l), and relative expression of TF *TBX21*, *STAT1*, *STAT3*, *STAT4*, *STAT6*, *RORC*, *GATA3*, *FOXP3*, and *NR4A2* was measured. CD4+ Th subsets were obtained by

incubating samples with a cocktail of anti-human CD4, CXCR3 (CD183), CCR4 (CD194), and CCR6 (CD196) antibodies for the identification of Th1, Th2, Th17, and Th1/17. A cocktail of antihuman CD4, CD25, CD127, and CD45RA antibodies was used to determine total Treg (tTreg), naïve Treg (nTreg), and activated Treg (aTreg) cells.

Statistical analyses

Variables were expressed as counts (percentages) when categorical and as mean (± standard deviation) when continuous. The normality of data distribution was assessed using the Shapiro-Wilk test and parametric or non-parametric tests were used as appropriate. Categorical variables were compared through Fisher's exact test, whereas the T-test for independent samples was used to test for differences in means between groups. To explore the relationship between clinical, immunological, and SPECT semiquantitative data, Spearman and partial correlation analyses were conducted. All tests were two-tailed and the significance level was set to $P < 0.05$. The Benjamini–Hochberg procedure was used to correct for multiple testing using a false discovery rate at level $q = 0.10$ [17]. Assuming a medium size effect of 0.5, a sample size of 23 patients had 70% power with an α level of 0.05. All analyses were performed using SPSS Version 25 (IBM Corporation, Armonk, USA).

Results

23 PD patients (12 males and 11 females), aged 66.04 ± 7.6 years and with an average disease duration of 1.57 ± 0.8 years were enrolled. Full details of clinical, demographic, immunological, and imaging assessments are summarized in Table 1.

Table 1: Demographic, clinical, immunological, and imaging data of PD patients

Characteristics	**Mean ± SD/ n (%)**
Clinical and demographic data	
23 patients	11 women/12 men
Age, years	66.04 ± 7.6
Disease duration, years	1.57 ± 0.8
UPDRS-III score	11.57 ± 4.7
Hoehn & Yahr stage	
- Stage 1	21 (91.3%)
- Stage 2	2 (8.7%)
Tremor dominant phenotype	16 (69.5%)
Side of onset, right	13 (56.5%)
MMSE score	28.02 ± 2.4
ACE-R score	92.39 ± 6.9
Immunological data	
WBC (10^3/µl)	6.38 ± 0.94
Lymphocytes (10^3/µl)	1.64 ± 0.39
Lymphocytes (%)	25.65 ± 4.90
CD4+ lymphocytes (10^3/µl)	0.71 ± 0.23
Th1 (% of CD4+)	13.84 ± 5.01
Th1 (10^3/µl)	0.10 ± 0.04
Th2 (% of CD4+)	8.34 ± 3.84
Th2 (10^3/µl)	0.06 ± 0.04

Th17 (% of CD4+)	11.69 ± 11.08
Th17 (10^3/µl)	0.07 ± 0.04
Th1/17 (% of CD4+)	10.96 ± 4.71
Th1/17 (10^3/µl)	0.07 ± 0.03
tTreg (% of CD4+)	8.56 ± 2.76
tTreg (10^3/µl)	0.07 ± 0.03
nTreg (% of Treg)	28.19 ± 8.77
nTreg (10^3/µl)	0.08 ± 0.15
aTreg (% of Treg)	27.88 ± 6.74
aTreg (10^3/µl)	0.05 ± 0.08
TBX21 (2-ΔCt)	3,781E-007 ± 1,389E-007
STAT1 (2-ΔCt)	1,947E-004 ± 1,080E-004
STAT3 (2-ΔCt)	3,671E-004 ± 1,659E-004
STAT4 (2-ΔCt)	2,489E-005 ± 2,783E-005
STAT6 (2-ΔCt)	5,205E-006 ± 7,681E-006
RORC (2-ΔCt)	2,135E-007 ± 9,105E-008
GATA3 (2-ΔCt)	9,542E-005 ± 7,904E-005
NR4A2 (2-ΔCt)	1,460E-004 ± 2,359E-004
FOXP3 (2-ΔCt)	7,776E-005 ± 7,240E-005
DAT-SPECT Imaging data	
maC uptake	2.616 ± 0.574
maP uptake	1.602 ± 0.430
laC uptake	2.851 ± 0.671
laP uptake	1.917 ± 0.474
tP/C	0.632 ± 0.130

tSBR	2.247 ± 0.455

Abbreviations: *UPDRS*, Unified Parkinson's Disease Rating Scale; *MMSE*, Mini-Mental State Examination; *ACE-R*, Addenbrooke's cognitive examination-revised; *tTreg*, total Treg; *nTreg*, naïve Treg; *aTreg*, activated Treg; *maC*, most affected caudate; *maP*, most affected putamen; *laC*, least affected caudate; *laP*, least affected putamen; *tP/C*, total putamen-to-caudate ratio; *tSBR*, total striatal binding ratio.

