Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE SPECIALISTICHE

Ciclo XXVIII

Settore Concorsuale di afferenza: **NEUROLOGIA 06/D6**
Settore Scientifico disciplinare**: NEUROLOGIA MED/26**

Identification and Characterization of MicroRNAs Involved in Parkinson's Disease: Potential Role as Diagnostic Biomarkers.

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Esame finale anno 2016

Acknowledgments

I would like to thank Prof. Pietro Cortelli, my PhD Tutor, who has always supported me, encouraged with energy and advised in giving the best, and guided me along my educational and professional way.

I would like to thank Dot. Christine Schwienbacher, Senior Researcher at Center of Biomedicine EURAC (The European Academy of Bolzano), who has followed my laboratory work with confidence, dedication and scientific curiosity and urged me to independence and to have a researcher's vision.

I would also thank Luisa Foco, Anne Picard and Alessandra Zanon.

ABSTRACT

Background. MicroRNAs (miRNAs) are small non-coding RNAs of 20-22 nucleotides, involved in transcriptional and post-transcriptional regulation of gene expression. MiRNAs function via base-pairing with complementary sequences within target mRNA molecules, usually resulting in gene silencing via translational repression or target degradation. Research on miRNAs is becoming an increasingly attractive field, as these small RNA molecules are involved in several physiological functions and diseases. To date, only few studies have assessed the expression of peripheral blood mononuclear cells (PBMCs) and plasma miRNAs related to Parkinson's disease (PD) using microarray and quantitative real-time polymerase chain reaction.

Objective. The aim of the present study is to profile the expression of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p and assess their potential role as biomarker for Parkinson's disease. In order to define their potential as biomarker, we planned our study according to the following points (i) identification of a suitable set of commonly used small RNAs as normalizers to be considered reliable reference genes in qRT-PCR expression analyses on PBMCs samples from Levodopa (L-dopa) treated PD patients and disease-free matched controls and evaluate the consequence in expression's direction of the target miRNAs (miR-29a-3p and miR-30b-5p) using different combinations of reference gene set; (ii) evaluation of the expression levels of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p first in PBMCs and then in plasma samples from L-dopa treated PD patients and disease-free matched controls; subsequent comparison of their expression profiles in order to assess whether the observed difference in miRNAs expression is present in both of them, and verify if plasma is a suitable alternative/additional easy accessible resource for PD miRNA biomarker; (iii) verify if the observed difference in miRNAs expression could be the consequence of treatment rather than the PD pathogenesis per se, performing an expression analysis on PBMCs and plasma samples from drug-naïve PD patients and disease-free matched controls; (iv) perform an exploratory deep sequencing analysis on 4 PBMCs samples from L-dopa treated PD patients and disease-free matched controls using Illumina MiSeq Next Generation Sequencing platform in

order to discover new miRNAs; (v) interpretation of the miRNAs expression data in a biological context through a more sophisticated *in silico* target prediction.

Methods. Plasma and PBMCs miRNAs coming from L-dopa treated PD patients, drug-naïve PD patients, and unaffected controls matched 1:1 by sex and age, were analyzed. The expression analysis for miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p was performed by qRT-PCR and the data normalized using RNU24 and Z30 for PBMCs samples and the C.elegans synthetic oligos spike-in cel-miR-39-3p, cel-miR-54-3p and cel-miR-238-3p regarding plasma samples. The statistical significance of miRNA expression differences was calculated by computing a two-tailed paired t-test. NGS analysis was done using MiSeq Illumina platform. To detect putative miRNA targets, several miRNA resources were combined to generate an overall score for each candidate gene using weighted rank aggregation.

Results. RNU24 and Z30 have been identified as reliable and stable set of reference genes to be use as normalizers for expression analysis in PBMCs samples from L-dopa treated PD and drug-naïve PD patients. The effect on the relative expression values of miR-29a-3p and miR-30b-5p has been tested using different combinations of reference genes. The normalization of the microRNAs expression using different reference gene sets results in a modification or in an opposite direction of the miRNAs expression.

We revealed for the first time an over-expression of miR-103a-3p ($p<0.0001$) in 36 PBMCs samples from L-dopa treated PD patients and matched controls. Moreover, we found an up-regulation of miR-29a-3p ($p=0.005$) and miR-30b-5p ($p=0.002$). The analysis on 31 plasma samples from L-dopa treated PD patients indicated a trend of over-expression of miR-30a-5p ($p=0.054$), whereas the miR-29b-3p previously found expressed in peripheral blood samples could not be analyzed in plasma due to its extremely low expression level. The trend over-expression of miR-30a-5p was confirmed in an independent secondary experiment on 50 plasma samples from L-dopa treated PD patients and controls matched 1:1 by sex and age. Thus, indicating different miRNA expression profiles between PBMCs and plasma.

