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AN EXPLORATIVE ASSESSMENT OF POTENTIAL NOVEL DIAGNOSTIC AND PROGNOSTIC BIOMARKERS FOR IDENTIFICATION OF PRODROMAL PARKINSON'S DISEASE

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Summary

ABSTRACT

Background - Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer, primarily affecting about 6 million people worldwide.

An early identification of PD is one of the main challenges in neurological research because to date, its diagnosis is still largely based on the clinical assessment of cardinal motor signs (bradykinesia, rigidity, resting tremor and postural instability) resulting by a progressive degeneration of dopaminergic neurons of the substantia nigra and locus coeruleus. However, impaired motor function appears when over 60% of the dopaminergic neurons are degenerated in the brain. In recent years, several evidence indicates that the onset of PD happens years to decades before the occurrence of classic motor symptoms. Pathological and imaging studies, for example, suggest that signs of nigrostriatal lesion can be detected 5–10 years before this clinical stage, and various observational prospective studies reveal that several non-motor symptoms (NMS) occur in this pre-diagnostic phase. Actually NMS such as olfactory impairment, cardiovascular dysautonomia as orthostatic hypotension (OH) and rapid eye movement (REM) behaviour disorder (RBD) are currently being studied as features of prodromal PD and seem to be correlated to the early neuropathological process of disease.

Beside these clinical manifestations, other biological alterations such as elevated oxidative stress and pro-inflammatory response have been involved in the cascade of events leading to degeneration of dopaminergic neurons. Recently, microRNAs (miRNAs) have been recognized as potent post-transcriptional regulators of PD-related gene expression.

Consequently, the characterization of several NMS together with the assessment of molecular biomarkers linked to inflammation and oxidative damage, could be a potential methodological approach for the early identification of PD patients.

Objectives - The main objective of my study was to explore potential novel diagnostic and prognostic biomarkers of PD. Specific study aims were, in patients with prodromal and established PD: a) to evaluate clinical markers such as olfactory and cardiovascular autonomic functions; b) to measure circulating mediators of oxidative stress and inflammatory response as early biomarkers of organ failure; c) to correlate biological findings with clinical functional alterations; d) to characterize specific circulating miRNA profiles in plasma samples.

Methods - For this purpose, we recruited 15 patients with overt PD (Hoehn and Yahr stage I-III, on L-DOPA and dopamine agonists combination therapy), 11 subjects diagnosed with idiopathic RBD (iRBD) confirmed by lack of atonia during the REM sleep phase on polysomnography and 12 age- and gender-matched controls (CTRL).

All enrolled subjects underwent the following assessments: total olfactory score (TOS) using Sniffin' Sticks Extended Test; autonomic function by measuring heart rate variability during deep breathing (DB) test, which expresses parasympathetic function, lying to standing (LS) test and the Valsalva manoeuvre (VM), that gives information about both sympathetic and parasympathetic function; antioxidant/oxidative stress mediators [glutathione (GSH), the most important endogenous scavenger, assessed in total and reduced form and in plasma and blood samples according to a high performance liquid chromatographic (HPLC) method; plasma malondialdehyde (MDA), a marker of lipid peroxidation, assayed by HPLC with fluorescence detection; 8-hydroxy-2-deoxyguanosine (8-OHdG), index of oxidative DNA damage, and 3-nitrotyrosine (3-NT), a stable end product of peroxynitrite oxidation, analyzed by commercial ELISA kits]; inflammatory response [plasma concentrations of tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL1β), the most important inflammatory cytokines, measured by ELISA commercial kits; urine neopterin levels, a sensitive marker of cellular-mediated inflammation, by an isocratic HPLC method]. Biochemical parameters were than correlated with clinical functional results.

The miRNA profiling was performed in a subpopulation of the enrolled subjects (4 PD, 4 iRBD and 4 CTRL) by small RNA Sequencing, using Miseq sequencer (Illumina). The differentially expressed (DE) miRNAs analysis, based on the negative binomial distribution, was performed with DE Seq2 by performing three comparisons:1) iRBD versus CTRL; 2) PD versus iRBD; 3) PD versus CTRL. Subsequently, the relative expressions of specific miRNAs were validated in all study population by quantitative real-time (qRT) PCR using miScript PCR System kit (Qiagen).

Results - A significant worsening trend was observed in total olfactory score, blood reduced GSH, LS and VM ratio and neopterin from the reference controls to iRBD and PD groups. In the multivariable ordinal logistic regression model, only low blood reduced GSH levels ($p=0.037$, OR=0.994; 95% CI 0.988 – 1.000), adjusted by history of hypertension, total olfactory score, LS ratio and VM ratio, were associated to PD status. Functional anosmia was similarly prevalent in iRBD (36%) and PD (33%) patients, but

was absent in CTRL (p= 0.097). OH was more common among iRBD (73%) and PD (60%) than in controls (25%) (p=0.055), independently of antihypertensive treatment.
A direct correlation was observed between total olfactory score and blood reduced GSH concentrations (R=0.034, p=0.037) and with VM ratio (R=0.43 p=0.015). Conversely, an inverse relation was found between total olfactory score and urine neopterin levels (R=-0.39 p=0.016).

The results on circulating miRNA profiles found about 889 thousand sequenced reads mapped to mature miRNA sequences annotated in miRBase v21, by small RNA sequencing analysis. After data processing, no statistically significant DE miRNA was observed in the PD versus CTRL, whereas we found 33 DE miRNAs (18 downregulated, 15 upregulated, p-value <0.005) in the comparison between PD and iRBD and 6 (3 downregulated, 3 upregulated, p-value <0.005) in iRBD versus CTRL.
Four common DE miRNAs (miR-101, miR-1260a, miR-142, miR15a) were dysregulated between the two different comparisons. In the PD patients, three miRNAs (miR-101, mir-142 and miR15a) were downregulated (Fold Change < -0.5) and only mir-1260a was upregulated (Fold Change > 0.5) with respect to iRBD. Conversely, miR-101, miR-142 and miR15a were upregulated and miR-1260a downregulated in iRBD compared to CTRL. The NGS results have not been validated by RT-PCR analysis till now because these miRNAs are poorly expressed in plasma. This condition makes very difficult, from a methodological point of view, their extraction and quantification.
Discussion - The main findings of the present study are that reduced systemic antioxidant capacity is independently associated to overt PD and iRBD, a condition now established as prodromal PD, and correlates with olfactory and sympathetic dysfunction. Moreover, progressive cardiovascular autonomic dysfunction, expressed as altered sympathetic (VM ratio, OH) or parasympathetic (LS ratio) response to testing, is found from prodromal state to overt disease and correlates with olfactory dysfunction. Increased concentrations of neopterin, an inflammatory biomarker, are associated with worse olfactory dysfunction.
The NGS analysis highlights a miRNA profiling in PD and iRBD subjects that needs to be verify, by changing and modifying the methodological approach for miRNA quantification.
Conclusions - Reduced systemic antioxidant capacity is found in prodromal and overt PD and may represent, in association with olfactory loss and cardiovascular autonomic dysfunction, a useful additive biomarker of disease. Moreover, the present miRNA profiling study allowed to identify a set of differentially modulated miRNAs, in the overt

PD with respect to prodromal phase despite the analysis was no validated by RT-PCR. Our pilot findings need to be confirmed in a larger population to establish their actual clinical value for an early diagnosis of PD.

1. BACKGROUND

1.1 Parkinson Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer, primarily affecting about 6 million people worldwide [1].

Pathologically, PD is characterized by the significant degeneration of dopaminergic neurons in the substantia nigra pars compacta of the midbrain. This degeneration is accompanied by reactive gliosis in specific areas (ie, vulnerable) of the nervous system and by the presence in the remaining substantia nigra neurons of eosinophilic intra-cytoplasmic inclusions, known as Lewy bodies (LB), of which misfolded α-synuclein is a major component [2].

The dopamine loss within the basal ganglia, a cluster of deep nuclei that participate in the initiation and execution of movements, leads to typical movement disorders of PD patients [3]. Cardinal motor manifestations for PD include: bradykinesia, rigidity, resting tremor and postural instability [4].

The prevalence of PD in industrialized countries is generally estimated at 0.3% of the entire population and increases with age from 1% in people over 60 years to 4% of those over 80, and is projected to double by 2030 in parallel with an increasing aging population. Ten% of cases are classified as young onset, occurring between 20 and 50 years of age, and may represent a distinct disease group. Reported standardized incidence rates of PD are 8–18 per 100.000 person-years [5].

The molecular mechanism of neurodegeneration in PD is mostly unknown. Recently, the discovery of a number of monogenetic mutations in several causative loci explain the origin of familial cases of PD, although they consider only 5–10% of patients. Over 90% PD cases are in fact idiopathic [4]. At present, it seems likely that the vast majority of sporadic cases are due to a complex interaction among genes, environmental factors and brain aging [6]. In idiopathic PD, mitochondrial dysfunction, oxidative stress, and protein damage induced by non-genetic factors, probably in interaction with susceptibility genes, are currently considered to have a central pathogenetic role in PD [7].

To date, its diagnosis is still largely based on the clinical assessment of cardinal motor signs [8]; however, by the time impaired motor function appears, over 60% of the dopaminergic neurons are degenerated in the substantia nigra [3].

Unfortunately, clinical PD features, especially in early stages of the disease, are common with those of other neurodegenerative and movement disorder, resulting with frequent misdiagnosis [9].

A number of clinical rating scales are used to evaluate the motor impairment and disability in PD patients, but most of them have not been fully assessed for validity and reliability [10,11]. The Hoehn and Yahr scale is commonly used to compare groups of PD patients and to provide overall assessment of symptoms progression, ranging from stage 0 (no signs of disease) to stage 5 (wheelchair bound or bedridden unless assisted) [12]. The Unified Parkinson's Disease Rating scale (MDS-UPDRS) is the most well established scale used to follow the longitudinal course of the disability and impairment of the disease [13]. Studies making use of UPDRS scale to track the severity of PD suggest that the PD progression is not linear and that the rate of deterioration is variable and more rapid in the early phase of the disease and in patients with the postural instability gait difficulty [14-16].

However, PD can only be definitely confirmed through its pathological hallmark of LB and Lewy neurites located in residual neurons or axons, respectively, upon post-mortem analysis [17].

Therefore, an early identification of PD is one of the main challenges in neurological research to better understand, characterize, and identify features of the preclinical phase of PD.

Currently, no reliable and clinically validated biomarker has been yet identified to refine the PD diagnosis and to objectively monitor the severity and the rate of progression of PD dysfunction and neurodegeneration in the substantia nigra [18].

1.2 Preclinical phase of PD

Several evidence indicates that the onset of Parkinson's disease happens years to decades before the occurrence of classic motor symptoms. Pathological and imaging studies, for example, suggest that signs of nigrostriatal lesion can be detected 5–10 years before this clinical stage (19), and various observational prospective studies [20,21] reveal that several non-motor symptoms (NMS) occur in this prediagnostic phase [22].

The early presence of NMS in the majority of PD patients may suggest an opportunity for early diagnosis and early treatment of PD, with consequent benefits to patient quality of life and potential treatment cost savings [23].

Beside the cardinal motor symptoms, PD patients also show a broad spectrum of NMS. These include disorders of mood, depression, cognitive dysfunction and hallucinosis, as well as sensory dysfunction with hyposmia and pain, disturbances of sleep–wake cycle regulation. Also autonomic alterations, including orthostatic hypotension, urogenital dysfunction and constipation are present to some degree in PD patients [24].

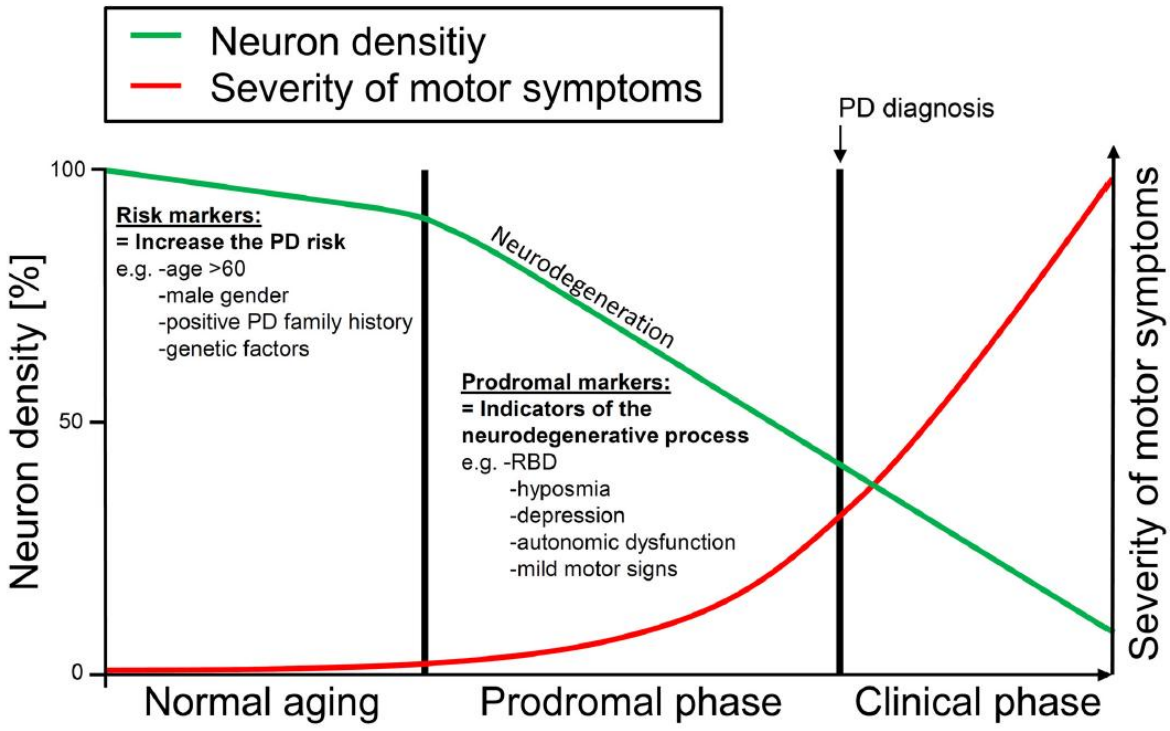

Figure 1. Graphical illustration of the prodromal phase occurring years or even decades before the clinical diagnosis of PD. Adapted from ref. [25].