Continuous variables are expressed as mean ± standard deviation, categorical variables as counts (percentages).

Results of complete blood count analysis are shown in Supplementary Table S1.

Supplementary Table S1: Complete blood count of PD patients

Blood count (units)	**Mean ± SD**
RBC (10^6/µl)	4.80 ± 0.29
Hemoglobin (g/dL)	14.46 ± 0.84
Hematocrit (%)	43.27 ± 2.44
MCV (fL)	90.24 ± 3.37
MCH (pg)	30.18 ± 1.41
MCHC (g/dL)	33.43 ± 1.19
Platelets (10^3/µl)	229.27 ± 50.43
WBC (10^3/µl)	6.38 ± 0.94
Lymphocytes (10^3/µl)	1.64 ± 0.39
Lymphocytes (%)	25.65 ± 4.90
Monocytes (10^3/µl)	0.50 ± 0.16
Monocytes (%)	7.87 ± 2.46
Neutrophils (10^3/µl)	4.11 ± 0.67
Neutrophils (%)	64.47 ± 5.32

Eosinophils (10^3/µl)	0.09 ± 0.08
Eosinophils (%)	1.42 ± 1.12
Basophils (10^3/µl)	0.04 ± 0.02
Basophils (%)	0.62 ± 0.34

Abbreviations: *RBC*, red blood cells; *MCV*, mean corpuscular volume; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *WBC*, white blood cells.

Spearman correlation analysis was carried out to establish the relationship between demographic/clinical variables and immunological data. The UPDRS-III score correlated with tSBR (r = - 0.467, *P* = 0.025), the uptake in the maP (r = - 0.548, *P* = 0.007) and the total number of tTreg (r = 0.469, *P* = 0.037), nTreg (r = 0.503, *P* = 0.028), and aTreg (r = 0.493, *P* = 0.032). A direct correlation between disease duration and the total number of CD4+ (r = 0.539, *P* = 0.017), tTreg (r = 0.458, *P* = 0.042), and aTreg (r = 0.477, *P* = 0.039) was also found. Since gender and age may notably affect immunological assessment [18, 19], they were used as control variables in partial correlation analysis (see Table 2). We observed an inverse relationship between *FOXP3* mRNA levels and the uptake in the maC (r = - 0.542, *P* = 0.011), laP (r = - 0.467, *P* = 0.033), and tSBR (r = - 0.483, *P* = 0.027). Furthermore, we found an inverse correlation between the total number of peripheral CD4+ T cells and the uptake in the maP (r = - 0.490, *P* = 0.046). Concerning CD4+ T helper subsets, significant correlations between tP/C and the percentage of Th1 (r = 0.627, *P* = 0.009) and the total number of Th2 (r = - 0.517, *P* = 0.040) as well as between total count/percentage of Th2 and the uptake in the laC (respectively r = 0.586, *P* = 0.017; r = 0.543, *P* = 0.030), were detected. Regarding Treg subsets, we found significant inverse

correlations between the total number of nTreg/aTreg and the uptake in the maP (respectively, r = - 0.717, *P* = 0.001; r = - 0.691, *P* = 0.002) as well as between the count and percentage of nTreg and tP/C (respectively, r = - 0.601, *P* = 0.011; r = - 0.536, *P* = 0.026). However, after applying the Benjamini-Hochberg correction for multiple comparisons, significant correlations were confirmed only between the maP uptake values and the number of circulating nTreg and aTreg, see Table 2.