No differences in miRNAs expression levels in 10 PBMCs samples from drug-naïve PD patients were found, whereas a borderline significant over-expression of miR-30b-5p (p=0.0858) was detected in 10 plasma samples from drug-naïve PD patients, thus indicating a potential involvement of the L-dopa treatment in the miRNAs expression.

The very preliminary result of the subgroup analysis in plasma and blood, stratifying the samples by gender, suggests that there is a trend of differentially miRNA expression according to the gender.

It has been defined the method for the library preparation for NGS analysis. Albeit the number of reads generated and the number of samples sequenced in this pilot phase, the data were not sufficient to generate sequencing results in a meaningful biological analysis.

A sophisticated approach has been elaborated to obtain a promising *in silico* target prediction model, identifying interesting candidate target genes, including genes related to neurodegeneration and PD.

Discussion. miRNAs expressed in peripheral blood and plasma are promising candidate diagnostic biomarkers due to the easy accessibility of the blood tissue. Our study shows for the first time an over-expression of miR-103a-3p in L-dopa treated PD patients and replicates a documented deregulation in PD, albeit opposite to published data, of miR-29a-3p and miR-30b-5p. Although the exploratory expression analysis performed on plasma samples from 10 drug-naïve PD patients and matched controls, showed a trend of higher expression of miR-30b-5p, suggesting an involvement of the L-dopa treatment in the miRNA expression, no difference in expression levels of miRNAs was revealed in PBMCs samples from drug-naïve PD patients, deriving from the same individuals analyzed in plasma samples. So, further expression studies in a larger sample size should be performed to elucidate the putative role of L-dopa in the deregulation of the investigated miRNAs, in patients before and after drug administration.

The *in silico* analysis identified putative candidate target genes, including genes related to neurodegeneration and PD, such as *LRRK2* for miR-30b-5p and miR-103a-3p, *PARK7/DJ-1* for miR-29a-3p, *Bcl-2* as common target for all miRNAs

(miR-30b-5p, miR-29a-3p, miR-103a-3p, and miR-30a-5p), or the involvement of miR-103a-3p in the insulin resistance.
Despite the preliminary character of the study, the results provide a rationale for further clarifying the role of the identified miRNAs in the pathogenesis of PD, for validating their diagnostic potential, and future use as biomarkers to monitor or prevent the motors symptoms induced by L-dopa treatment.

The obtained results have been published in two differents manuscripts, the first in Neurology (Serafin *et al., "Overexpression of blood microRNAs 103a, 30b, and 29a in L-dopa-treated patients with PD."*, 2015) and the second in BMC Research Notes (Serafin *et al., "Identification of a set of endogenous reference genes for miRNA expression studies in Parkinson's disease blood samples.",* 2014) and a third one is under elaboration.

1. INTRODUCTION

The aim of the present study is to profile the expression of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p and assess their potential role as diagnostic and/or prognostic biomarkers for Parkinson's disease (PD). The study was conducted on peripheral blood mononuclear cells (PBMCs) and plasma samples from Levodopa (L-dopa) treated PD patients, drug-naïve PD patients and not affected controls matched 1:1 by sex and age.

The subsequent chapter will give an introduction on Parkinson's disease, microRNAs and their possible role in the pathogenesis of idiopathic Parkinson's disease and their potential role in early diagnosis and disease progression.

1.1 Parkinson's disease

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. PD affects 1% of population beyond 65 years of age with high prevalence in men. Although approximately 10% of cases affect people under age 40, PD is primarily a disease of the elderly, only a small percentage of PD patients, mostly with the genetic forms of PD, develop parkinsonism before the age of 45. PD is the most frequent form of parkinsonism, a term used to describe movement disorders with parkinsonian features independent of their etiology. Parkinson's disease is a progressive neurodegenerative disease, which is generally considered a multi-factorial disorder that arises due to a combination of genes and environmental factors. Most cases are idiopathic or late-onset PD (> 85% of all cases), and a monogenic cause can be detected in about 2–3 % of the patients (Klein and Schlossmacher, 2007). Since our study only focuses on idiopathic PD, genetic forms of PD will not be described here.

The majority of PD cases are sporadic, about 10-15% of patients report a positive family history. Although familial and idiopathic forms of the disease differ in several clinical aspects, it is clear that common molecular pathways underlie the neurodegeneration. These include oxidative stress, mitochondrial dysfunction, energy production imbalance and disruption of the ubiquitin-proteasome system.

The study of these pathways gives us some information about the mechanisms underlying PD, but the causative factors for idiopathic PD remain still unknown.