Although NMS can occur at the same time or follow the onset of motor abnormalities, many of them often predate the onset of motor symptoms by many years and are currently being studied as features of prodromal PD [3]. However, many NMS are common in the general population even unrelated to underlying PD, so they might not be, at least in isolation, good markers for early PD identification. Conversely orthostatic hypotension (OH) and rapid eye movement (REM) behaviour disorder (RBD) are less commons and might be more specific clinical markers of prodromal PD (Figure 1).

Whereas the causes of motor dysfunction in PD are reasonably well understood, the origin of NMS has been largely related to pathology outside of the basal ganglia [26].

In fact, postmortem evaluation of PD brains has revealed more widespread degeneration in non-dopaminergic systems including several brainstem nuclei [raphe nucleus, locus

coeruleus (LC), dorsal vagal nucleus], limbic and neocortical structures, as well as the peripheral autonomic system [27,28].

The degenerative process, proposed by Braak and colleagues, reflecting the stepwise progression of LB pathology in the brain (Figure 2), begins at 'induction sites' with degeneration of the olfactory bulb and the anterior olfactory nucleus (clinically manifest as olfactory dysfunction) at stage 1, while stage 2 exhibits progression of the pathological process to the lower brainstem [29] such as the raphe nucleus, LC and pedunculo pontine nucleus that may be responsible for prominent early sleep, autonomic and mood disturbances [26].

The typical motor signs of PD is only apparent at stages 3 and 4 when the SN and mid and forebrain nuclei are affected. Stages 5 and 6 are associated with Lewy bodies in the limbic and mature neocortex and clinical correlates include the development of hallucinations and dementia [30].

Consequently, autonomic physiology in PD may provide additional clarification on the nature of PD as a multi-level widespread neurodegenerative process, as PD pathology may directly lead to autonomic dysfunction [31]. These neuropathological changes might be responsible for the occurrence of dysautonomic symptoms, hyposmia, and RBD before the onset of parkinsonism.

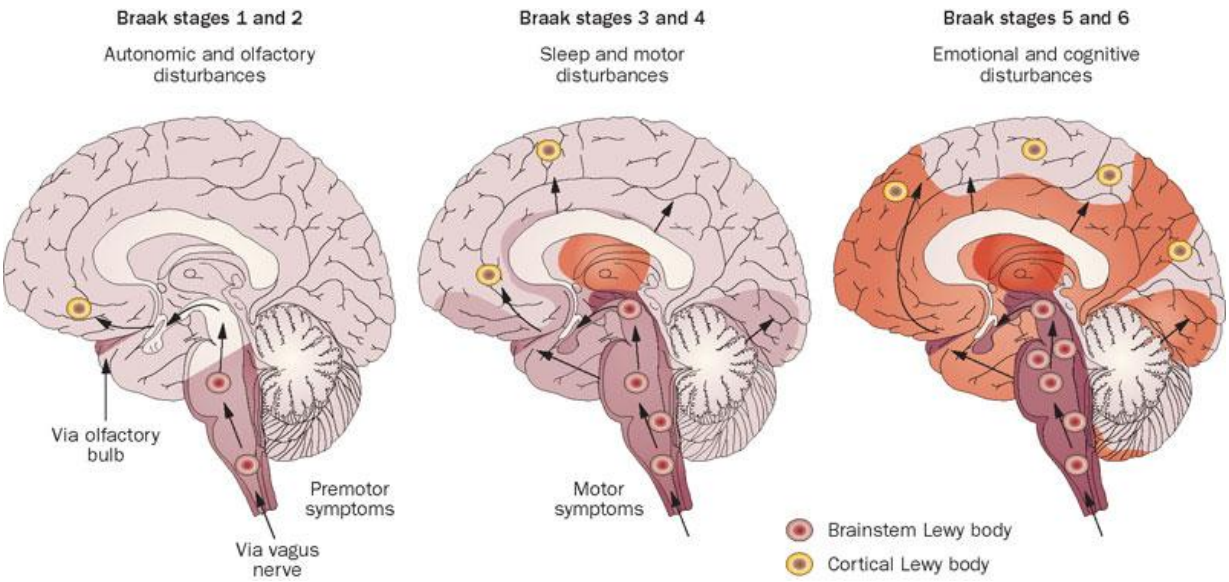

Figure 2. Stepwise progression of Lewy Bodies in the Brain. Adapted from ref. [29].

1.3 Clinical biomarkers

1.3.1 Olfactory dysfunction

Neurodegeneration in PD has been shown to occur with earliest involvement of the olfactory bulbs. Recent findings of decreased olfactory bulb volume in volumetric magnetic resonance imaging (MRI) scans of PD patients lent *in vivo* support to this neuropathological hypothesis [32]. It is hypothesized that neurons within the microglia-rich olfactory bulb, that have high metabolic activity and reduced antioxidant capacity, may be more susceptible to mitochondrial function impairment, oxidative stress, and excitotoxicity [33].

Olfactory dysfunction of PD is well characterized phenotypically, with validated olfactory tests able to differentiate PD patients from controls better than clinical motor tests [34]. Clinically, olfactory dysfunction has a prevalence of up to 90% in established PD, and smell loss is relatively stable over time, and unrelated to disease stage or duration. It is not improved by drugs that control motor symptoms. Furthermore, losses in both olfaction and cardiac sympathetic and parasympathetic function as well as vascular sympathetic dysfunction appear to be closely related [35].

Longitudinal studies demonstrated that olfactory dysfunction often predates motor signs by several years and holds therefore potential as an early marker for subjects at risk to develop PD [36,37] and its accompanying non-motor features [33].

1.3.2 Cardiovascular dysautonomia in PD

Clinical manifestations of dysautonomia, common NMS in PD, are linked to differential involvement of the autonomic nervous system components as recently reviewed [38,39]. The sympathetic noradrenergic system is the sympathetic nervous system (SNS) component responsible for reflexive constriction of blood vessels and stimulation of the heart. The sympathetic cholinergic system mediates sweating. The parasympathetic nervous system (PNS) is responsible for different phenomena including respiratory sinus arrhythmia, gastrointestinal and urinary bladder tone, salivation, lacrimation, and pupillary constriction [38].

Cardiac and extra-cardiac noradrenergic denervation and baroreflex failure occur independently of striatal dopamine depletion in PD. Loss of cardiac sympathetic noradrenergic nerves is virtually universal in PD, and seems to be independent of the

movement disorder in individual patients and is correlated with olfactory dysfunction, also an early sign of neurodegenerative disorders [40,41].

Plasma levels of the sympathetic neurotransmitter, norepinephrine and its main neuronal metabolite, dihydroxyphenylglycol (DHPG), are normal in PD. Some evidences [42] about bases for cardioselectivity of sympathetic noradrenergic denervation in PD may be that the myocardium contains a high tissue concentration of norepinephrine (NE), implying relatively dense innervation; cardiac sympathetic nerves remove circulating catecholamines from the coronary arterial blood; and there is greater production of DHPG, the product of NE oxidation, than other organs, suggesting a high rate of production of potentially toxic quinones, aldehydes, and other oxidation products [38].

Postmortem studies as well as in vivo imaging with ^{123}I-metaiodobenzylguanidine (MIBG) in single-photon emission computed tomography (SPECT) studies suggest that there exist an early, cardioselective, postganglionic denervation in PD patients [43,44].

Although extra-cardiac noradrenergic denervation also occurs and results in inadequate vasoconstriction, among PD patients there is greater loss of noradrenergic innervation in the heart than in the body as a whole, as documented by normal plasma levels of both NE and dihydroxyphenylglycol [24].

The coexistence of cardiac and extra-cardiac noradrenergic and arterial baroreflex failure in PD result in a syndrome that includes OH, which found in about 30-40% of PD patients [45], post-prandial hypotension, blood pressure lability, supine hypertension, and possibly fatigue and exercise intolerance [35].

Measurement of heart rate variability (HRV), a physiological phenomenon where the time interval between heart beats varies, is a simple non-invasive method to study changes in cardiovascular autonomic control. HRV reflects the relationship between the PNS and SNS. In PD patients, spectral components of HRV are lower during wakefulness and appear to have an inverse correlation with disease severity. Abnormalities in cardiovascular autonomic controls elicited by head tilting including blunted heart rate and LF/HF ratio increase have been demonstrated in PD patients with and without OH [46].

Interestingly, one study reported that cardiac autonomic denervation in PD patients, as measured by HRV, seems to be predominantly associated with the presence of RBD [47]. Decreased HRV has also been observed in premotor PD patients [38] and particularly in patients with RBD [48].

1.3.3 REM sleep Behaviour Disorder (RBD)

RBD is a parasomnia characterised by loss of normal skeletal muscle atonia during REM sleep and associated with vivid dreaming and complex motor activity. The prevalence of RBD in the general population is approximately 500 per 100 000 (0.5%) [49].

RBD may be idiopathic or may precede a neurodegenerative disease: about half of RBD cases are associated with neurological disorders, most often the α-synucleinopathies: Parkinson disease, dementia with Lewy bodies, and multisystem atrophy. RBD is a NMS that occurs in the early stages of PD as it tends to manifest prior to the onset of parkinsonism and then decreases in frequency and severity over time [50]. In patients with isolated RBD, imaging studies have indicated a small but significant symmetrical reduction in striatal dopaminergic uptake, which may be suggestive of preclinical Parkinson's disease [51].

The prevalence of probable clinically significant RBD associated with PD is 15 per 100 000. In PD patients, RBD is associated with male gender, less parkinsonism and higher levodopa equivalent dose.

In addition, it has been shown that several iRBD patients eventually developed Parkinsonian diseases: after 4-5 years since the iRBD diagnosis, between 28% to 45% of patients developed PD or multisystem atrophy [49,52-54]

Reported rates of neurological-disease-free survival from time of iRBD diagnosis are 65.2% at 5 years and 26.6% at 10 years [52] and the median interval between iRBD diagnosis and diagnosis of a defined neurodegenerative syndrome was 6 years [49].

Cross-sectional studies in iRBD patients have shown an impressive convergence of cardiovascular and neurological alterations: substantial proportion of them have detectable abnormalities on measures of smell testing, colour vision and discrimination, cardiac autonomic activity, cardiac metaiodobenzylguanidine imaging, motor and gait functioning, neuropsychological testing, electroencephalography, transcranial sonography, MRI or magnetic resonance spectroscopy, dopamine transporter imaging single-photon emission computed tomography (SPECT), and fluorodeoxyglucose and dihydrotetrabenazine positron emission tomography (PET) [49, 55].

The study of patients with iRBD gives us the opportunity to investigate early disease events and changes using clinical, imaging, and biochemical biomarkers before the onset of the cardinal motor and cognitive manifestations of Lewy body disorders.

About half of patients with PD do not have RBD, in those with RBD, only 18% report that dream-enacting behaviours preceded the onset of parkinsonism [49].
RBD is a potential candidate for the study of early events and progression of this phase and to test disease-modifying strategies to slow or stop the neurodegenerative process.

1.3.4 Depression and mood alterations

Mood disorders such as depression, anxiety and apathy are the most common neuropsychiatric symptoms in PD [56]. Depression affect 40–50% of PD patients and can adversely impact their quality of life. Depressed patients with PD have greater frontal lobe dysfunction and involvement of dopaminergic and noradrenergic systems than non-depressed PD patients [57]. A cross-sectional study has recently reported an association between elevated plasma homocysteine levels, depression and cognitive impairment in PD [58]. Other studies have suggested that depression, like RBD and hyposmia, may precede the development of PD [59,60].
Although the relationship between the pathophysiology of PD and depression remains unclear, dysfunction of a combination of dopaminergic, serotoninergic and norepinephrinergic pathways in the limbic system is likely [61].
Recently Tan et al. [62] reported that mesencephalic dopaminergic neurons (mDA) and serotonergic (5-hydroxytryptamine; 5-HT) neurons are involved in depression. Degeneration of mDA is associated with PD; and defects in the serotonergic signalling are related to depression, obsessive–compulsive disorder, and schizophrenia. Although these neuronal subpopulations reveal positional and developmental relationships, the physiological events that manage specification and differentiation of mDA or 5-HT neurons revealing missing determinants are not yet understood exactly. However, the serotonergic system is markedly affected in the parkinsonian brain with evidence of loss of axons as well as cell bodies in the dorsal and median raphe nuclei of the midbrain. However, it remains unclear whether alteration of the serotonergic system alone is sufficient to confer vulnerability to depression [62].

1.4 Biochemical markers

Analysis of brain tissue, blood, cerebrospinal fluid (CSF) or plasma markers is becoming increasingly accepted as an aid for diagnosis of neurological disorders. There is increasing attention in performance characteristics of markers for which our knowledge of Parkinson's disease pathogenesis provides an underlying rationale, and candidate pathways include protein processing, dopamine function, inflammation, transcriptional dysregulation, and oxidative stress and mitochondrial function.

Individual proteins related to pathogenesis of PD in CSF and in brain tissue such as α-synuclein [63], DJ-1 [64], and brain derived neurotrophic factor [65] have been considered as candidate biomarkers.

Assessment of these biomarkers and sample collection on PD patients are either invasive (CSF) or quite impossible (brain tissue). Moreover, conflicting results among studied CSF proteins have been reported due to assay differences and/or blood contamination [63,66].

Conversely, peripheral blood still remains an ideal candidate for a potential biomarker due to its propensity to contain biological and chemical signals from relevant sources [67] and its large availability and ready accessibility [68].

The characterization of NMS, together with the evaluation of molecular markers linked to inflammation and oxidative damage, could therefore be a valid methodological approach for the early identification of PD patients and the study of potential neuroprotective drugs at a stage when they may actually prevent the development of the motor features of PD.

1.4.1 α-Synuclein

One of the pathological hallmarks of PD is the presence of LB in surviving neurons. α-synuclein, the major structural component of LB, is present in aggregated and insoluble filaments that are hyperphosphorylated and ubiquitinated [69]. Thus, the detection of this protein may enable correlation with the risk and progression of the disease. Recently, plasma α-synuclein levels were found significantly lower in advanced PD and in early-onset PD patients respect with age-matched controls measured by western blotting [70]. In contrast, El-Agnaf et al. have shown a significant increase in oligomers form of plasma α-synuclein in PD patients compared with controls [71].