Table 2: Partial correlation analysis between immunological and DAT-SPECT measures

	[123I]FP-CIT SPECT measures					
Immunological measures	**maC (r; p)**	**maP (r; p)**	**laC (r; p)**	**laP (r; p)**	**tP/C (r; p)**	**tSBR (r; p)**
CD4+ T cell TF mRNA levels						
TBX21 (2-ΔCt)	0.207; 0.368	0.139; 0.549	0.260; 0.254	0.428; 0.053	-0.187; 0.416	0.298; 0.190
STAT1 (2-ΔCt)	-0.111; 0.632	-0.119; 0.606	0.139; 0.548	0.038; 0.871	-0.042; 0.858	-0.003; 0.990
STAT3 (2-ΔCt)	-0.062; 0.790	0.221; 0.336	-0.019; 0.606	0.040; 0.862	0.128; 0.582	-0.002; 0.992
STAT4 (2-ΔCt)	-0.318; 0.159	-0.075; 0.747	-0.111; 0.632	0.019; 0.934	0.337; 0.135	-0.152; 0.509
STAT6 (2-ΔCt)	-0.341; 0.130	-0.200; 0.385	-0.234; 0.307	-0.213; 0.354	0.193; 0.401	-0.291; 0.201
RORC (2-ΔCt)	-0.013; 0.957	0.155; 0.501	-0.005; 0.985	0.044; 0.851	-0.124; 0.594	0.040; 0.862
GATA3 (2-ΔCt)	-0.031; 0.893	0.049; 0.832	-0.016; 0.945	-0.108; 0.642	0.041; 0.642	-0.054; 0.817
NR4A2 (2-ΔCt)	-0.064; 0.784	0.050; 0.829	-0.057; 0.806	0.105; 0.652	0.222; 0.334	-0.002; 0.992
FOXP3 (2-ΔCt)	*-0.542; 0.011*	-0.286; 0.209	-0.361; 0.108	*-0.467; 0.033*	-0.055; 0.812	*-0.483; 0.027*
CD4+ T cell subsets						
CD4+ (10^3/µl)	0.057; 0.827	*-0.490; 0.046*	0.221; 0.395	-0.198; 0.446	-0.390; 0.064	-0.059; 0.822
Th1 (10^3 cells/µl)	-0.033; 0.904	-0.126; 0.641	-0.117; 0.665	-0.134; 0.621	0.139; 0.609	-0.115; 0.672
Th1 (% of CD4+)	-0.139; 0.609	0.268; 0.316	-0.343; 0.193	0.080; 0.768	*0.627; 0.009*	-0.104; 0.701
Th2 (10^3 cells/µl)	0.092; 0.734	-0.448; 0.082	*0.586; 0.017*	0.241; 0.369	*-0.517; 0.040*	0.231; 0.389
Th2 (% of CD4+)	0.128; 0.638	-0.153; 0.570	*0.543; 0.030*	0.353; 0.180	-0.355; 0.177	0.315; 0.235

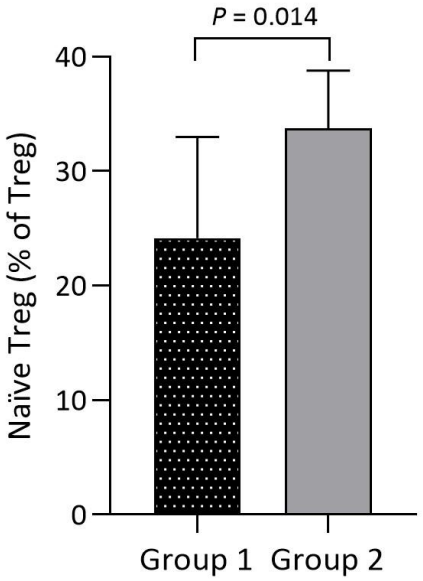
Th17 (10^3/µl)	-0.124; 0.646	0.050; 0.855	-0.117; 0.666	0.047; 0.863	0.301; 0.257	-0.075; 0.782
Th17 (% of CD4+)	-0.130; 0.631	0.164; 0.544	-0.147; 0.587	0.070; 0.798	0.370; 0.159	-0.059; 0.829
Th1/17 (10^3/µl)	0.008; 0.975	-0.039; 0.886	-0.220; 0.412	-0.295; 0.268	0.041; 0.880	-0.170; 0.528
Th1/17 (% of CD4+)	0.023; 0.931	0.337; 0.202	-0.293; 0.270	-0.118; 0.663	0.408; 0.117	-0.072; 0.790
tTreg (10^3/µl)	0.114; 0.653	-0.227; 0.364	0.018; 0.942	-0.125; 0.622	-0.231; 0.357	-0.040; 0.874
tTreg (% of CD4+)	0.125; 0.621	0.079; 0.756	-0.088; 0.728	0.020; 0.936	0.074; 0.771	0.028; 0.912
nTreg (10^3 cells/µl)	-0.115; 0.661	**-0.717; 0.001**	0.380; 0.132	-0.198; 0.446	*-0.601; 0.011*	-0.103; 0.693
nTreg (% of Treg)	0.076; 0.772	-0.471; 0.056	0.419; 0.094	0.013; 0.961	*-0.536; 0.026*	0.084; 0.748
aTreg (10^3 cells/µl)	-0.217; 0.402	**-0.691; 0.002**	0.052; 0.843	-0.397; 0.114	-0.455; 0.066	-0.308; 0.230
aTreg (% of Treg)	0.192; 0.460	0.384; 0.128	-0.212; 0.413	0.035; 0.893	0.236; 0.363	0.079; 0.764