PD is characterized clinically by restring tremor, rigidity, reduced motor activity (bradykinesia), and postural instability (Hoehn and Yahr, 1967), and pathologically by loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of alfa-synuclein positive inclusions in the cytoplasm of neurons, termed Lewy bodies (Forno et al., 1996; Goedert et al., 2013; Spillantini et al., 1998b). The fundamental components of these inclusions are alfa-synuclein, neurofilament proteins, and ubiquitin (Forno et al., 1996). Although the majority of idiopathic PD has Lewy body pathology at autopsy, this feature is neither exclusive to this disease nor common to all cases (Halliday et al., 2011). Motor impairment is often associated to non-motor features such as autonomic, cognitive, and psychiatric problems, sensory loss and sleep disturbance, which are difficult to treat and often manifest before motor disability (Langston et al., 2006). Moreover, these motor dysfunctions become apparent when 70–80% of nigrostriatal nerve terminals have undergone degeneration, implicating a compensatory mechanism in the first stages of the disease (Bernheimer et al., 1973). Mild cognitive impairment and subsequent dementia is also common (Litvan et al., 2012).

To date, there is no treatment to arrest or retard the progression of neurodegeneration. In fact, dopamine (DA) therapy can improve motor symptoms, but does not influence the rate of clinical disease progression and has serious side effects such as motor fluctuations and dyskinesias, due to the fluctuation in L-dopa concentration (Fahn, 2000,Schapira, 2007). Since DA administered orally is not able to cross the blood brain barrier, current therapy is based on administration of the dopamine precursor, named L-dopa. L-dopa crosses the blood-brain barrier and replaces the lack of dopamine in the striatum after conversion in dopamine, increasing the DA content in the brain. L-dopa is the most effective drug that acts on the symptoms of PD but it has a short period of half-life. In fact, the main issue related to L-dopa treatment is represented by its low bioavailability in the central nervous system, due to the rapid peripheral decarboxylation to dopamine (Colamartino et al., 2012). For that reason, it is necessary to combine the

treatment with drugs which inhibit the amino acid decarboxylase (AADC) such as Carbidopa, which increases the L-dopa half-life in central nervous system (CNS). Many patients with the progression of disease severity require higher doses of L-dopa, causing motor complications. Because of the side effects in L-dopa treatment it would be important to be able to monitor its levels in blood, so that an individual personalized therapy could be developed, and could be possible predict and avoid significant motor fluctuations. Indeed, each patient requires a different dosage of drug and the use of miRNAs as biomarker could represent an important variable for therapy improvement.

In the absence of reliable diagnostic markers, the clinical diagnosis of PD is based on the presence of characteristic features. Several diagnostic criteria have been developed for PD and in 1999 Gelb and colleagues proposed a set of diagnostic criteria for PD, based on a review of the literature regarding the sensitivity and specificity of the characteristic clinical features (Gelb et al, 1999). The reliability of the different diagnostic criteria, however, has not been vigorously tested by autopsy examination, that is commonly considered the gold standard. Two separate clinical-pathologic studies concluded that only 76% of the patients with a clinical diagnosis of PD actually met the pathologic criteria; the remaining 24% had evidence of other causes of parkinsonism. The clinical diagnosis of PD is mainly based on physical examination findings, from moderate to high accuracy (sensitivity and specificity of 88% and 95.4% respectively, with a positive predictive value of 85.7%). Much of the difficulty in the diagnosis of PD is in differentiating it from other disorders that cause parkinsonism. Moreover, predicting which patients with PD will have a relatively benign versus a more severe course of disease, such as the development of dementia, is also very difficult based solely on clinical grounds.

Neuroimaging techniques have acquired an important role in the last decade. For example conventional magnetic resonance imaging (MRI) and advanced MRI techniques such as magnetic resonance spectroscopy (MRS), diffusion-weighted and diffusion tensor imaging and functional MRI, seems to be promising methods

in differential diagnosis of early PD with initial motor symptoms from atypical parkinsonian disorders, thus, making easier early diagnosis. Thus, the development of new potential therapeutics or predictable biomarkers for PD is essential for patient care.

1.2 MicroRNAs

Small RNAs are a variety of non-coding RNAs classes of 20-30 nucleotides long. Each class of small RNA differs in its origin and biogenesis, the proteins they interact with, the mechanism of action of the RNPs (RNA-protein complexes), and the nature of their targets.

MiRNAs act as endogenous post-transcriptional regulators of gene expression by base-pairing with their target, the RNA messenger (mRNA). The AGO proteins play a role as effectors recruiting factors, that cause translational repression, mRNA deadenylation, and mRNA decay (Huntzinger, 2011). The miRNA-binding site is situated normally at the 3' untranslated region (UTR) of mRNAs. MiRNA genes represent one of the most abundant gene families. To date, they have been annotated into a database called miRBase, 2588 human miRNAs and the miRNA number is constantly increasing (www.mirbase.org).

1.2.1 MicroRNAs structure, biogenesis and mechanism of action

The so-called miRNA-seed region represents the domain at the 5'end of the miRNA, which is extended from nucleotide position 2 to 7, and plays a key role in the target recognition. In human more than 60% of the genes coding for proteins contain at least one conserved miRNA binding-site, and considering the fact that also numerous not conserved sites exist, most protein-coding genes may be under the control of miRNA (Ha and Kim, 2014). The function of the miRNAs is tightly regulated, and their participation in the regulation of physiological cellular processes such as development and differentiation pathways, proliferation, stress response, metabolism and apoptosis has been demonstrated, also their

deregulation is often associated with human diseases such as cancer, autoimmune diseases and some nervous system disorders.