In a later study, plasma immunoassays for total and oligomeric forms of both normal and phosphorylated (at Ser-129) α-synuclein were performed in 32 PD patients (sampled at months 0, 1, 2, 3), as well as single plasma samples from 30 healthy controls. the mean level of total phosphorilated α-synuclein was found to be higher in the plasma of PD patients than controls. No difference in the mean levels of total, oligomeric and oligomeric phosphorylated α-synuclein between PD cases and controls were be found [72]. These conflictual results may be due to case definition and disease duration large individual-to-individual variation, as well as the types of control cases included, and in the laboratory methods in detecting the various forms of α-synuclein [73].

1.4.2 Oxidative Stress

It is well known that oxidative stress, a condition of free radical overproduction not adequately counterbalanced by endogenous antioxidant defence systems, plays a pivotal role in the pathogenesis of PD, favouring the initiation and progression of neurodegenerative processes [75] (Figure 3). In the brain there are many sources of free radical production. The autoxidation of dopamine in the dopaminergic neurons may produce reactive oxygen species (ROS). Normally, these species are eliminated by intracellular antioxidant systems, which might be impaired by aging or by specific alterations due to the disease pathogenesis [76].

Recent studies have provided evidence that serum uric acid, a natural antioxidant that exerts its scavenger action in many cell populations including neurons, could be a useful biomarker of PD diagnosis and disease progression [77].

8-hydroxydeoxyguanosine (8-OHdG) produced when ROS react with guanine residues in DNA, is a suitable marker of oxidative damage and had been demonstrated to follow with good accuracy the progression of disease; moreover, the increase of 8-OHdG is apparently not influenced by dopaminergic therapy [78]. Bolner et al. studied the ratio between 8-OHdG and 2-dG (which is related to the efficacy of the DNA repairing mechanisms) indicating that, only the 8-OHdG/2-dG ratio but not the 8-OHdG level was significantly higher in plasma samples of PD patients compared to healthy controls, suggesting that the ratio of 8-OHdG/2-dG might be a reliable diagnostic tool [79].

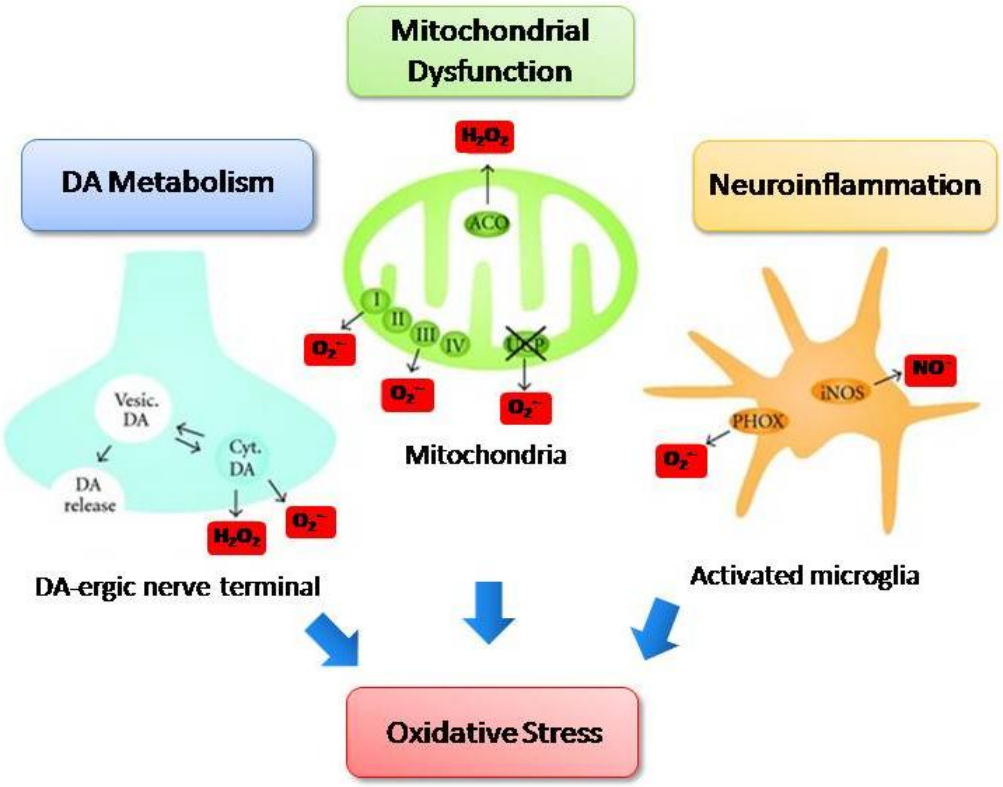

Figure 3. Pathways leading to oxidative stress in PD. Abbreviations: ACO: aconitase, CYT: cytosolic, DA: dopamine, iNOS: inducible nitric oxide synthase, PHOX: NADPH oxidase, VESIC: vesicular. Adapted from ref. [74].

The most important scavenger of free radicals in brain is the intracellular antioxidant glutathione (GSH), whose function depends on two enzymes, glutathione peroxidase and glutathione S-transferase (GST), which are responsible for the transition from reduced to oxidized state of the molecule. As a consequence of improved oxidative stress in PD, increased levels of oxidized GSH and GST had been found not only in the substantia nigra [80] but also in peripheral blood of PD patients, suggesting the potential role of these antioxidant agents as reliable biomarkers for PD [81].

1.4.3 Inflammation markers

Neuroinflammation, comprising microglial activation and astrogliosis in the substantia nigra of PD patients has been shown to be a key contributor to the pathogenesis of PD [82]. Increased levels of pro-inflammatory cytokines particularly interleukin (IL)-1β, IL-6 and tumor necrosis factor alpha (TNF-α), are molecular indicators of systemic inflammation and are correlated with chronic neurodegeneration [83,84]. Furthermore, it was found that high-sensitivity C-reactive protein (hs-CRP) levels in the early PD group were higher than those in healthy controls [85]. Increased TNF-α is associated with cognitive decline in PD and Alzheimer's disease [84,86]. Additionally, plasma levels of both soluble TNF-α receptors are increased in PD patients [87], and certain single nucleotide polymorphisms in the TNF promoter are associated with PD [88]. Increased levels of IL-6 are also associated with poorer performance in cognitive tasks in multiple sclerosis patients [89] and an increased risk of all-cause dementia [90]. Thus, it is reasonable to consider that profiling of blood plasma cytokines and other inflammatory markers could provide a valid and non-invasive means of assessing neuroinflammation in relation to PD.

1.5 Genomic biomarkers

Increasing evidence suggests that both genetic susceptibility and environmental factors contribute understanding of the etiopathogenesis of PD [91].

Only 10-15% of PD patients with the typical clinical parkinsonian state have a positive family history compatible with a mendelian (autosomal dominant or autosomal recessive) inheritance [92].

To date, several genetic loci have been identified in PD through genome wide association studies [93,94] but these explain only a small percentage of the heritability.

Mutations described for familial forms of PD include autosomal dominant mutations of SNCA (PARK1, PARK4), UCHL1 (PARK5), LRRK2 (PARK8), HTRA2 (PARK13) or autosomal recessive mutations of Parkin (PARK2) (the most common), PINK1 (PARK6), DJ-1 (PARK7) and ATP13A2 (PARK9). A list of the PARK loci associated with familial PD and their probable function is provided in Table 1.

However, it is established that genetic susceptibility study may allow the identification of individuals at risk for disease prior to the onset of motor symptoms.

Table 1. Gene loci identified for PD

Locus	Gene	Chromosome	Inheritance	Probable function
PARK1 and PARK4	α-Synuclein	4q21	AD	Presynaptic protein, Lewy body
PARK2	Parkin	6q25.2-27	AR	Ubiquitin E3 ligase
PARK3	Unknown	2p13	AD	Unknown
PARK4	Unknown	4p14	AD	Unknown
PARK5	UCH-L1	4p14	AD	Ubiquitin C-terminal hydrolase
PARK6	PINK1	1p35-36	AR	Mitochondrial kinase
PARK7	DJ-1	1p36	AR	Chaperone, Antioxidant
PARK8	LRRK2	12p11.2	AD	Mixed lineage kinase
PARK9	ATP13A2	1p36	AR	Unknown
PARK10	Unknown	1p32	AD	Unknown
PARK11	Unknown	2q36-37	AD	Unknown
PARK12	Unknown	Xq21-q25	Unknown	Unknown
PARK13	HTRA2	2p12	Unknown	Mitochondrial serine protease

Abbreviations: UCHL1, Ubiquitin C-terminal hydrolase L1; ATP13A2, ATPase type 13A2; HTRA2, HtrA serine peptidase 2; IGF-1, insulin-like growth factor 1; LRRK2, leucine-rich repeat kinase 2; PINK1, PTEN-induced putative kinase 1; AD, Autosomic dominat; AR Autosomic recessive. Adapted from reference [95].

1.5.1 microRNA

Recent advances in OMICS technologies have opened new opportunities in the detection of genetic, epigenetic and transcriptomic biomarkers. Very promising are mainly small non-coding microRNAs (miRNAs). A number of studies have shown changes in distribution profiles of circulating miRNAs (c-miRNAs) associated with various diseases and disorders including neurological disorders such as PD [96].

miRNAs are endogenous, conserved small non-coding RNAs and an important class of post-transcriptional regulators (18 to 22 nucleotides in length) [97].

miRNAs play important roles in the regulation of target genes by binding to complementary regions of messenger transcripts (mRNAs) and repressing protein translation or promoting mRNAs degradation. The process may result in decreased mRNA stability and/or translation. Based on computational prediction, it has been estimated that more than 60% of mammalian mRNAs are targeted by at least one miRNA [98].

The biogenesis of miRNAs starts in nucleus where long primary molecules are transcribed by RNA polymerase II. The primary transcript, structured with characteristic stem-loop configuration, is processed by the nuclear RNase III-type enzyme Drosha into a shorter miRNAs precursor (pre-miRNAs). After the initial processing in the nucleus, pre-miRNAs are then exported to the cytoplasm by the complex of Exportin-5 [99]. A Dicer enzyme cleaves the molecules, which then form RNA-induced silencing complex (RISC). By attaching themselves to complementary sequences of the target RNA, the RISC complexes improve their stability and help in mRNA translation. miRNA inhibits protein synthesis by interacting with partially complementary regions near the 3'-end, which do not undergo translation. Upon binding, the miRNA initiates a pathway that either degrades the transcripts or suppresses their translation [100] (Figure 4).

miRNAs have been proven to regulate neuronal processes such as brain morphogenesis, neuronal cell fate and differentiation, and transcription of neuronal-specific genes regulate the expression of many genes in neuronal processes such as brain morphogenesis, neuronal cell fate and differentiation, and transcription of neuronal-specific genes [101]. More recently it has been proposed that miRNAs may also be involved in the pathogenesis of several diseases such as cancer and neurodegenerative disease [102,103].

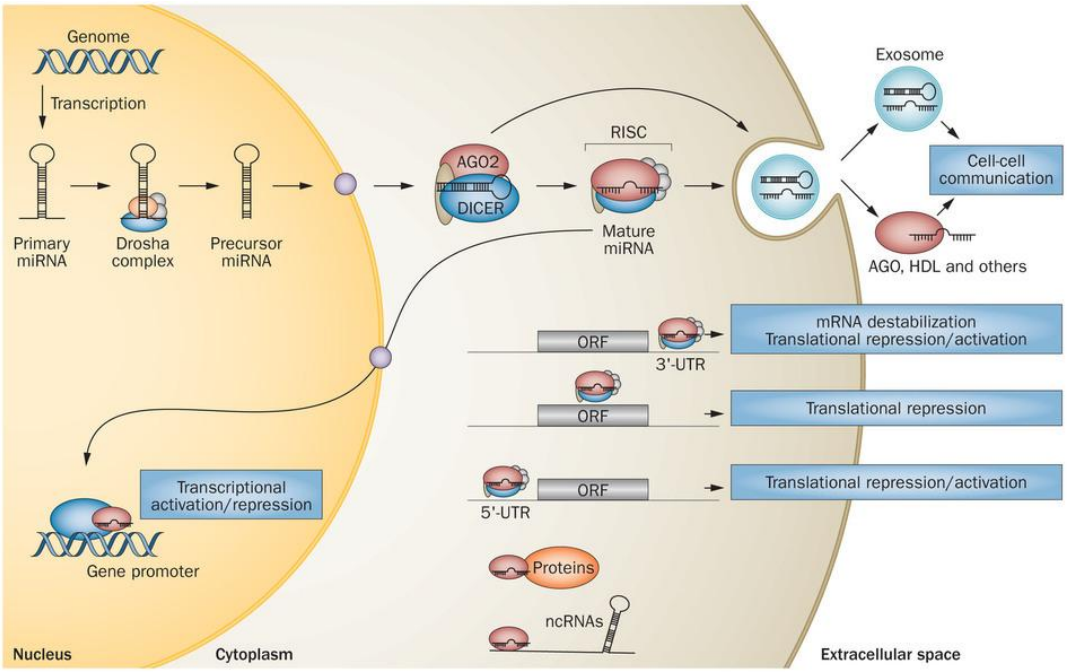

Figure 4. Biogenesis and different functions of miRNAs. Adapted from ref. [104].

Importantly, miRNAs can also be detected in bio fluids such as blood plasma and serum, suggesting their biological function outside of the cell as paracrine signalling molecules [105]. This fraction of miRNAs is regarded cell-free circulating molecules residing in various extracellular vesicles such as microparticles, exosomes and apoptotic bodies or conjugated with RNA binding proteins or lipoprotein complexes [106,107].

Recently, a group of reports demonstrate that the expression of miRNA profiles is tissue specific and that several miRNAs are dysregulated in brain tissue [108, 109]. For example, miR-133b has been reported to be specifically expressed in normal dopaminergic neurons and reduced in midbrain tissue of PD patients [110]. Early deregulation of miR-34b/c in PD brain samples triggers alterations underlying mitochondrial dysfunction and oxidative stress, which at last, compromise cell viability in affected brain areas [111]. Cho et al. showed that miR-205 was significantly underexpressed in the frontal cortex of sporadic PD patients. They also demonstrated that miR-205 binds to LRRK2-3'UTR, thus leading to its down-regulation [112].