Abbreviations: *maC*, most affected caudate; *maP*, most affected putamen; *laC*, least affected caudate; *laP*, least affected putamen; *tP/C*, total putamen-to-caudate ratio; *tSBR*, total striatal binding ratio; *tTreg*, total Treg; *nTreg*, naïve Treg; *aTreg*, activated Treg. Partial correlation analysis was conducted controlling for age and gender. Significant *P*-values are in italics, *P*-values that remained statistically significant after Benjamini-Hochberg correction for multiple comparisons are marked in bold; r=correlation coefficient.

Patients were then stratified into two groups based on the number of striatal regions affected. Group 1 (*n* = 14) consisted of patients with unilateral or bilateral putaminal involvement whereas Group 2 (*n* = 9) consisted of patients with three or four striatal regions affected. When comparing means between groups, we found in Group 2 a significantly higher percentage of circulating nTreg (33.74 ± 5.04) than in Group 1 (24.15 ± 8.82), *t* (17) = - 2.752, *P* = 0.014; see Figure 1.

Fig. 1: Percentage of naïve Treg in PD patients stratified according to the number of striatal regions affected.

Group 1 (*n* = 14): patients with unilateral or bilateral putaminal involvement; Group 2 (*n* = 9): patients with three or four striatal regions affected. Data are plotted as means with standard deviations as error bars.



Discussion

This study unravels novel correlations between [^{123}I]FP-CIT SPECT measures and the levels of peripheral CD4+ T lymphocytes in newly-diagnosed drug-naïve PD patients.

Concerning flow cytometry analysis of circulating CD4+ T cell subsets, we found a significant relationship between tP/C, caudate uptake, and the levels of both Th1 and Th2. Indeed, an impaired balance of peripheral CD4+ T cell subgroups characterized by a pro-inflammatory shift towards the Th1 subset [6, 20] and a markedly increased production of interferon-γ and tumor necrosis factor-α [6] was observed in previous studies in PD patients compared with healthy controls. It has been hypothesized that the differentiation towards the Th1 lineage is favored by an altered anti-inflammatory

response promoted by reduced and functionally impaired Th2 and Treg [6, 21, 22]. In particular, aTreg exert their immunosuppressive activity by secreting anti-inflammatory cytokines and killing autologous target cells in a perforin-dependent manner [23], whereas nTreg are thymus-committed cells with a high degree of self-reactivity continuously replenishing the aTreg pool [24]. Decreased effectiveness of the Treg compartment could promote chronic neuroinflammation through the disruption of immune tolerance. Indeed, reduced levels of circulating Treg [6] and their inability to suppress effector T cell function [22] have been previously reported.

In light of this background, the results of the present study further highlight the role of Treg in PD. Firstly, the strong negative correlations between levels of circulating nTreg and aTreg and the uptake in the maP were confirmed after controlling for the confounding effect of age and gender and after the Benjamini-Hochberg correction for multiple comparisons. Secondly, we found that patients with a more widespread reduction of striatal uptake have significantly higher levels of circulating nTreg than subjects with unilateral or bilateral putaminal involvement. Taken together, these findings suggest that the relationship between the Treg subset and DAT-SPECT denervation measures is stronger than the one observed with other immune cells. Nonetheless, whether the increase of Treg in more severe DAT loss may be compensatory has yet to be established.