Here below will be described the canonical biogenesis pathway of miRNAs (Ha and Kim, 2014; Winter et al., 2009; Wilfred et al., 2007). MiRNA genes are generally transcribed by RNA polymerase II producing a double-strand stem loop structure primary transcript, called pri-miRNA (over 1kb) characterized by a local hairpin structure. Tanscription factors, such as p53, MYC, MYOD (myoblast determination protein 1), and ZEB1 and ZEB2 associated to the RNA-Pol-II determine the positive or negative expression of miRNAs (Ha and Kim, 2014). Only pri-miRNA, which contains an appropriate stem, a large flexible terminal loop and the capability of producing a single stranded RNA overhangs at the 5' and 3' sides, will be efficiently processed and mature into functional miRNA. The stem-loop sequence of the pri-miRNA is recognized by a series of enzymes that orchestrate a tightly controlled maturation process (Ha and Kim, 2014). The pri-miRNA is processed by Drosha and cofactor DGCR8 (double-stranded RNA-binding protein) called Pasha. Drosha is a nuclear protein of 160kDa, belonging to the family of the RNase III-type endonucleases, that acts specifically on double-stranded RNA (dsRNA). The nuclear RNase Drosha together with cofactor DGCR8, initiate the maturation of the pri-miRNA, cutting the pri-miRNA stem loop in order to release a small hairpin-shaped RNA of ~60-70 nucleotides long, called pre-miRNA.

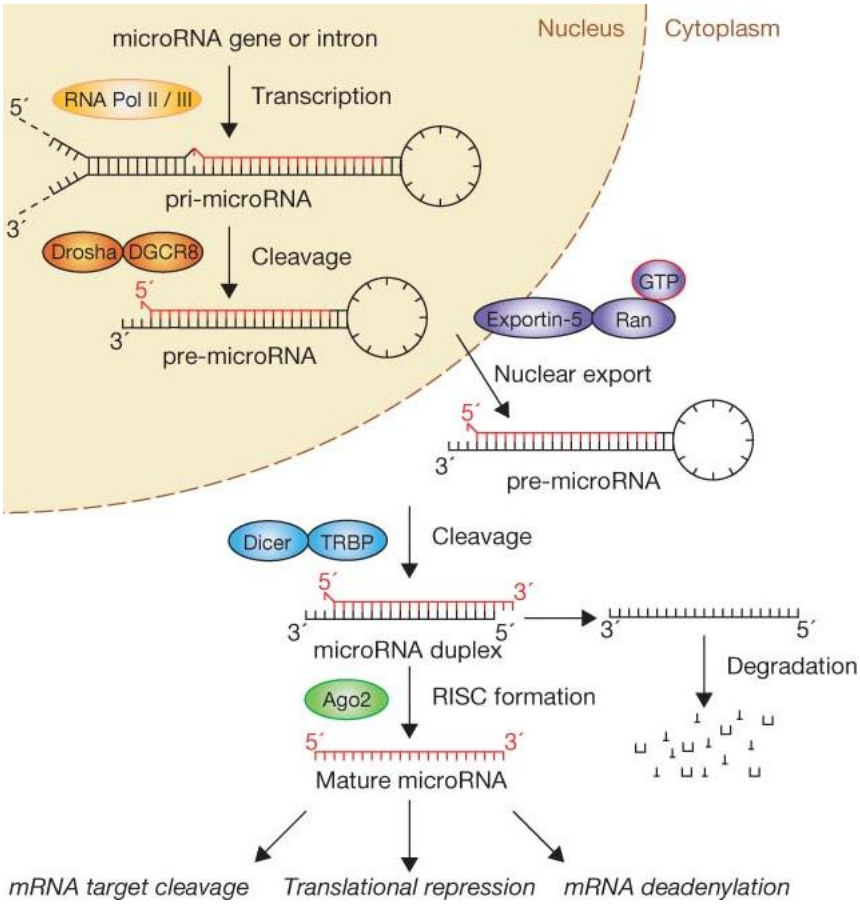
Pre-miRNA is exported to the cytoplasm in order to complete its maturation. The translocation through the nuclear pore is regulated by protein exportin 5 (EXP5), which binds Ran-GTP and pre-miRNA forming a transport complex. The hydrolysis of GTP results in the disassembly of the complex and the release of the pre-miRNA into the cytosol. The pre-miRNA transport through the nuclear membrane is specific due to the characteristic of EXP5 which recognizes a dsRNA stem of >14bp long that presents a short 3' overhang (that is 1-8 nucleotides in length) (Ha and Kim, 2014).