Margis et al [113] recently found that miR-1, miR-22-5p and miR-29 expression levels in total peripheral blood allow to distinguish non-treated PD from healthy subjects, and that miR-16-2-3p, miR-26a-2-3p and miR30a differentiate treated from untreated patients. Recent study was performed by qRT-PCR in 31 plasma samples from early onset PD patients and 25 healthy controls and reported one significantly up-regulated miRNA, miR-

331-5p [114]; a second plasma-based study identified miR-1826, miR-450b-3p, miR-626, and miR-505 in 32 PD patients and 32 controls [115]. Soreq et al. [116] recognized by Next Generation Sequencing (NGS) 16 miRNAs significantly dysregulated in blood leukocytes from PD patients compared to healthy controls, including miR-16, miR-20a and miR-320. Eleven miRNAs were modified following deep-brain stimulation (DBS) treatment, five of which were changed inversely to the disease-induced changes.

MiRNAs might also be involved in iRBD, since a role in sleep regulation and disturbances has been postulated from experimental models: sleep loss is associated with changes in miRNA expression in specific brain regions [117] and miR-132 appears to play a regulatory role in sleep [118].

Because of their characteristics, extracellular miRNAs detectable in the blood have been proposed to be used as early biomarkers suitable markers of PD. Identification of specific miRNA signature not only may be helpful for diagnosis, but would be possible to use miRNAs as target for personal and molecular drug treatment.

Association studies for disease-specific miRNA generally couple two approaches. The first approach based on analysis of thousands of miRNA by high throughput methods such as microarray, RT-PCR Array platforms and Next Generation Sequencing (NGS) with subsequent validation of miRNA expression biomarkers by quantitative RT-PCR (qRT-PCR). This method is suitable for screening since it has low sensitivity and high variability, but it is less suitable for the analysis of cell-free circulating miRNA in plasma or serum because concentrations of many miRNAs in plasma are low, and important changes in miRNA levels should not be expected for a chronic pathology. The second approach is based on analysis of miRNAs biomarkers candidates, whose expression level changes due to a pathology development. This approach also has certain limitations due to potential involvement of the same miRNA in diseases of various organs and because higher expression of miRNA in an affected organ is not necessarily accompanied by an increase in its plasma level [119,120]. Both approaches have been used in the present study.

2. AIMS OF THE STUDY

Finding a non-invasive and reliable biomarker in the Parkinson's disease would be necessary to better monitor and to early identify diagnostic features of the preclinical phase of PD so that people at high risk for progressing to the clinical phase can be recognised.

The general aim of this study was to explore potential novel diagnostic and prognostic biomarkers of PD.

Specific study objectives were, in patients with prodromal and established PD:

a) to evaluate clinical markers such as olfactory and cardiovascular autonomic functions;

b) to measure circulating mediators of oxidative stress and inflammatory response as early biomarkers of organ failure;

c) to correlate biological findings with clinical functional alterations;

d) to characterize specific circulating miRNA profiles in plasma samples.

3. MATERIALS AND METHODS

3.1 Study population

The study population includes 15 patients diagnosed with idiopathic PD (PD group), 10 subjects diagnosed with idiopathic RBD (iRBD group) and 10 healthy controls (CTRL group).

Eligible subjects aged ≥50 e ≤80 years consecutively recruited between May 2014 and May 2015 from the Centre of Sleep Medicine (iRBD group) and by the Neurology outpatient clinic (PD group) of Niguarda Cà Granda Hospital and age and gender-matched controls (CTRL group) who attended our Institute for laboratory assessment.

The study was approved by Niguarda Hospital Ethics Committee and conducted in accordance with the Declaration of Helsinki [121].

All subjects expressed their written informed consent to participate.

All subjects presented none of the exclusion criteria listed below:

- neurological disorder (other than PD for PD group),
- major mental disorder,
- cognitive impairment (Mini–Mental State Examination (MMSE) <26);
- previous myocardial infarction, heart failure, pacemaker, atrial fibrillation;
- beta-blockers therapy;
- glomerular filtration rate <15 ml/min;
- hepatic insufficiency
- long standing (>10 years) diabetes.

PD group

Subjects with clinically diagnosed idiopathic PD, confirmed by a neurologist with expertise in movement disorders based on EFNS/MDS-ES recommendations for PD diagnosis [122], had to meet the following criteria:

- Hohen & Yahr stage ≥1 and ≤3 [123].
- on L-DOPA and dopamine agonists combination therapy, titrated to maintenance doses since at least 1 month.

PD patients with the following exclusion criteria were not enrolled into the study:

- genetic aetiology or familial clustering;
- atypical or secondary parkinsonism;

- history of cerebrovascular events;
- treatment with COMT inhibitors.

iRBD group

iRBD subjects, enrolled by the Sleep physician according to the International Classification of Sleep Disorders criteria [124] had to meet the following criteria:

- repeated episodes of sleep-related vocalization and/or complex motor behaviours;
- video-polysomnographic (PSG) documentation of occurrence during REM sleep or history suggestive of dream enactment;
- polysomnographic observation of REM sleep without atonia;
- exclusion of other sleep disorder, medication, or substance use.

CTRL group

The control cohort consisted of ten age and gender-matched healthy subjects with a cardiovascular risk factor profile similar to the cases enrolled in the study.

3.2 Study design

Eligible subjects attended the CNR Clinical Physiology Institute in the morning in the fasting state to undergo the baseline visit that included the following activities:

- explanation of the study purpose, procedures, potential risks and benefits, and informed consent signing;
- review of the subject's medical and drug history;
- review of concomitant medications;
- general physical and neurological examination;
- REM Sleep Behavior Disorder Screening Question [125];
- olfactory testing using Sniffin' Sticks Extended Test [126];
- blood pressure and heart rate assessment;
- autonomic function testing;
- blood sampling for miRNA analysis, oxidative stress profile and inflammation markers.
- urine sampling for neopterine and creatinine assessment.

PD subjects were administered the Movement Disorder Society -Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Parts I- Non-Motor Aspects of Experiences of Daily Living (nMEDL score), for evaluation of mentation, behaviour, and mood and Part II- Motor Aspects of Experiences of Daily Living (MEDL score) for self-evaluation of the activities of daily life including speech, swallowing, handwriting, dressing, hygiene, falling, salivating, turning in bed, walking, and cutting food [127].

3.3 Sample processing

After an overnight fast, an antecubital vein was cannulated and blood was drawn into different Vacutainer tubes. Immediately after collection, blood samples in EDTA for miRNA evaluation and for measurement of oxidative stress and inflammation markers were centrifugated at 2000 rpm for 10 minutes in order to separate plasma aliquots. Urine samples were drawn into a Vacutainer tubes and immediately stored at -80°C until the time for the neopterin determination

Blood samples for reduced and total GSH determination were treated (see above) and then centrifugated at 4000 rpm, for 2 minutes at 4°Cto prevent thiols oxidation.

Blood samples, collected in serum separator tubes, were kept at room temperature for 30 minutes to allow samples coagulation before centrifugation at 4000 rpm for 15 minutes. Serum samples were used for routine biochemical assessment.

All samples were stored at -80°C until the time for the determination of circulating parameters.

3.4 Biochemical assessments

3.4.1 Total and reduced glutathione

Glutathione (GSH), the most important endogenous scavenger, was assessed in total and reduced form in plasma and blood samples according to a high performance liquid chromatographic (HPLC) method, validated in our laboratory [128].

Sample preparation - Blood and plasma **reduced GSH** were assessed by mixing whole blood or plasma with 10% tri-chloro acetic acid (1:1 v/v), and immediately frozen in liquid nitrogen. This step is required to rapidly acidify the medium, avoiding alterations in redox state, and remove the aminothiols component linked to proteins. The unfrozen sample was centrifuged at 10,000 rpm for 2 min; clear supernatant (100 μL) was mixed with 100 μL of

1 M borate buffer, pH 11 containing 4 mM EDTA, 10 µL of 1.55 M NaOH and 10 µL of SBD-F acting as a fluorescent derivatization agent able to bound functional sulphydrilic –SH groups. NaOH and borate buffer assure a basic condition (pH 9.2-9.5) needed by SBD-F function ability. The mixture was incubated for 60 min at 60 °C before chromatographic analysis.

Because plasma levels of reduced GSH are low (1–2%), blood reduced GSH concentrations may come close to GSH content inside the circulating cells (red and white blood cells, platelets).

Blood and plasma **total GSH** were assayed by mixing 100 µL of whole blood or plasma with 10 µL of 10% Tris(2carboxyl-ethyl)-phosphine solution, a reducing agent. In this way all the oxidized form of aminothiols have been converted into reduced forms. After 30 minutes at room temperature, 100 µL of 10% tri-chloro acetic acid were added and samples were centrifugate at 10,000 rpm for 2 minutes. Clear supernatant (100 µL) was mixed with 1 M borate buffer/4 mM EDTA pH 11, 1.55 M NaOH and SBD-F, as described above.

Standard Curve Preparation: an individual stock solution of GSH reduced forms, was prepared by dissolving the GSH powder in a 0.1 M HCl solution to have a final concentration of 120 mM (point 1 of curve). From point 1, five successive dilutions (1:2) were performed (point 2,3,4,5,6). Each standard point (100 µL) was then treated with the same procedure used for blood and plasma samples (Figure 5).

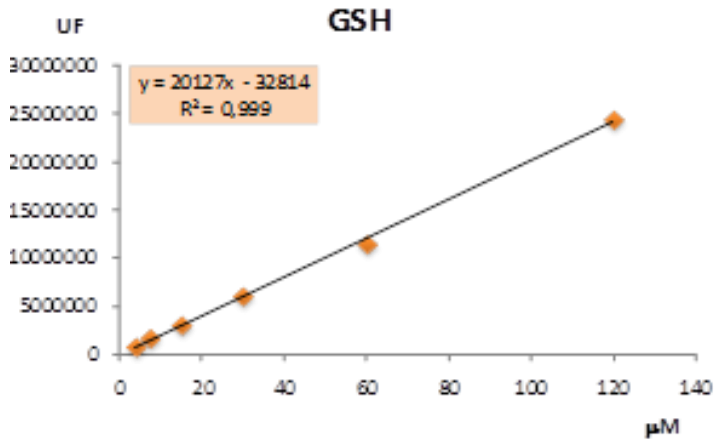

Figure 5. Calibration curve of GSH standard.

Chromatographic conditions - GSH separation was performed using a Varian ProStar HPLC system (VARIAN, Agilent) equipped with a pump (240 model) and a refrigerated

autosampler 410 model), on a Discovery C-18 column (250×4.6 mm I.D, Supelco, Sigma-Aldrich), eluted with an isocratic mobile phase of 0.1 M phosphate buffer and 8% acetonitrile pH 2.1, at a flow rate of 1 mL/min. Fluorescence intensities were measured using a with a λ excitation at 385 nm and λ emission at 515 nm, using a JASCO fluorescence detector (FP-4025 model).

The retention time of GSH was 7.76 minutes (Figure 6).

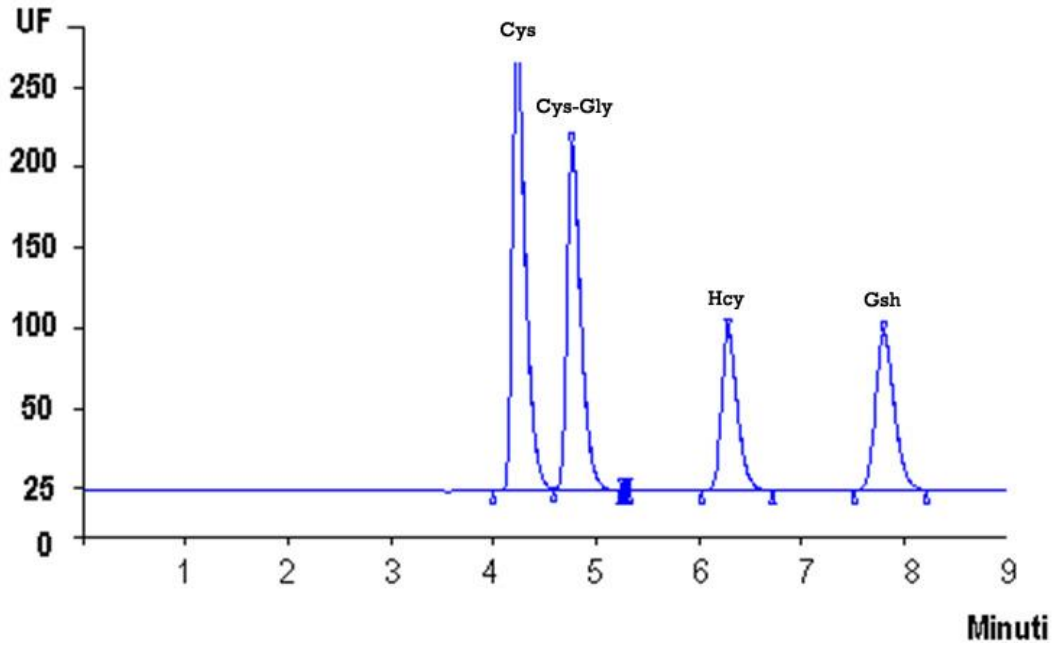

Figure 6. Aminothiols Chromatogram.

3.4.2 3-Nitrotyrosine (3-NT)

3-NT is a stable end product of peroxynitrite oxidation. Plasma 3-NT is measured using an ELISA Kit from Hycult Biotech (Uden, The Nederland) according the manufacturer's instruction. It is a ready-to-use solid-phase enzyme-linked immune-sorbent assay (ELISA) based on the sandwich principle (Figure 7). Samples and standards were incubated in micro titer wells coated with antibodies recognizing 3-NT. Biotinylated tracer antibody binds to captured 3-NT. Streptavidin-peroxidase conjugate binds to the biotinylated tracer antibody and reacts with the substrate, tetramethylbenzidine (TMB) producing a color proportional to the amount of substrate bound. The absorbance at 450 nm was measured

with a spectrophotometer. A standard curve was obtained by plotting the absorbance (linear) versus the corresponding concentrations of the 3-NT standards log. The 3-NT concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

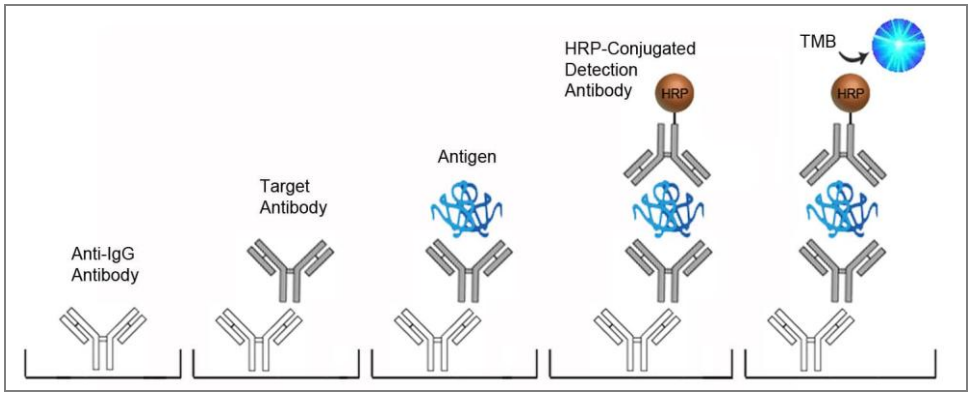

Figure 7. A sandwich ELISA schematic principle.