Furthermore, our results support the role of CD4+ T cell subsets in the early stages of the disease. Several studies have observed in PD that the reduction of striatal uptake does not follow a linear model and is more rapid at the beginning [25]. Thus, the initial

phase may represent the best point to evaluate the contribution of inflammatory mechanisms to neurodegeneration. Since the Th1 pro-inflammatory bias is amplified by a dysfunction of the Treg compartment, a timely enhancement of this subpopulation could be crucial. For example, granulocyte-macrophage colony-stimulating factor (GMCSF) induced the production of protective Treg in the mouse model of PD [26], and Sargramostim (a human recombinant GM-CSF) determined motor improvement in 20 PD patients [27].

To the best of our knowledge, this is the first study providing evidence of significant correlations between DAT-SPECT measures and the involvement of peripheral CD4+ T lymphocyte subsets. This research involved a cohort of drug-naïve PD patients, thus excluding the potential interference of dopamine replacement therapy [28, 29]. Another strength is represented by the thorough immunological assessment, which included a detailed analysis of CD4+ T cell subsets and TF profile. Furthermore, patients recruited in this study entered a longitudinal protocol to evaluate the predictive value of peculiar immunological signatures on motor and non-motor outcomes, intending to establish at the same time their relationship with baseline DAT-SPECT measures. Nonetheless, some limitations should be highlighted as well: i) the cross-sectional design could not determine any causal relationship; ii) the small sample size limited the power of statistical analysis and prevented the investigation of different clinical subgroups.

Conclusion

This research, though exploratory, further strengthens the link between peripheral inflammation and neurodegeneration, pointing to the prominent contribution of Treg in the early stages of PD.

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Chapter 3. Discussion

Several studies showed that the immune system plays a key role in the pathophysiology as well as in the development of both motor and non-motor symptoms in PD.

Among non-motor symptoms, cognitive impairment (CI) is more common in PD patients compared with healthy controls [1] and contributes greatly to the disease burden. CI can significantly affect the quality of life (QoL) of PD patients and even at the early stages may determine substantial economic consequences [2, 3], thus representing a priority for both patients and caregivers. The spectrum of CI occurring in PD ranges from subjective cognitive decline (SCD) and mild cognitive impairment (PD-MCI) to dementia (PDD). SCD is a self-reported decline in cognitive function with overall normal cognitive tests [4], in PD-MCI there is a subjective impairment of cognitive abilities associated with objective cognitive deficits on formal neuropsychological testing or a scale of global cognitive abilities [5], and PDD is defined as CI with deficits in at least two of four cognitive domains that are severe enough to significantly affect daily activities beyond the impairment caused by other disease-related motor and non-motor symptoms [6]. Multiple cognitive domains can be affected in PD, including memory, attention, executive functions, and visuospatial abilities [7].

From the neuropathological perspective, all patients with PD display early loss of dopaminergic neurons in the substantia nigra (SN) and abnormal deposition of αsynuclein in Lewy bodies, but in PD-related CI coexisting Alzheimer's disease (AD) pathology can be observed as well, together with proteinopathy in limbic rather than brainstem regions [8]. Several mechanisms have been implied in the pathogenesis,

including the degeneration of neurotransmitter systems (dopaminergic [9], noradrenergic [10, 11], cholinergic [12], and serotoninergic [13]). Furthermore, genetic factors may considerably impact cognition in PD as well. For instance, carriers of *SNCA* mutations exhibit younger age at onset along with a faster disease progression in terms of both motor and cognitive decline and a greater burden of non-motor symptoms [14]. Severe *GBA* gene variants determine impaired cognitive status and more rapid cognitive deterioration [15]. In addition, progression and increased cognitive deterioration in PD are associated with the *APOE* (encoding apolipoprotein E) ε4 allele [16, 17], which may predispose to β-amyloid deposition over time.

Many of the pathologies associated with CI can be identified *in vivo* using imaging and biological markers. These biomarkers can be used to provide a more extensive knowledge of the mechanisms underlying CI in PD, thus aiding the identification of patients with an increased risk of early and fastly progressing cognitive decline. Temporo-parietal atrophy on MRI is one of the first identified markers [18], together with basal forebrain atrophy [12]. Hypometabolism in the medial frontal and parietal regions using FDG-PET [19] and more recent MRI techniques (such as diffusion tensor imaging) are also providing valuable associations with cognitive function [20]. Other biomarkers, such as CSF markers of AD pathology, have been proved useful as well. Indeed, low levels of amyloid-$\beta_{1\text{-}42}$ were associated with the development of MCI or dementia in PD [21], and CSF tau levels in combination with CSF Aβ42 and caudate [^{123}I]FP-CIT uptake were useful in predicting the development of CI [22]. On the other hand, α-synuclein as a biomarker of PD-related-CI has yielded conflicting results,