In the cytoplasm, a second RNase III enzyme of ~200kDa called Dicer, its cofactor

TRBP (transactivation response RNA-binding protein), and PACT (protein activator of PKR) mediate the conversion of pre-miRNA to mature miRNA, by cleaving the pre-miRNA near to the terminal loop, in an ATP-independent manner, generating a double-stranded miRNA duplex (miRNA:miRNA*) of ~22 nucleotides long consisting of the most abundant mature miRNA and the less abundant anti-sense strand miRNA* (also called passenger strand). Each strand of the miRNA complex originates from opposite arms of the stem-loop. The miRNA duplex is loaded into Argonaute 1-4 proteins (Ago 1-4) complex as a double-strand RNA, forming a pre-RISC complex (RNA-induced silencing complex). This step is ATP-dependent and requires the Hsc70/Hsp90 chaperone machinery (Kawamata et al., 2011). Normally the second filament is degraded (miRNA*). However there are many cases in which also the second filament becomes a mature miRNA and plays an active role in the regulation of the target mRNA. The pre-RISC complex (in which the Ago protein associate with the miRNA duplex) quickly removes the passenger strand to generate a mature RISC complex. The core components of the RISC complex are Dicer, Ago2, PACT and TRBP, and both TRBP and PACT are critical for efficient miRNA processing, because their deletion results in decreased mature miRNA levels and target gene silencing (Redfern et al., 2013). The mechanism of the human miRNA-RISC assembly is still unclear.

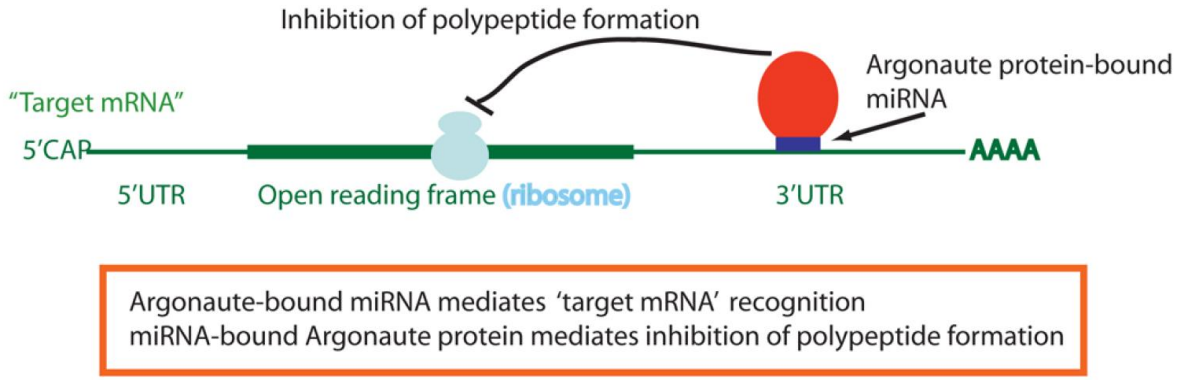
The mature miRNA is responsible for aligning the RISC complex to target mRNA by binding at complementary seed sequences (position 2-8) in the 3'UTR. This association of target mRNA with the miRNA-containing RISC most commonly results in down-regulation of gene expression by either repression translation or recruitment of protein complexes causing deadenylation and degradation of target mRNA. MiRNA bind to its target mRNA, repressing the translation or cleaving the target when is present a perfect complementarity, even if normally miRNAs bind in a non perfect complementary way to the 3' UTR of the target mRNAs blocking the translation. Through this mechanism miRNAs regulate the translation of more than 60% of protein-coding genes. The target silencing requests the presence of the Ago 4 proteins suggesting that the expression levels of miRNAs are directly related to the expression levels of Ago proteins. All Ago proteins are capable of inducing translational repression and decay of target mRNAs through interaction with the translation machinery and mRNA decay factors, called slicer-independent

silencing (Ha and Kim, 2014).



Canonical pathway of microRNA processing (Winter et al., 2009).

Most of the regulation processes in which miRNAs are implicated, are not known or clear; the difficulty lies in the fact that a single miRNA can regulate up to few hundreds of different mRNA, and therefore able to regulate the expression of multiple proteins involved in different biological processes.



MiRNAs recognition of target mRNA and impairment of the formation of a polypeptide from that mRNA (Wilfred et al., 2007). After miRNAs recognition of the target mRNA, the Argonaute protein helps to mediate translational inhibition and/or mRNA sequestration and degradation. MiRNAs (blue/purple); Argonaute proteins (red); target mRNA (green).

1.3 MicroRNAs and Parkinson's disease

In recent years has been highlighted that epigenetic and genetic defects in miRNAs and their processing machinery are a common hallmark of human diseases, albeit other non-coding RNAs, such as small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs), and the heterogeneous group of long non-coding RNAs (lncRNAs) might also affect the development of many different human disorders.