3.4.3 Malondialdehyde (MDA)

MDA is formed by lipid peroxidation of unsaturated fatty acids and is a index for oxidative degradation of cellular membranes. In order to measure this molecule, we use a commercial kit of ChromSystems (Gräfelfing, Germany) that allows the reliable chromatographic determination of MDA on a simple, isocratic HPLC system with fluorescence detector. Sample preparation is based on an efficient protein precipitation step followed by derivatisation. The resulting fluorophore is specific and detectable at λ excitation of 515 nm and λ emission of 553 nm. The flow rate of mobile phase was 1 mL/minute. Briefly, the protocol implemented the following steps:

- 100 µl plasma were mixed with 500 µl Precipitation Reagent.
- Centifugated for 5 min at 16 000g.
- 500 µl of the supernatant were transfer into a dark derivatization vial.
- 100 µl Derivatization Reagent were added and mixed briefly.
- The reaction was incubated for 60 min at 95 °C, and cooled down immediately.
- 500 µl Neutralisation Buffer were added and mixed briefly.
- 20 µl of each sample was injected into the HPLC system.

3.4.4 8-Hydroxy-deoxiguanine (8-OHdG)

The 8-OHdG, a biomarker of oxidative DNA damage, is removed from DNA by the base excision repair pathway, and subsequently transported into saliva, urine and plasma. Plasma 8-OHdG levels were assessed according HT 8-OHdG ELISA kit II recommendations from TREVIGEN (Gaithersburg, MD, USA). It is a fast and sensitive immunoassay for the detection and quantitation of 8-OHdG in plasma. This assay employs a 96 strip well pre-coated with 8-OHdG, an anti-8-OHdG monoclonal mouse antibody, an HRP conjugated secondary antibody, and colorimetric detection substrate. The 8-OHdG monoclonal antibody binds competitively to 8-OHdG immobilized on pre-coated wells and in solution. Antibody bound to 8-OHdG in the sample is washed away with PBST buffer (1X PBS + 0.1% Tween 20) while antibody bound to 8-OHdG attached to the well is retained. Detection was performed with horseradish peroxidase (HRP) conjugate and a colorimetric substrate, using a microplate reader with a spectrophotometer set at 450 nm. HRP binds to the biotinylated tracer antibody and reacts with the substrate, tetramethylbenzidine (TMB) producing a blue colour product that changed into yellow after adding acidic stop solution. Product formation is inversely proportional to amount of 8-OHdG present in the sample.

3.4.5 Inflammatory markers

Interleuchin 1 beta (IL-1β) is an important mediator of the inflammatory response produced by activated macrophages. Plasma IL-1β level was measured by PicoKine™ ELISA kit from Boster Biological Technology (Pleasanton, USA) according the manufacturer's instruction. Briefly, a monoclonal antibody from mouse specific for IL-1β has been precoated onto 96-well plates. Standards and test samples were added to the wells, a biotinylated detection polyclonal antibody from goat specific for IL-1β was added subsequently and then followed by washing with PBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS buffer. HRP binds to the biotinylated tracer antibody and reacts with the substrate TMB producing a blue colour product that changed into yellow after adding acidic stop solution. The optical density, determined using a microplate reader with spectrophotometer set at 450 nm, was proportional to IL-1β amount present in the sample.

Tumor necrosis factor alpha (TNF-α) is another important cytokine involved in acute phase inflammation. Plasma TNF-α levels were performed according Human TNF-

αELISA kit from Cayman Chemical Company (Ann Arbor, MI, USA). Each well of micro well plate has been precoated with a monoclonal antibody specific for TNF-α. Standards and test samples were added to the wells, An acetylcholinesterase:Fab' Conjugate (AChE:Fab'), which binds selectively to a different epitope on the TNF-α molecule, is also added to the well. When TNF-α (standard or samples) is added to the well, the two antibodies form a "sandwich" by binding on opposite sides of the TNF-α molecule. The "sandwiches" are immobilized on the plate so the excess reagents may be washed away. The concentration of analyte is the determined by measuring the enzymatic activity of the AChE by adding Ellman's Reagent (that contains the substrate for AchE) to each well. The product of the AChE-catalyzed reaction has a distinct yellow colour which absorbs strongly at 412 nm. The intensity of this colour, determining spectrophotometrically, is directly proportional to amount of bound Conjugate which in turn is proportional to the concentration of TNF-α.

Urine neopterin (Neo) is a sensitive marker of cellular-mediated inflammation. Urine Neo levels were measured by an isocratic HPLC method and normalized by urine creatinine concentrations [129]. Briefly, urine samples, stored at −20 °C, were thawed and centrifuged; the surnatant was then adequately diluted with chromatographic mobile phase (15 mM of K_2HPO_4, pH 3.0). Neopterin and creatinine levels were measured using a Kontron instrument (pump 422-S, autosampler 465) coupled to a fluorimetric detector (JASCO FP-1520, λ excitation = 355 nm and λ emission= 450 nm) for neopterin detection and to a UV–VIS detector (BIO-RAD 1706, λ=240 nm) for creatinine determination. Neopterin and creatinine separations were performed at 50 °C on a 5 μm Discovery C18 analytical column (250×4.6 mm I.D., Supelco, Sigma-Aldrich) at flow rate of 0.9 mL/min. The calibration curves were linear over the range of 0.125–1 μmol/L and of 1.25–10 mmol/L for neopterin and creatinine levels, respectively.

3.5 Functional testing

3.5.1. Olfactory test

Olfactory function was assessed by the Sniffin' Sticks Extended Test (Burghart, Medizintechnik, GmbH, Wedel, Germany) [126]. The olfactory threshold is the minimum concentration of an odorant (n-butanol) that can be detected by a subject when presented with 16 different dilutions in felt tip pens. Olfactory discrimination assesses the ability to discriminate between different odorants in 16 different triplets. Olfactory identification evaluates the ability to correctly identify an odorant among four possible odours for each of 16 trials (Figure 8). The total olfactory score (TOS) was calculated as sum of the 3 sub-scores for olfactory threshold, discrimination and identification and reclassified as normal olfaction (between 31 and 48), hyposmia (between 16 and 30) and anosmia (≤15).

Figure 8. Sniffin' Sticks Extended Test.

3.5.2 Autonomic testing

Autonomic function [130] was tested in the supine position at a comfortable ambient temperature. Heart rate was recorded via standard 12-lead electrocardiogram (Norav PC ECG-1002). Blood pressure was measured non-invasively by a manual sphygmomanometer.

Deep Breathing (DB). After 10-minute rest, subjects performed one minute DB (6 inspiratory and expiratory cycles of 5 seconds each) during continuous ECG recording. The DB Expiration/Inspiration (E/I) ratio was calculated as ratio of averages of the three

longest RR intervals during expiration and the three shortest RR intervals during inspiration.

Lying to standing (LS). After 10-minute rest, patients were instructed to stand up and remain standing for 5 min. Changes in systolic/diastolic blood pressure and heart rate were assessed after 1 and 5 minutes standing from the supine position. Orthostatic hypotension (OH) was defined as a drop ≥20 mmHg in systolic and/or ≥10 mmHg in diastolic blood pressure. The LS 30:15 ratio was the ratio between the longest RR interval measured between the 25th and 35th beat after active standing and the shortest RR interval between the 10th and 20th beat.

Valsalva manoeuvre (VM). Sitting patients were instructed to blow into a tube connected to a manometer to maintain the pressure at 40 mmHg for 15 seconds, during continuous heart rate and blood pressure monitoring. VM ratio was the ratio of the shortest RR interval (tachycardia) during expiration to the longest RR interval (bradycardia) after expiration.

3.6 Circulating miRNA profiling study

The circulating miRNA study was performed in a subpopulation of the enrolled cohort of subjects (4 PD, 4 iRBD and 4 CTL), using the Next Generation Sequencing (NGS) method able to study a complete profile of miRNA expression.

Plasma circulating RNA extraction.

Circulating RNA extraction was performed using QIAamp Circulating Nucleic Acid kit (Qiagen) following the manufacturer's instruction. Plasma volume used for each samples was 2 mL. Quality control of total RNAs was performed with 2100Bioanalyzer (Agilent Technologies).

small-RNA libraries preparation

small-RNA libraries was construction constructed using TruSeqSmall RNA sample preparation kit (Illumina) according to the manufacturer's instruction. Briefly, the protocol implemented the following steps to generate a library product, shown in Figure 9:

- RNA ligation with 3' and 5' adapter;

- reverse transcription (RT),
- 15 cycles of PCR amplification,
- gel purification

The RNA 3' adapter was specifically modified to target microRNAs and other small RNAs that have a 3' hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. The adapters were ligated to each end of the RNA molecule. An RT reaction was used to create single stranded cDNA by SuperScript Reverse Transcriptase (Life Technologies) using a primer complementary of RNA 3' adapter.This process selectively enriches those fragments that have adapter molecules on both ends. The resulting cDNA was then PCR amplified using two primers: one common to the ends of the adapters and a primer containing one of 48specific sequence (Index primer) that allow the subsequently identification during sequencing. Each cDNA thus obtained constitutes the sample library, and each library is characterized by a different Index primer: in this way it is possible to sequence more samples simultaneously. The small-RNA cDNA libraries were heterogeneous in size below 200 bp.

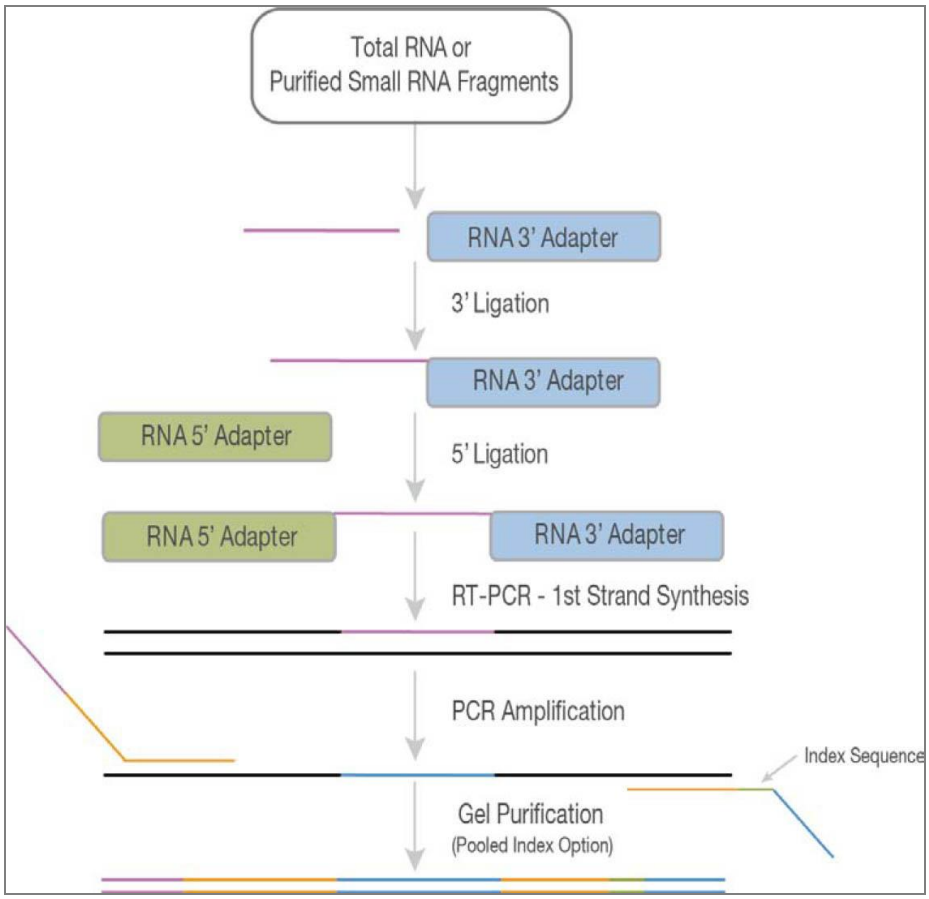

Figure 9. TruSeq Small RNA Sample Preparation Workflow.

To isolate only the mature miRNA fraction, a gel purification was performed. For each library, cDNA was size fractionated on a 5% tris-borate-EDTA (TBE) polyacrylamide gel (Bio-Rad) and the 147 base pair fraction, corresponding to miRNA (20bp) plus two primers, was selected and excised. After gel purification, the cDNA libraries were eluted in 10 µl of Tris-HCl 10 mM pH 8.5. At the end, each library was run on Bioanalyzer 2100 (Agilent Technologies) in order to check the library quality. A good library should present a single peak around the length of 147-150 bp (Figure 10). Six purified libraries were mix to obtain two libraries pools (6plex).

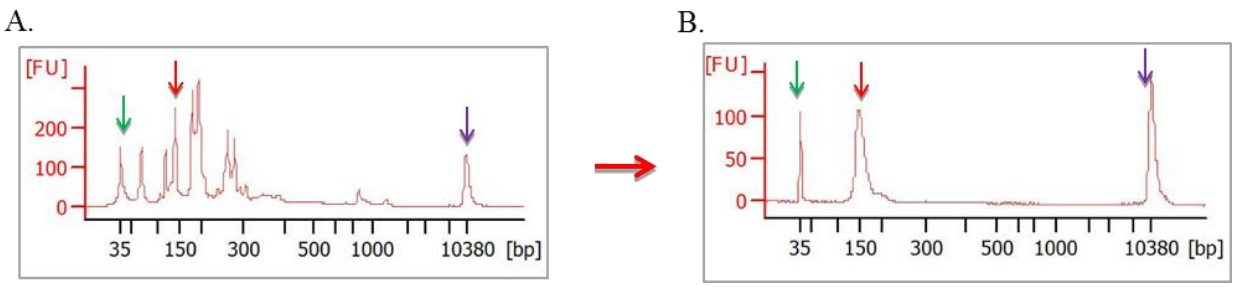

Figure 10. Library quality check assessed by 2100 Bioanalyzer (Agilent Technologies) A. Electropherogram of a library before size selection. B. Electropherogram of a library after size selection. The red arrows indicate mature miRNA fraction. The green and purple arrows indicate the 35 bp and 10380 bp reference markers, respectively.