probably because of the central role of α-synuclein in PD itself. CSF levels of total αsynuclein have been inconsistently associated with cognitive deterioration, as both reduced and increased concentrations have been reported [23], even though studies using novel seed technologies may provide in the future more accurate predictors of cognitive decline [24]. Concerning plasmatic biomarkers, recent research [25] found that plasma neurofilament light chain (NfL) levels predicted Mini-Mental State Examination (MMSE) decline over time in PD, whereas no significant association between plasma ptau181 concentration and baseline or longitudinal cognitive performance was found.

There is growing evidence also supporting the role of quantitative electroencephalography (EEG) as a diagnostic marker: background slowing and spectral power analysis performed with machine learning techniques have been suggested as feasible biomarkers of cognitive decline in PD [26]. Concerning other neurophysiological markers, Zhang et al. found higher latency and lower amplitude of P300 (an eventrelated potential component involved in active cognitive processing, especially attention and memory) in 53 drug-naïve PD patients compared with healthy controls [27]. Furthermore, a meta-analysis showed that PDD patients exhibit prolonged P300 latency at the Cz site compared to non-demented PD patients, thus suggesting the valuable role of P300 and other event-related potential components as valuable markers of CI in PD [28].

In this complex scenario, the immune system may be involved in the mechanisms underlying PD-related CI. Research involving animal models and patient studies suggests a connection between neurodegenerative diseases, neuroinflammation, and the

activation of the immune system [29]. This process is triggered by microglia and astrocytes and leads to a subsequent release of pro-inflammatory and immune mediators, such as cytokines and chemokines, and the production of reactive oxygen species (ROS) and nitric oxide (NO). The belief that the central nervous system (CNS) is immune-privileged and thus protected from the infiltration of immune mediators from the periphery has been challenged over recent years. Indeed, in addition to the presence of activated microglia and astrocytes, neurodegenerative diseases often exhibit lymphocyte infiltration from the periphery [29].

As reported in our studies, PD patients with cognitive deficits display an altered peripheral immune phenotype characterized by higher levels of circulating lymphocytes, lower neutrophil-to-lymphocyte ratio (NLR) [30], and an altered Treg compartment [31]. A profound decline in adaptive immunity, compared to the innate immunity response, has been observed in neurodegenerative disorders, including PD and AD. The concept of “inflammaging” (age-dependent inflammation) describes the chronic, low-grade inflammation that occurs during aging [32], and is regarded as one of the main contributors to age-related diseases. Inflammaging is triggered by the accumulation of microbiota products and damaged or misfolded molecules [33] and leads to the activation of both pro-inflammatory and anti-inflammatory processes. Inflammaging is tightly connected to immunosenescence, and this interaction leads to increased vulnerability to foreign pathogens and the onset of tumors, altered naïve T cell/memory cell ratio, a decrease in the immune responses, and a steady state of low-grade inflammation [34, 35].

We found that Treg cells are particularly crucial in this impaired immune network and significant associations with both CI and the degeneration of the nigrostriatal pathway were observed, thus implying the prominent role of this subset in the pathophysiology of PD. Since Tregs are central to immune tolerance against autoimmunity, the proportion and function of these cells have been investigated in the context of inflammaging in many studies. The production of thymically derived Tregs declines with age [36], and in mucosal compartments of aged mice, Tregs were reported to be dysfunctional [37]. They expand in response to interleukin (IL)-6, upregulate the effector cytokine interferon (IFN)-γ, and lack immunomodulatory functions. Therefore, dysbiosis in mucosa may contribute to the dysregulation of Tregs, ultimately contributing to the worsening of inflammation in elderly individuals. PD patients, in particular, exhibit reduced Treg numbers and/or function as well as increased number~~s~~ of effector T cells compared with matched controls [38–40]. In animal models, immunization of mice with *α*-synuclein reduces Treg function and induces *α*-synuclein-specific effector T cells, whereas adoptive transfer of Tregs restored immunological homeostasis~~,~~ and attenuated neuroinflammation with amelioration of nigrostriatal dopaminergic neurodegeneration [41].