The miRNAs are found in high abundance within the nervous system, around 70% of all miRNAs expressed by all human tissues are expressed in the brain. These miRNAs are found to be co-expressed with their target and often display a brain-specific expression pattern. The miRNAs are physiologically implicated in different biological functions, often playing a regulatory role, including synaptic plasticity and neurogenesis. Deregulation or dysfunction of miRNAs in the neurodegenerative disorders and their emerging role in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease pathogenesis is increasingly recognized.

1.3.1 MiRNAs as regulators of PD genes

The first evidence of miRNAs implication in Parkinson's disease is to attribute to Kim and his group (Kim et al., 2007) showing a reduction of locomotion and symptoms reminiscent of human PD as a consequence of a deletion of Dicer in dopaminergic neurons in transgenic mice. Another example is miR-133b, which was shown to be downregulated in PD patients and be involved in regulation of maturation processes and function of dopaminergic neurons in midbrain (Kim et al., 2007). The mRNA expression levels of alfa-synuclein are repressed by miR-7, which shows decreased levels in PD-associated neurotoxin cell and rodent models (Junn et al., 2012). Furthermore, associated with PD, is the disruption of the binding site for miR-433 in FGF20 gene and the over-expression of FGF20 is correlated with high levels of SNCA (Wang G et al., 2008). MiR-34b/c has been found down-regulated in PD brains, underlying mitochondrial dysfunction and oxidative stress (Miñones-Moyano et al., 2011). Alfa-synuclein is negatively regulated by mirR-7 and miR-153, suggesting a possible approach to limit or solve PD progression.

The groups of Maciotta and Maffioletti (Maciotta et al., 2013; Maffioletti et al., 2014) elaborated an interesting and exhaustive summary of the miRNAs implicated in PD. Even if to date there are limited findings on deregulated miRNAs associated to PD, they are a promising source of new diagnostic, prognostic and therapeutic tools which could also help to understand more clearly its pathophysiology.

NDDs	miRNAs	Target genes	References
PD	miR-7	DP	Junn et al., 2009; Doxakis, 2010
	miR-153	E2F1	Doxakis, 2010
	let-7	LRRK2	Junn et al., 2009; Gehrke et al., 2010
	miR-184*	LRRK2	Gehrke et al., 2010
	miR-433	FGF20	Davis et al., 2005; Wang et al., 2008a,b
	miR-205	LRRK2	Cho et al., 2013

MiRNAs and specific target genes involved in PD. Table modified by Maciotta et al., 2013. NDDs (neurodegenerative disorders).

NDD	miRNAs	Source	Changes	Patients (P) and controls (C)	References
PD	miR-133b	SNC	Down-regulation	3P, 5C	Kim et al., 2007
	miR-34b/c	SNC	Down-regulation	11P, 6C	Minones-Moyano et al., 2011
	miR-1, -22*, -29a	Peripheral blood	Down-regulation	8P, 8C	Margis et al., 2011
	miR-181c, -331-5p, -193a-p, -196b, -454, -125a-3p, -137	Plasma	Over-expression	31P,25 C	Cardo et al., 2013

MiRNAs deregulation in PD. Table modified by Maciotta et al., 2013. NDDs (neurodegenerative disorders).

1.3.2 MicroRNAs as potential biomarker for Parkinson's disease

The Biomarkers Definition Working Group (Biomarkers Definition Working Group, 2001) has defined as biomarker "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". It is possible to think to a biomarker as a trait biomarker when indicates susceptibility to a disease, a state biomarker if it defines the diagnostic of a disease, or a rate biomarker, when it tracks progression of the disease (Chahine et al., 2014). It means that a biomarker is represented by a biochemical or molecular element expressed during a physiological or pathological condition in a specific stage of life, that can be used to diagnose, prognosticate and monitor the disease itself before and/or during the therapy. The identification of specific biomarkers at early stages of the disease could lead to early drug treatment, delaying the progression of the disease, and monitoring the treatment. Moreover a biomarker should meet requirements such as easy accessibility, sufficiently high specificity and sensitivity, low costs and standard applicability in the laboratories.

Most of the methods aimed to discover new biomarkers include proteomics (study of structure and function of specific proteins), metabolomics (study of chemical metabolic processes through the measurement of small-molecule metabolites), and gene expression profiling.

Regarding the proteomics, there are several studies applied to cerebrospinal fluid for PD. Challenges for developing new protein-based biomarkers include the complexity of protein composition in body fluids, the diversity of post-translational

modifications, which can affect the accuracy of measurement, the low relative abundance of many proteins of interest, the sequence variations among different clinically relevant species, and the difficulties in developing suitable high-affinity detection agents, even if this method is expensive and time-consuming (Etheridge et al., 2011).
Microarrays, next generation sequencing (NGS) and quantitative real-time PCR (qRT-PCR) are widely used in the gene expression profiling; to date NGS remains expensive and labor intensive, both for the sample preparation and for data analysis, a microarray approach generally require more starting material than qRT-PCR.