Small-RNA-sequencing

After libraries quality check, the library pools were quantified using fluorescence-based detection, by Qubit 2.0 Fluorometer (Invitrogen, Life Technologies),and then, were correctly diluted with NaOH 0.2 N. Sequencing was performed using MiSeq sequencer (Illumina). As a control for sequencing runs, a fixed amount of PhiX Control was added for each sample.

Primary data analysis

Raw sequenced reads were stored in FASTQ files and were analyzed through the following steps:

A. *Sequences quality assessment:* the quality of produced reads and the presence of adapters sequences were checked using FASTQC v0.10.1, a bioinformatics software. Primary sequences reads were initially trimmed off adapter sequences using Cutadapt v.1.2.1 [131];

B. *miRNA mapping:* to identify expressed miRNAs, reads were mapped to known human pre-miRNA and mature miRNA sequences that are annotated in miRBase database (release 21) using miRExpress tool (v2.1.3) [132]. The miRBase database is a public available repository containing all known human miRNAs. The number of clean reads that mapped on mature miRNAs was reported in an output file.

C. *miRNA profiling:* the expression level of each miRNA is proportional to the number of reads that are sequenced; for this reason, miRNA expression profiles were built by calculating the sum of read counts for each miRNA, according to the alignment criteria.

Secondary Analysis

A. *Exploratory data analysis*: the overall similarity between all samples was investigated by calculating the Euclidean distance of the miRNA expression profiles, followed by hierarchical clustering. More specifically, the cluster analysis is an explorative analysis that tries to identify homogenous groups of cases, showing a similar expression of a specific microRNA pattern.
B. *Differentially expressed miRNAs identification*: from the output file the differential expression analysis, based on the negative binomial distribution, was performed with DESeq2 (R/Bioconductor package) comparing:
   1) CTRL versus RBD samples
   2) RBD versus PD samples
   3) CTRL versus PD samples

The output file of each comparison contained the following items for each miRNA selected:

- baseMean: average of the normalized count values
- log2FoldChange: effect size estimate
- the P value and the P value adjusted calculated by BH multiple testing

3.7 miRNA validation by Quantitative Real-Time PCR

Expression analyses of specific miRNAs were validated by qRT-PCR. This study was performed in all recruited subjects.

miRNA extraction

Total RNA that included miRNAs was isolated from plasma using the miRNeasy Serum/Plasma Kit (QIAGEN). Each plasma sample was thawed and centrifugated at 14,000 rpm for 15 minutes at 4°C to obtain cell-free plasma. Briefly, the procedure consisted of the following steps (Figure 11):

1. plasma samples were lysed in QIAzol Lysis Reagent, a monophasic solution of phenol and guanidine thiocyanate that facilitates cell lysis, inhibits RNases, and also removes most of the cellular DNA and proteins from the lysate by organic extraction. After addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase.
2. the upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides upwards. The sample was then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants were efficiently washed away with buffers included in kit. High-quality RNA is then eluted in a 14 μL of RNase-free water.

During miRNA purification, the miRNeasy Serum/Plasma Spike-In Control from C. Elegans (cel-miR-39) was added to samples at the lyses phase after the addition of QIAzol Reagent.

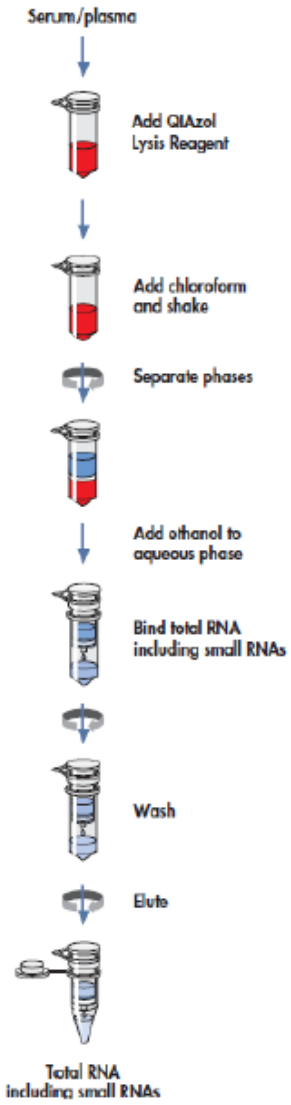

Figure 11. miRNeasy Serum/Plasma Procedure.

cDNA synthesis

Reverse transcription (RT) reaction was prepared in a total volume of 20 µL using miScript II RT Kit (QIAGEN) adding the following reagents (Figure 12):

- 10 µL of each total RNA
- 4 µL of miScript HiSpec Buffer,5x (in order to retrotrascribed only mature miRNA)
- 2 µL of miScript Nucleics Mix,10x
- 2µL of miScript Reverse Transcriptase Mix, 10x
- 2µL of RNase-free H_2O

Thermal cycler conditions were as follows: one step of 37°C for 90 minutes, one step of 95°C for 5

minutes to inactivate miScript Reverse Transcriptase, followed by 1 step of 4°C for 5 minute. The RT reaction product was diluted with 200 μL of RNase-free water and then stored at -20°C until its use.

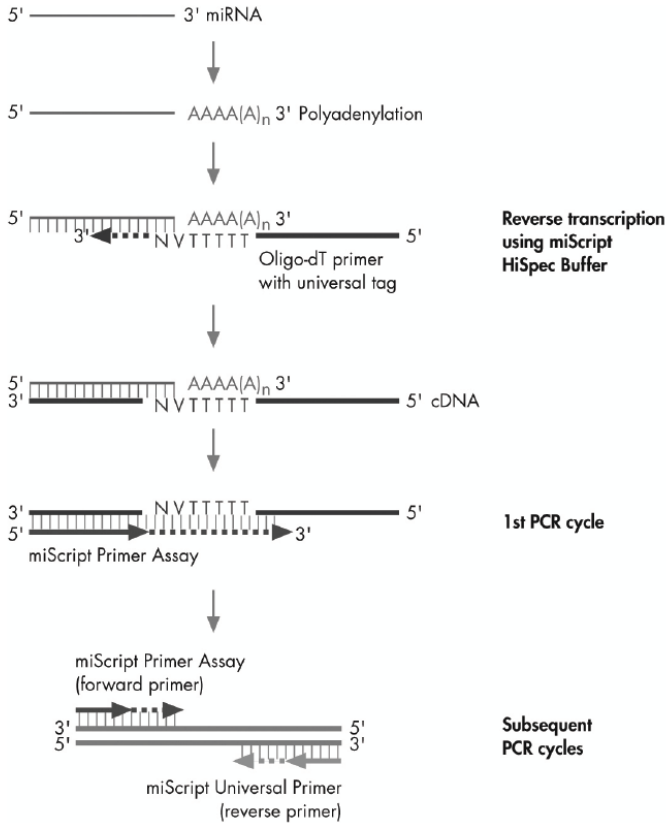

Figure 12. miScript II RT Kit (QIAGEN)

Quantitative Real Time PCR

Expression analysis of specific miRNAs (let-7, miR-101, mir-1260, miR-142, miR15a) were assessed by qRT-PCR. qRT-PCR was performed according to miScript SYBR Green PCR kit (QIAGEN) recommendation, using CFX96 thermal cycler, (Biorad, Hercules, CA). miRNA quantification was detected by evaluating the level of fluorescence emitted by SYBR® Green at every reaction cycle. During each cycle, cDNA molecules are doubled since they reach a plateau when all the reagents are depleted and enzymes activity is drastically reduced. All RT PCR reactions were performed in a total volume of 20μL,

and prepared in duplicate. For each miRNA-specific assay, the following reagents were added:

- 10 μL of QuantiTec SYBR Green PCRMaster Mix, 2x
- 2.5 μL of miScriptUniversal Primer(UP), 10x
- 2.5 μL of miScriptPrimer Assay, 10x
- 2.5 μL of Template cDNA
- 2.5μL of RNase-free H_2O

Reactions were incubated at 95°C for 15 min, then 40 cycles of 94°C for 15 sec, 55°C for 30 sec, 70°C for 30 sec, then a dissociation curve analysis of the PCR products to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers.

Relative expression levels were calculated with the comparative threshold cycle (Cq) method using the formula: 2^-ΔΔCq [133], using the exogenous spike-in cel-miR-39 as reference for normalization. Threshold for expression was set as Cq<37, and miRNAs not expressed under these criteria were removed from consideration.

3.8 Statistical Analysis

Data are presented as median (interquartile range) or frequency (percentage). Categorical variables were compared by the chi-square test. Pearson's R correlation coefficient or Spearman's rho index were used to correlate continuous clinical, biochemical and functional variables (age, symptom duration, nMEDL, MEDL, GSH, Neopt, MDA, 3-NT, 8-OHdG, TNFα, IL1β, TOS, LS 30:15 ratio, VM ratio, DB-ratio). Associations with the dependent variable “group” were tested by univariable ordinal logistic regression; variables with p<0.10 were entered in a multivariable model.

miRNA expression values coming from RT-PCR quantification have been compared among groups by ANOVA analysis.

A P value <0.05 was considered statistically significant. Statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS Inc, Chicago, Illinois, USA), version 17.0 for Windows.

4. RESULTS

4.1 Clinical, biochemical and functional characteristics of the study groups

The clinical characteristics and biochemical and functional parameters of the three study groups are summarized in Table 2. We enrolled 38 subjects: 15 patients with overt PD, 11 subjects with PSG-confirmed iRBD and 12 age- and gender-matched CTRL between May 2014 and May 2015.

The average age of the overall population was 70 ± 6 years. Gender distribution was similar in the three different groups. The cognitive impairment, expressed as MMSE, was 28 in iRBD and CTRL and 29 in PD.

The clinical features of PD patients with respect to disease severity are summarized in Table 3. Patients had long standing disease with mild to moderate burden of motor and non motor symptoms; only 2 had complicated disease with on-off phenomena and dyskinesia. The duration of disease correlated directly with L-DOPA dose (r 0.650, p=0.012) and inversely with the DB E/I ratio (r -0.642, p=0.021). Conversely, no correlation was observed either with the burden of NMS or with the olfactory score. nMEDL score was inversely related to VM ratio (R=-0.726, p=0.017) and with TOS (R=-0.873, p<0.001). Conversely, MEDL did not correlate with any biochemical or functional variable but only with age (R=0.523, p=0.045). Six PD patients (40%) reported sleep behaviours compatible with RBD at the screening question; no differences were observed between patients with and those without suspect RBD either in clinical findings or in functional and biochemical characteristics.

Table 2. Clinical, biochemical and functional characteristics of the study groups

	Controls (n=12)	IRBD (n=11)	PD (n=15)
Age, years	71 (59-74)	71 (64-73)	70 (58-76)
Gender (M), n%	7 (58%)	10 (91%)	7 (47%)
Smoking habit, n%	2 (17%)	4 (36%)	1 (7%)
Hypercholesterolemia, n(%)	4 (33%)	2 (20%)	2 (13%)
Hypertension, n(%)	7 (58%)	4 (36%)	3 (20%)
Anti-hypertensive drugs, n(%)	7 (58%)	4 (36%)	2 (13%)
MMSE	28 (27-28)	28 (27-29)	29 (27-30)
Systolic Blood pressure mmHg	120 (113;129)	140 (105;160)	135 (115;140)
Diastolic Blood pressure mmHg	70 (65;70)	70 (65;70)	70 (70;80)
Heart rate, bpm	64 (60;70)	58 (49;64)	69 (61;78)
Blood reduced GSH, μmol/L	759 (650;833)	582 (380;715)	519 (467;639)
Blood total GSH, μmol/L	1364 (1205;1521)	1091 (923;1563)	1070 (860;1743)
Plasma reduced GSH, μmol/L	1.13 (0.88;1.57)	1.05 (0.72;1.61)	0.86 (0.69;1.01)
Plasma total GSH*, μmol/L	6.95 (6.35;8.66)	5.60 (4.25;10.90)	6.10 (4.57;7.60)
Plasma MDA, μmol/L	0.16 (0.14;0.17)	0.15 (0.14;0.16)	0.14 (0.13;0.16)
Plasma 3-NT, nmol/L	12.7 (6.1;29.3)	19.7 (17.1;48.6)	18.2 (10.0;34.5)
Plasma 8-OHdG, nmol/L	279 (229;348)	227 (180;307)	280 (209;357)
Urine neopterin,(μmol/molcreat)	127 (93;188)	141 (114;185)	164 (125;226)
Plasma TNFα, pg/mL	26 (21;46)	34 (22;66)	28 (22;60)
Plasma IL1β, pg/mL	10.9 (8.9;13.6)	15.0 (12.0;16.1)	10.9 (8.4;14.5)
Total olfactory score	28.7 (22.2;33.0)	17.5 (11.0;21.5)	18.7 (15.0;24.0)
LS 30:15 Ratio	1.22 (1.05;1.37)	1.13 (1.07;1.19)	1.03 (1.01;1.18)
SBP drop at 1 minute standing	-2.5 (-16.2;-12.5)	-15.0 (-20.0;-10.0)	-20.0 (-30.0;-5.0)
DBP drop at 1 minute standing	0 (-7.5;5.0)	-10.0 (-15.0;-5.0)	-5.0 (-15.0;0)
VM Ratio	1.50 (1.31;1.73)	1.38 (1.26;1.45)	1.27 (1.14;1.53)
DB E:I ratio	1.17 (1.11;1.27)	1.14 (1.09;1.21)	1.15 (1.11;1.26)

Data are expressed as median and interquartile range (I-III) or as frequency (%).DB, deep-breathing; E:I expiration/inspiration; IL, interleukin; LS, lying to standing; GSH, glutathion, MDA, malondialdehyde; TNF, tumor necrosis factor; VM, Valsalva manoeuvre.