In this scenario, the altered balance between the Th1 and Treg subsets is essential for the establishment of the so-called “Th1 pro-inflammatory bias” [38]. We observed that mRNA levels of transcription factors (TF) STAT1 and NR4A2, involved in the development of these lymphocyte subpopulations, are altered in PD patients without motor complications (MC).

It is known that MC may arise after continued treatment with levodopa, and are characterized by reduced duration of response, dose failures, dose variability, and dyskinesias [42]. The etiology of the shortened duration of effect and inconsistency of response to levodopa is probably due to pharmacokinetics and the altered absorption of levodopa through the duodenum [43]. In the earliest phases of the disease, when dopaminergic cell loss is less severe, the efficacy is maintained over time because levodopa is taken up into the dopaminergic cells and then released physiologically. As the disease progresses, the brain loses this buffering capacity, and the clinical effect follows the pharmacokinetics of the drug. The mechanisms underlying drug-induced dyskinesia are even more complex: apart from presynaptic dopaminergic cell loss, phenomena of striatal neuronal plasticity are involved, including changes in gammaaminobutyric acid (GABA) receptors. Total levodopa dose and the modality of levodopa administration may influence the occurrence of dyskinesia, along with non-motor features and demographic factors (e.g., younger age at PD onset and lower body mass index) [44]. Management of MC can be challenging for physicians, and several pharmacological and surgical interventions are available. Imaging measures have been proposed as indicators of MC in advanced PD: Hong et al. reported that lower DaT activity on ^{18}F-FP-CIT positron emission tomography (PET) might be a significant predictor of the development of dyskinesia [45]. Furthermore, the size of neuromelanin-positive substantia nigra pars compacta area (NM-SNc) on neuromelanin magnetic resonance imaging (NM-MRI) is significantly associated with MC occurrence, reflecting the status of advanced PD more accurately than DaT-SPECT [46, 47].

Nonetheless, a reliable biomarker to identify patients who will develop more severe trajectories of motor fluctuations is still an unmet need in PD.

Future perspectives

As the field of neurodegeneration moves forward in understanding the role of central and peripheral inflammation in the pathogenesis and progression of PD, one major challenge is the identification of immune-based therapies holding meaningful translational potential.

Studies targeting the immune system with a disease-modifying approach in PD are rapidly accumulating, and the repurposing of existing drugs is being explored. For instance, fingolimod and glatiramer acetate are disease-modifying drugs employed in the treatment of multiple sclerosis (MS). Fingolimod is a molecule sharing with pramipexole the activation of an intracellular antiapoptotic pathway involving the sphingosine-1- phosphate (S1P). The use of fingolimod reduced the myeloid major histocompatibility complex (MHC)II response to α-synuclein in a PD mouse model [48]. Glatiramer acetate antagonistically binds to MHCII complexes, thus blocking antigens from presenting to T cells, and alters the differentiation of T cells preferentially stimulating T-helper 2 (Th2) over T-helper1 (Th1) subsets [49]. Glatiramer acetate administration in the parkinsonian mouse model restored tyrosine hydroxylase positive neurons through the increase of brain-derived neurotrophic factor (BDNF) levels and counteracted the expression of microglial marker (IBA1) and α-synuclein accumulation, leading to clinical improvement [50]. Conversely, intravenous immunoglobulins (IVIG), a blood-derived product commonly used in autoimmune diseases, did not exert a

restorative effect on the nigrostriatal system in the 1-methyl-4-phenyl-1,2,3,6tetrahydropyridine (MPTP)-treated mice [51]. Other immunosuppressant drugs were investigated as well: azathioprine is an immunosuppressor commonly used in autoimmune disorders and inhibits nucleic acid synthesis, thus reducing lymphocyte proliferation [52]. AZA-PD is an ongoing phase II trial evaluating whether the suppression of the peripheral immune system using azathioprine has a diseasemodifying effect in PD [53].