MicroRNAs are strong and specific gene regulators and therefore promising candidates to be diagnostic biomarkers and as well potential therapeutic targets.
They are stable in the bloodstream and their expression levels changes as a consequence of cellular damage or tissue injury. In fact, because of the proximity of the biological fluids to cells, the biochemical content of the fluids reflects the biological conditions of the cells and gives us information about the pathological status. For the present study it was decided to consider peripheral blood and plasma as potential miRNAs diagnostic sources as they are readily obtainable and as they reflect dynamically a system-wide biology (Liew et al., 2006). MiRNAs are highly abundant, tissue-specific (some miRNAs are specific to tissues or to biological stages), quantifiable, stable, with no post processing modifications. In the laboratory, the use of miRNAs as biomarkers could be advantageous, as they are stable in different extreme conditions, like for example high temperatures, high or low pH, extended storage, freeze-thaw cycles, and resistant to degradation by RNase-rich blood environment (Jin et al., 2013). Once blood samples are processed and plasma is collected, the miRNAs are stable at room temperature up to 24h, but it is not still clear whether the time duration between blood collection and the processing of plasma can affect miRNAs expression levels. For that reason in our study were standardized the processing conditions, defined after different time-tests of plasma processing (data not shown).

MicroRNAs have a lower complexity than proteins, which facilitates their exploration. Last but not least an important factor to consider, is that blood and plasma sample collection, containing miRNAs, is a minimal invasive process and costly efficient, and the levels of specific miRNAs can be measured by commonly standardized laboratory methods. All these characteristics make miRNAs attractive candidates biomarkers.

In order to develop a precise and sensitive PBMCs/plasma-miRNA based diagnostic biomarker it's necessary to focus on different technical aspects, such as the miRNA qRT-PCR assay, specific extraction methods in order to minimize the degradation process, quantification analysis, and the usage of correct endogenous genes for the expression data normalization.

1.4 Common miRNAs normalization procedures in blood

The importance of the miRNAs normalization is currently broadly recognized. To date do not exist an established and standardized reliable reference gene set to use as normalizers for miRNA profiling study in peripheral blood and plasma PD samples. The normalization process consist in the comparison of the expression levels of the investigated miRNAs (case-control groups) with the expression of an endogenous reference gene in order to obtain a relative quantification of the investigated miRNAs (Wotschofsky et al., 2011).

The common methods applied for normalization are described as follow (Sheinerman et al., 2013) (i) the use of synthetic miRNAs spiked in the sample before the miRNA extraction (e.g. C. Elegans or plant miRNAs which are not endogenous in the analyzed sample). By adding the same amount of spiked-in RNAs with an equal volume of sample, a stable reference control is obtained. This approach allows compensating for variability caused by RNA extraction step and the possible presence of inhibitors during the qRT-PCR process. They can be used as well to monitor the efficiencies of single step in the miRNA extraction, retrotranscription and expression analysis; (ii) the use of stable concentrated endogenous miRNAs not involved either directly or indirectly in the pathology

under study. Ideally, the value of the endogenous miRNAs are used as reference by comparing their concentration between the control and affected samples group fluctuation; (iii) the use of endogenous small nuclear or nucleolar RNAs (snRNA and snoRNA) which are longer than the miRNAs and more stably expressed; (iv) the normalization per average of all investigated miRNAs (global mean approach); this approach is used mostly for miRNA microarrays studies, where a large number of miRNA are analyzed.

Furthermore, the factors that can affect the efficiency of endogenous reference genes chosen as normalizers, could be technical factors including variability during the sample collection and processing, as well the presence in body fluids of various factors affecting miRNAs purification and qRT-PCR analysis.

The normalization for endogenous snRNAs, snoRNAs and miRNAs is commonly used in blood based study, whereas in plasma based study are used miRNAs and/or synthetic spikes-in. At the moment different normalization approaches are described in literature, but at the same time no single standard normalization method is commonly accepted.

In our study was decided to investigate snRNAs, snoRNAs, miRNAs and miRNAs spike-in in order to measure their stability values in all samples and choose the best normalization reference gene set feasible for our designed study.

2. OBJECTIVE

The aim of the present study is to identify and characterize PBMCs and plasma microRNAs (miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p) as potential diagnostic biomarkers for PD and verify if plasma is a suitable alternative/additional easy accessible resource as PD miRNA biomarker.

The following specific aims were addressed:

1. A screening of the best reference genes set to be used in an expression profile study in PBMCs samples from L-dopa treated PD, and drug-naïve PD patients and evaluate the impact of different reference gene sets on interpretations of target miRNA expression profile.
2. Profile the expression of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p in PBMCs and plasma samples from L-dopa treated PD patients and matched controls and compare their profiles; verify if plasma is a interchangeable and easily accessible PD biomarker sources.
3. Verify if the observed difference in miRNA expression could be the consequence of L-dopa treatment rather than to the PD pathogenesis per se, performing an expression analysis on PBMCs and plasma samples from drug-naïve PD patients and matched control.
4. Perform a preliminary deep sequencing analysis on 4 PBMCs samples from L-dopa treated PD patients using Illumina MiSeq Next Generation Sequencing platform in order to discover new miRNAs.
5. Interpret the miRNAs expression data in a biological context through a more sophisticated *in silico* target prediction.