Table 3. Clinical characteristics of disease severity in PD group

	Median (I-III)
Hohen&Yahr Score	2.5 (2.0-2.5)
UPDRS Part I	2.0 (1.0-6.0)
nMEDL	9.0 (5.0-11.0)
MEDL	12.0 (7.0-14.0)
nMEDL + MEDL	19 (16-26)
DOPA dose (mg/die)	500 (300-850)
Rotigotine dose (mg/die)	9.0 (6.5-11.5)
Ropirinol dose (mg/die)	6.0
Pramipexol dose (mg/die)	2.10 (1.05-2.10)

MEDL, motor experiences of daily living; nMEDL, non-motor experiences of daily living

4.2 Functional and biochemical differences among PD, iRBD and CTRL groups

Results of univariable ordinal logistic regression analysis are shown in Table 4. Among clinical characteristics, a higher prevalence of a history of hypertension was found in CTRL than in iRBD and PD patients. A significant worsening trend was observed in total olfactory score, blood reduced GSH, LS 30:15 and VM ratio, and urine neopterin from the reference controls to iRBD and PD groups.

In the multivariable ordinal logistic regression model, only low blood reduced GSH levels (P = 0.037, OR = 0.994; 95% CI 0.988 – 1.000), adjusted by history of hypertension, total olfactory score, LS 30:15 ratio and VM ratio, was associated to PD status.

Table 4. Univariable ordinal logistic regression analysis

	P	OR	95% CI
Age, years	0.575	0.981	0.920 - 1.047
Gender (M), n%	0.365	0.566	0.165 - 1.944
Smoking habit, n%	0.473	0.573	0.125 - 2.622
Hypercholesterolemia, n(%)	0.216	0.392	0.089 - 1.726
Hypertension, n (%)	**0.043**	**0.267**	**0.075 - 0.960**
MMSE	0.620	1.147	0.668 - 1.970
Blood reduced GSH, μmol/L	**0.009**	**0.995**	**0.991 - 0.999**
Blood total GSH, μmol/L	0.892	1.000	0.998 - 1.002
Plasma reduced GSH, μmol/L	0.181	0.736	0.469 - 1.155
Plasma total GSH, μmol/L	0.132	0.852	0.692 - 1.049
Plasma MDA, μmol/L	0.229	0.000	0.001 – 8323
Plasma 3-NT, nmol/L	0.585	0.998	0.991-1.005
Plasma 8-OHdG, nmol/L	0.696	1.001	0.995 - 1.007
Urine neopterin, (μmol/molcreat)	**0.083**	**1.107**	**0.987 - 1.242**
Plasma TNFα, pg/mL	0.421	1.004	0.992 - 1.016
Plasma IL1β, pg/mL	0.582	1.043	0.897 - 1.213
TOS	**0.009**	**0.890**	**0.815 - 0.973**
LS 30:15 Ratio	**0.047**	**0.004**	**0.001 - 0.919**
SBP drop after 1 minute standing	0.100	0.977	0.951-1.004
DBP drop after 1 minute standing	0.116	0.952	0.896-1.012
MV Ratio	**0.047**	**0.045**	**0.002 - 0.961**
DB E/I ratio	0.958	0.879	0.007 – 111

For abbreviations see Table 2

4.3 Group comparison with respect to functional category

The distribution of olfactory abnormalities among groups is depicted in Figure 13, panel A. Although the findings did not achieve statistical significance (P=0.097), functional anosmia was similarly prevalent in iRBD (36%) and PD (33%) patients, but was absent in CTRL; conversely, a higher proportion of controls (36%) was normosmic compared to iRBD (9%) and PD (7%). Hyposmia was similarly prevalent in the 3 groups (64% in CTRL, 55% in iRBD and 60 % in PD group), as expected from the advanced age of the enrolled population.

OH was more common among iRBD (73%) and PD (60%) than in controls (25%), with borderline significance (p=0.055) (Figure 13, panel B), independently of antihypertensive treatment.

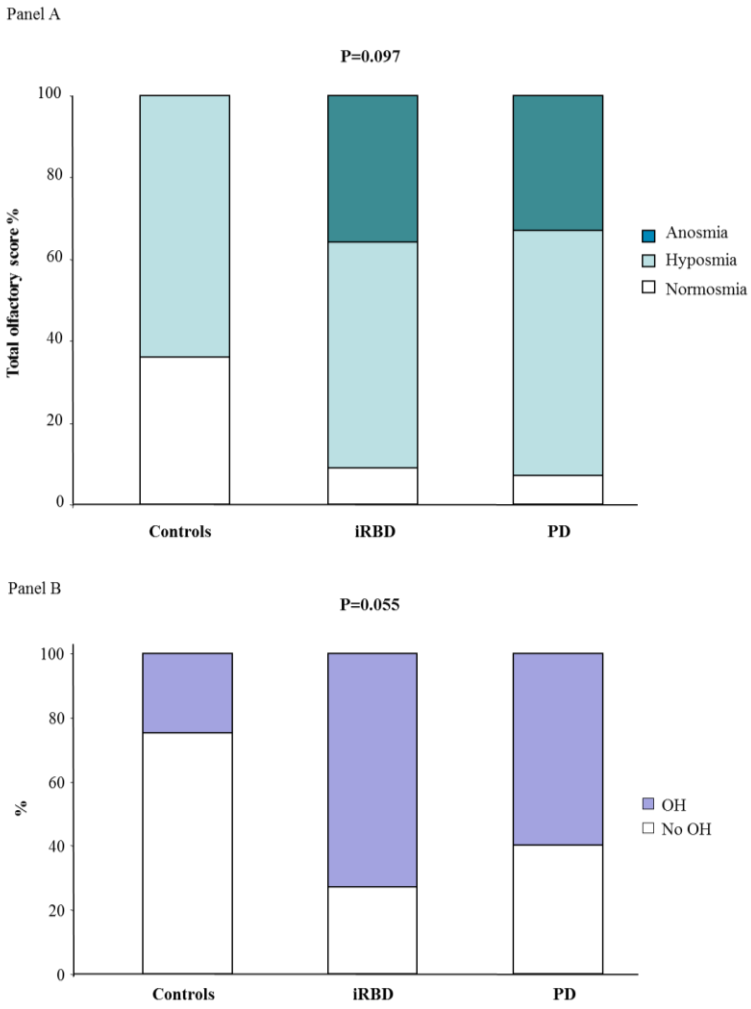

Figure 13. Comparison among CTRL, iRBD and PD groups with respect to olfactory function (Panel A) and orthostatic hypotension (OH) (Panel B).

4.4 Correlations among clinical, biochemical and functional variables

The relationship between functional and biochemical variables in the overall population is shown in Figure 14 A direct correlation was observed between TOS and blood reduced GSH concentrations (panel A) and VM ratio (panel B). Conversely, TOS was inversely related with urine neopterin levels (panel C).

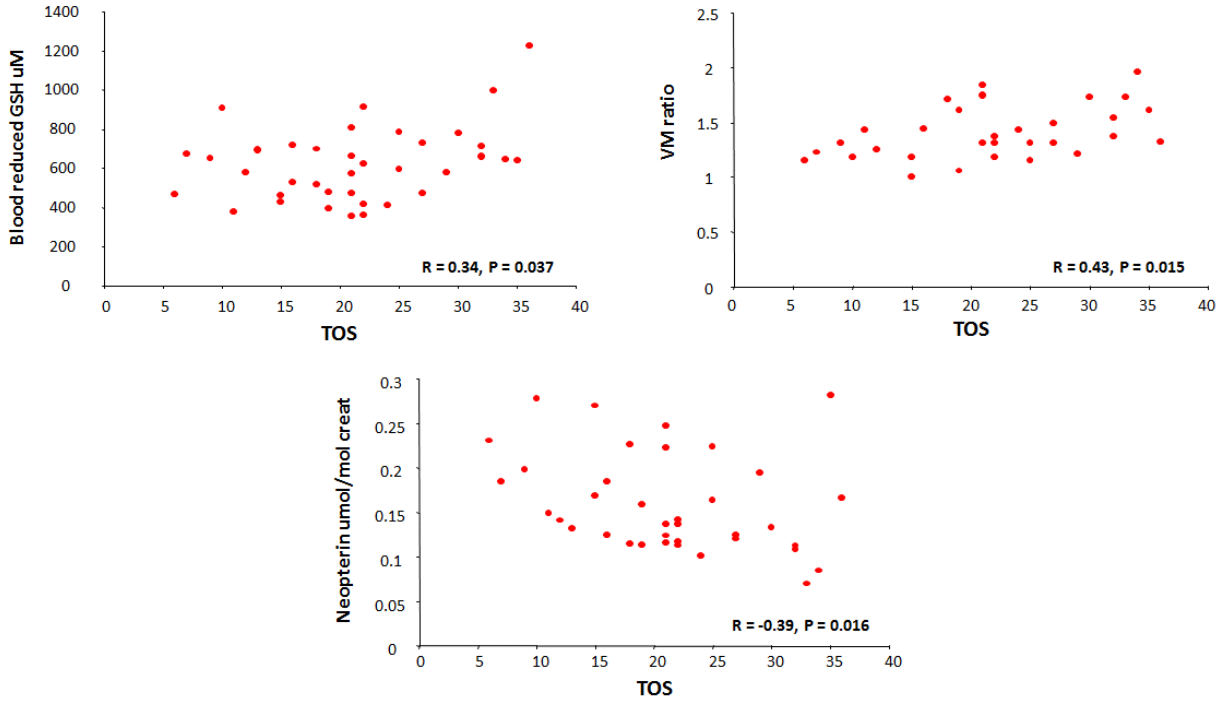

Figure 14. Correlation between (A) total olfactory score (TOS) and blood reduced glutathione (GSH), (B) Valsalva manoeuvre (VM) ratio, (C) urinary neopterin.

4.5 miRNA screening analysis

Primary analysis:

Overall, an average of 4 million of 36 bp single-end reads from each sample was obtained. After pre-processing and filtering steps, about 2,7 million reads per sample of clean sequences (minimum length of 17bp) were selected for downstream analysis. For each sample, miRNA expression profile was built by counting the number of sequenced reads for each identified miRNA that is present in miRBase v.21. About 889 thousand reads mapped to mature miRNA sequences annotated in miR Base v.21. The distribution of total reads counts in each sample is shown in table 5.

Table 5. Primary data analysis for each sample submitted to NGS

		Total raw reads	**Total clean reads**	**miRNAs reads**
Sample ID	**Group**			
MONPI	PD	4175567	3460919	1471225
PERSA	PD	6746074	3656277	655337
CORGI	PD	3837344	2566989	714984
FOIMA	PD	4765964	2998895	655740
GUAGU	RBD	4434625	3098705	1423235
HONGI	RBD	3072386	1885273	934277
ACQAN	RBD	3137508	2264316	723285
UGOMA	RBD	1661235	1358618	667221
PEDAN	CTRL	2810804	1493910	473819
CAMPA	CTRL	5918979	4944012	998772
CINGI	CTRL	5558930	3453483	1188206
FIOGRE	CTRL	2729471	1669040	756306
Average Counts		**4070741**	**2737536**	**888534**

Secondary analysis:

A. *Exploratory data analysis:* in order to retrieve insight into group similarities, the overall similarity between all samples was investigated by measuring the Euclidean distance between the miRNA expression profiles, followed by hierarchical clustering. Resulting heat map (Figure 15) suggested that, considering the global miRNA expression profile of all samples, samples were generally very similar each other. Samples belonging to RBD group were more homogeneous respect to samples of the other two groups.

Moreover, in the PD group as well as in the CTRL group two samples (MONPI and CAMPA respectively) were more different from the other members of the same group, even if not so much to exclude them from the differential analysis only on the base of these results. So we decided to perform the differential analysis with all samples.

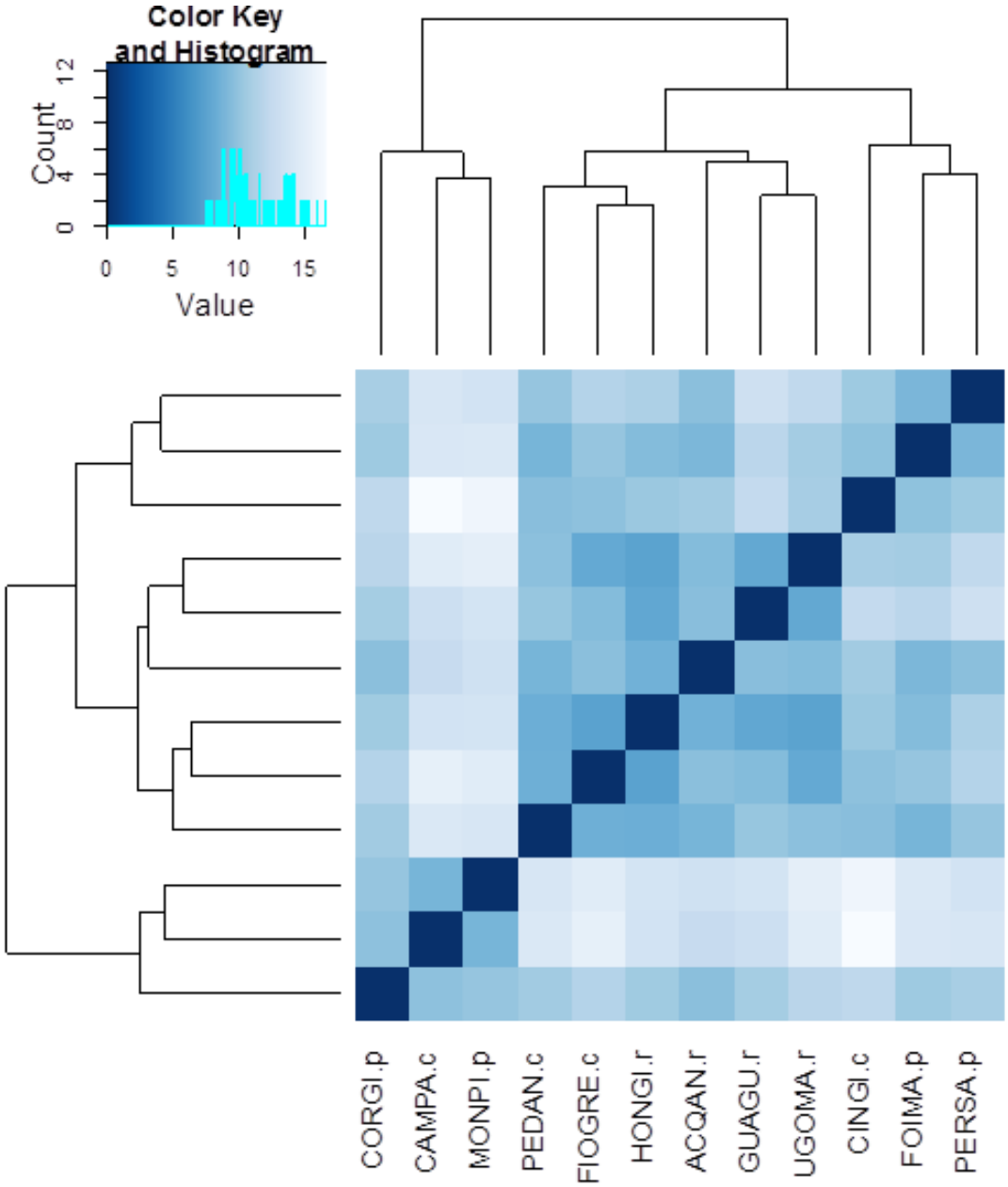

Figure 15. Sample clustering based on sequenced microRNA profiles. The heat map shows a blue scale false colour representation of the Euclidean distance matrix, and the dendrogram represents a hierarchical clustering.