Drug repurposing was not limited to immunomodulatory agents, as several compounds employed in other conditions (such as asthma and diabetes) have been explored as therapeutic strategies in PD. Peroxisome proliferator-activated receptor (PPAR) receptor agonists are used for the treatment of diabetes and have been shown to promote microglial phenotypic conversion from the pro-inflammatory M1 state to the anti-inflammatory M2 state [54]. Rosiglitazone, a PPARγ agonist, suppressed the degeneration in both the striatum and SN by reducing the production of tumor necrosis factor (TNF)-α and modifying microglial polarization in the PD mouse model [55]. Another PPARγ agonist, pioglitazone, could reduce microglial activation, the production of iNOS, and toxicity [56]. Nonetheless, a phase 2 clinical trial failed to prove any diseasemodifying effect at the doses of 15 and 45 mg in early PD patients [57]. Another compound, the glucagon-like peptide-1 receptor (GLP1R) agonist NLY01, was able to inhibit in the mouse model of PD the phenoconversion of astrocytes towards the proinflammatory A1 phenotype, known to exert detrimental effects [58].

Regarding the repurposing of asthma drugs, beta2 adrenoceptor (β2AR) agonists have been investigated for their role in the modulation of both α-synuclein gene expression and immune function [59]. Immune cells express β2ARs, and their activation may exert anti-inflammatory properties through the modulation of Th2 and Treg responses along with the reduction of ROS production [60]. Albuterol, administered as adjunctive therapy, determined an improvement in parkinsonian symptoms and motor fluctuations [61] and recently entered into a phase 2 randomized controlled platform trial of putatively protective, approved medications in manifest PD (ACTRN12620000560998) [62]. From an epidemiological perspective, chronic therapy with β2AR agonists was associated with a lower risk of developing PD [63, 64].

Lastly, one promising therapeutic avenue is represented by drugs that enhance Treg activation or recruitment. Immunomodulating agents acting on the Treg compartment include vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [65]. VIP promoted the restoration of Treg activity attenuating neuroinflammation [66], and PACAP preserved the dopaminergic neurons from neurodegeneration and ameliorated motor disorders in rat and snail animal models [67]. GM-CSF attenuated nigrostriatal degeneration through Treg induction and activation of protective pathways linked to the upregulation of IL-27 [68]. This intriguing preclinical evidence led to the use of sargramostim, a human recombinant GM-CSF, in human clinical trials: the subcutaneous administration of this drug at 6 μg/kg/day for 56 days increased the numbers of Tregs and determined modest improvement in the UPDRS-III after 6 and 8

weeks of treatment compared with placebo [69]. Since some adverse events were noticed, another study explored long-term sargramostim treatment at 3 μg/kg/day in 5 PD patients. Reductions in adverse events, as well as an increase in peripheral blood Treg numbers, function, and hypomethylation of upstream FoxP3 DNA elements, were observed [70]. Furthermore, there was no worsening of motor function scores for any subject during treatment.

Alternatively, another approach to enhance the Treg compartment is to isolate and purify Tregs from peripheral blood, expand them *in vitro*, and administer autologous infusions of expanded Tregs, as reported in a recent phase I trial involving patients with amyotrophic lateral sclerosis (ALS) [71].

Conclusions

Several lines of evidence converge on the significant role of the immune system in the pathophysiology and progression of PD, even though some questions are still unanswered. Interest in the role of the immune system is growing rapidly: in 2002 there were fewer than 50 studies published on PD and inflammation, and this number has increased more than tenfold over the last years [72]. The most intriguing advantage of exploring the relationship between immunity and PD is that, despite the multiple triggering factors (both genetic and environmental), inflammation represents a common pathway by which the disease progresses.

As in other neurodegenerative disorders, the outcome of anti-inflammatory drug trials in PD has been discouraging thus far, but substantial remarks in the approach to such trials should be considered. First of all, the selection of patient cohorts is a critical issue

for the outcome: if the inflammatory process begins early in the disease process, an antiinflammatory intervention starting after the onset of motor features is unlikely to have a disease-modifying effect. The immune system should be interrogated in the prodromal stage of the disease to provide successful target engagement. Secondly, to establish the contribution of the immune system in the prediction of disease trajectories, we need longitudinal evaluations of immune cells from subjects at genetic and environmental risk for PD, to perform comprehensive profiling. Furthermore, the use of comparable methodologies (i.e., standardized markers for the identification of different cell subsets) is warranted to avoid contradictory findings. Lastly, since immune-related genes may vary consistently across different populations, immune-based therapies may display different efficacy based on ethnicity and should therefore be tested in cohorts with distinct ethnic backgrounds.

If these issues will be correctly addressed, a deeper understanding of the immunological mechanisms underlying PD will open novel therapeutic avenues providing immunomodulatory therapies able to slow or even delay the progression of the disease.

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