B. Differentially expressed miRNAs identification: No statistically significant DE miRNA was observed in the PD versus CTRL, whereas we found 33 DE miRNAs in the PD versus iRBD comparison; among these, 18 were downregulated with FC<-0.5 and 15 upregulated with FC>0.5. Six DE microRNA (3 down- and 3 up-regulated) was detected in the iRBD group compared to CTRL one.

Only 4 DE miRNAs (miR-101, mir-1260a, miR-142, miR15a) were common in the two comparisons reported above. As shown in Table 6, three of these (miR-101, mir-142 and miR15a) were downregulated and one (mir-1260a) upregulated in the PD patients with respect to iRBD group. Conversely, miR-101, mir-142 and miR15a were upregulated and miR-1260a downregulated, in iRBD compared to CTRL subjects.

Table 6. Analysis of DE miRNA between PD versus RBD and RBD versus CTRL groups

miRNA ID	PD vs iRBD		iRBD vs CTRL	
	log2 FC	**p-value**	**log2 FC**	**p-value**
hsa-miR-101	-1,26	2,56E-04	2,77	5,56E-03
hsa-mir-142	-0,91	1,53E-03	2,84	4,47E-03
hsa-mir-15a	-1,41	4,76E-05	2,78	5,35E-03
hsa-mir-1260a	1,31	3,10E-04	-2,94	3,31E-03

4.6 miRNA validation by Quantitative Real-Time PCR

In order to validate the results observed in our miRNA sequencing analysis, we subsequently carried out the qRT-PCR in the overall recruited subjects (n=38). Specifically, miR-101, mir-1260a, miR-142, miR15a were chosen for validation test. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method, using the exogenous spike-in cel-miR-39 as reference for normalization. The crucial step of this analysis was the optimization miRNA extraction method to achieve adequate miRNA quality and quantity. In order to optimize the method of RNA extraction we primarily tried to increase the starting plasma volume from 100 ml to 200 ml modifying the manufacturers' protocol. In addition, we added for each sample, 5 μg of glycogen, a common co-precipitant for enhanced RNA recovery. To evaluate the miRNA recovery, we monitored the Cp of cel-

miR-39 spike-in control, added during the RNA extraction. We detected an increase of miRNA yields observing a reduction of the mean spike-in control Cp (data not shown). We performed new qRT-PCR assay for each miRNA but no statistical differences were still found between three groups (Figure 16).

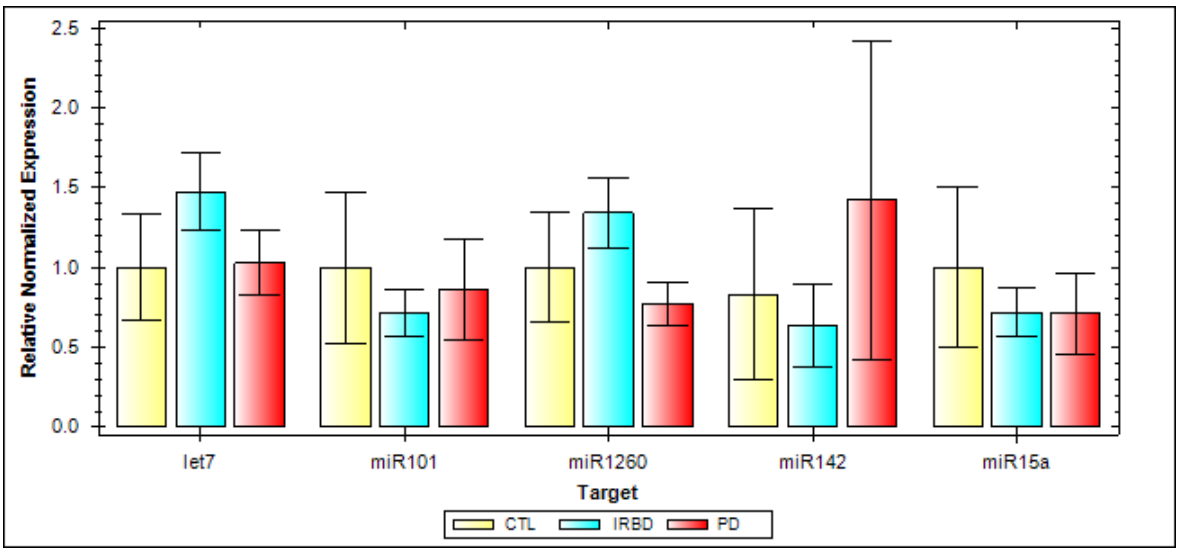

Figure 16. Relative Expression for let-7, miR-101, miR-1260, miR-142 and miR-15a in PD, iRBD and CTRL groups by qRT-PCR.

5. DISCUSSION

Development and validation of disease-specific biomarkers for the diagnosis of preclinical phase of PD represents one of the most urgent needs in neurological research [4]. The purpose would be to find a non-invasive and reliable biomarker, or a panel of biomarkers in PD in order to recognise and better monitor asymptomatic individuals during the early-stage of the disease.

The main findings of the present study are that reduced systemic antioxidant capacity is independently associated to overt PD and iRBD, a condition established as prodromal PD, and correlates with olfactory and sympathetic dysfunction. Moreover, progressive cardiovascular autonomic dysfunction, expressed as altered sympathetic (VM ratio, OH) or parasympathetic (LS ratio) response to testing, is found from prodromal state to overt disease and correlates with olfactory dysfunction. Increased concentrations of neopterin, an inflammatory biomarker, are associated with worse olfactory dysfunction.

GSH, the most abundant aminothiol in mammalial cells, has several functions in the brain, acting as an antioxidant and a redox regulator [134]. Several evidences, from experimental results in PD models and analyses of postmortem brain tissue from PD patients, point to a profound loss of GSH in the substantia nigra, that parallels the severity of disease and occurs prior to other hallmarks of tissue damage [135,136]. GSH depletion may affect mitochondrial function through the selective inhibition of mitochondrial complex I enzyme in the respiratory chain [137] which leads to an excessive production of ROS and a general decrease in ATP levels.

We found low GSH concentrations in blood cells of both PD patients and prodromal iRBD subjects. These results suggested that antioxidant depletion may be present not only in brain tissue, but also in systemic cellular districts, already at an early stage of disease. However, we did not find any difference in plasma GSH content and oxidative stress biomarkers, indicating that this perturbation might be confined to the intracellular space without involvement of the extracellular compartment.

In our study, blood reduced GSH concentrations paralleled the progression from prodromal to overt disease, as expressed by olfactory impairment and autonomic dysfunction. Moreover, blood reduced GSH levels were directly related to total olfactory score in the overall population, indicating that subjects with olfactory dysfunction have low GSH concentrations.

The long established observation of olfactory dysfunction as an early frequent feature of PD, which often precedes motor symptoms by several months or even years [138], is grounded in Braak's hypothesis of a six-stage pathological process in the development of PD [29]. The accumulation of Lewy bodies follows a predictable sequence, that begins in the anterior olfactory nucleus and bulb and in the dorsal motor nuclear complex of the glossopharyngeal and vagal nerves and gradually advances, through the brain stem toward the midbrain, in the late stage of the disease. Recent findings of decreased olfactory bulb volume in volumetric MRI scans of PD patients lent in vivo support to these neuropathological findings [32] Importantly, previous studies did not find a significant progression of olfactory dysfunction with PD duration [33,36]. We observed worsening olfactory dysfunction from control subjects to iRBD and PD patients, with functional anosmia being present in both iRBD and PD patients, but not in CTRL. While odour identification, discrimination, and threshold detection are unlikely to deteriorate in the course of PD, as confirmed by the lack of correlation between olfactory dysfunction and disease duration in our PD group, olfactory loss may be an important preclinical marker of disease, which may be assessed using non-invasive and low cost methods, such as the standardized “Sniffin’ Sticks” test. Conversely, hyposmia, which is strongly influenced by age, was unable to discriminate patients in the prodromal phase or with overt disease from CTRL. On the same line, worse olfactory dysfunction correlated with increased neopterin levels, which were not independently predictive of overt PD; aging per se is in fact a state of low-grade inflammation.

Cardiovascular dysautonomia in PD affects both the sympathetic and parasympathetic component of the autonomic nervous system [39,139,140]. OH is present in up to 52% of PD patients[141]. Mechanisms of OH in PD have been postulated to include baroreflex failure due to central lesions in the upper brainstem, that affect postural control of blood pressure, and loss of sympathetic innervation, e.g., post-ganglionic impairment, at the cardiac and peripheral vascular level. While the severity of parasympathetic dysfunction worsens with disease progression [36], as also confirmed in our patients by the inverse relation between PD duration and the DB E/I ratio, OH has been shown to occur early or predate disease development in PD [143] and to be associated with neuroimaging evidence of cardiac and extra-cardiac sympathetic denervation [139]. Among iRBD patients who converted to a neurodegenerative disease after a mean of approximately 3 years, Postuma et al. [143] observed a higher prevalence of OH (66%

versus 0%) and a larger systolic blood pressure drop from lying to standing than in non-converters. In a large multicenter series, patients with iRBD complained significantly more often of dizziness with postural changes than controls [144], while cardiovascular and gastrointestinal symptoms, together with older age, also predicted conversion from iRBD to neurodegenerative disease [145], which occurred in 41% of patients at 5 years.
Frauscher et al. [146] compared autonomic function testing among 15 iRBD, 12 PD patients and healthy controls. These authors found evidence of autonomic dysfunction in iRBD, with intermediate severity between controls and PD patients on blood pressure regulation during orthostatic standing test and symptoms, and similar to PD in Valsalva testing. In our cohort, OH prevalence overlapped among iRBD and PD patients at approximately 60% and was almost 3-fold higher than in age-matched controls. We likewise observed a lower Valsalva ratio and larger systolic and diastolic blood pressure drop in iRBD patients, of intermediate severity with PD, than in CTRL, as well as a progressive decline in the LS ratio from prodromal to overt disease.

Non-motor symptoms of PD such as anosmia, REM behaviour disorder, and OH, which were also associated in our series, have been suggested to indicate relatively greater involvement of noradrenergic than dopaminergic neurons [139]. The LC noradrenergic system influences olfactory function by directly modulating neurogenesis in the olfactory bulb, which has been implicated in olfactory learning and discrimination. Since an intact noradrenergic innervation is critical in maintaining normal levels of dopamine release in the mesolimbic and nigrostriatal systems, a deficient LC-noradrenergic system could be a key factor in the loss of nigrostriatal dopaminergic function and development of PD symptoms in this disease [149]

Recent evidences have suggested that miRNAs play a role in PD pathogenesis by regulating oxidative stress, mitochondrial dysfunction, α-synuclein aggregation and inflammatory neurodegeneration [147]. However, currently there are no known miRNA expression profiles rather than single miRNA levels that are specifically responsible for the development of individual neurological disease and that could be useful biomarkers for diagnosis of PD [148]. To date, few studies have been conducted in PD patients and healthy controls and the results presenting the circulating differentially expressed miRNAs are inconsistent [113-115,150]. For this purpose, we investigated the plasma miRNA profile in PD as potential biomarkers for overt and prodromal PD with respect healthy controls.

A next generation sequencing approach has been chosen to have a complete coverage of miRNA profiling and, even if the analysis was performed on a limited number of subjects, it reveals significant differences in the patterns of miRNA expression in the overt PD with respect to prodromal phase but not compared to controls. In particular, 33 miRNAs were deregulated between iRBD subjects and PD patients and 6 between prodromal state and control group. These results indicate that the number of deregulated miRNAs is greater in the prodromal subjects compared to established disease and controls. The NGS results have not been validated by RT-PCR analysis till now probably for a methodological problem and also because these miRNAs are expressed at very low levels in plasma. The methodological approach for plasma miRNA extraction, retrotranscription and amplification reactions needs to be improved.

This study presents, however, some specific limitations. Our series was relatively small, yet similar to those analyzed in previous studies comparing iRBD and PD for multiple domains [146]. There was some gender imbalance, which did not achieve statistical significance, between PD and iRBD groups; however, this finding is in accordance with the known male prevalence among iRBD patients [145].

We did not study drug-naive PD subjects, which might be important to trace the progression from prodromal to early disease. However, we recruited PD patients who were homogeneous for disease stage and drug therapy: all were in Hohen&Yahr stage 1 to 3 and on concomitant dopaminergic and DOPA treatment.

PD medications have shown differential effects on the autonomic responses to cardiovascular testing. In the study by Haapaniemi et al. [151] during chronic treatment dopaminergic agonists increased and levodopa reduced the orthostatic fall in blood pressure, whereas neither drug affected heart rate responses. Since all our PD patients were all on both drug classes, concurrent therapy might have mitigated orthostatic blood pressure changes. On the other hand, our results may not be generalizable to drug-naive patients or those on either drug class alone.

In conclusion, reduced systemic antioxidant capacity is found in prodromal and overt PD and may represent, in association with olfactory loss and cardiovascular autonomic dysfunction, a useful additive biomarker of disease. Moreover, the present miRNA profiling study allowed to identify a set of differentially modulated miRNAs, in the overt PD with respect to prodromal phase despite the analysis was no validated by RT-

PCR. Our pilot findings need to be confirmed in a larger population to establish their actual clinical value for an early diagnosis of PD.

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