3. MATERIAL AND METHODS

3.1 Standard protocol approvals, registrations, and patients consensus

Were enrolled 10 drug-naïve PD patients and 50 PD patients treated with L-dopa presenting at the Movement Disorders outpatient clinic of the General Regional Hospital of Bolzano (Italy). It was performed the PD diagnosis according to Gelb PD criteria (Gelb, Oliver, and Gilman, 1999) and were excluded all subjects with cognitive impairment or unable to sign the informed consent or affected by atypical parkinsonisms. Were enrolled 10+50 disease-free controls matched 1:1 by sex and age (range ± 3 years), recruited among spouses or unrelated caregivers. Demographic and clinical patients' features are summarized here below. The local ethics committee approved the study, and all enrolled participants provided written informed consent to participate.

Clinical details of 36 L-dopa treated PD patients, 10 drug-naïve PD patients, and disease-free matched controls.analyzed in PBMCs (Serafin et al., 2015).

Characteristic		Measure	Treated PD Patients (n=36)	Controls* (n=36)	Drug-Naïve PD Patients (n=10)	Controls** (n=10)
Gender	F	nr (%)	22 (61%)	22 (61%)	4 (40%)	4 (40%)
	M	nr (%)	14 (39%)	14 (39%)	6 (60%)	6 (60%)
AAE		Mean y ± SD	68 (11)	67 (10)	68 (7)	67 (7)
AAO		Mean y ± SD	60 (14)	/	64 (5)	/
Disease Duration		Mean y ± SD	7 (6)	/	4 (3)	/
IPD Phenotypic Subtype	Tremor Dominant	nr (%)	19 (53)	/	10 (100%)	/
	Akinetic-rigid	nr (%)		/	0 (0%)	/
UPDRS III	scale 0-30	Mean y ± SD	10 (7)	/	17 (10)	/
Hoen & Yahr modified stage	stage 1	nr (%)	17 (47)	/	4 (40%)	/
	stage 1,5	nr (%)	3 (9)	/	0 (0%)	/
	stage 2	nr (%)	11 (31)	/	4 (40%)	/
	stage 2,5	nr (%)	2 (6)	/	2 (20 %)	/
	stage 3	nr (%)	2 (6)	/	0 (0%)	/
	stage 4	nr (%)	0 (0%)	/	0 (0%)	/
	stage 5	nr (%)	0 (0%)	/	0 (0%)	/
LID		Mean y ± SD	11 (31)	/	/	/

*Legend: AAE: age at evaluation; AAO: age at onset; LID: Levodopa-induced dyskinesia; * age and sex matched controls for Not Treated IPD Patients;** age and sex matched controls for Drug-Naïve IPD Patients;*

Clinical details of L-dopa treated PD patients, drug-naïve PD patients and matched unaffected controls analyzed in plasma and drug-naïve PD patients and matched unaffected controls analyzed in PBMCs.

Characteristic		Measure	Plasma cases (n=50)	Plasma controls (n=50)	Plasma + PBMC cases (n=31)	Plasma + PBMC controls (n=31)	Drug-naive PD Plasma + PBMC cases (n=10)	Drug-naive PD Plasma + PBMC controls (n=10)
Gender	Female	n(%)	24 (48)	24 (48)	19 (61.3)	19 (61.3)	4(40)	4(40)
	Male	n(%)	26 (52)	26 (52)	12 (38.7)	12 (38.7)	6(60)	6(60)
AAE		Mean y (SD)	65.2 (10.0)	65.2 (10.4)	66.5 (10.7)	67 (11)	68(7)	67(7)
AAO		Mean y (SD)	58.5 (12.7)	-	59 (14)	-	64(5)	/
Disease Duration		Mean y (SD)	6.7 (5)	-	7.1 (5.9)	-	4(3)	/
PD Phenotipic Subtype	Tremor Dominant	n(%)	24 (48)	-	14 (45.2)	-	10(100)	/
	Akinetic-rigid	n(%)	26 (52)	-	17 (54.8)	-	0(0)	/
UPDRS III	scale 0-30	Mean y (SD)	9.3 (6.7)	-	10.1 (7.3)	-	17(10)	/
Hoen & Yahr modified stage	stage 1	n(%)	26 (53)	-	13 (43.3)	-	4(40)	/
	stage 1.5	n(%)	4 (8.2)	-	3 (10.0)	-	0(0)	/
	stage 2	n(%)	13 (26.5)	-	10 (33.3)	-	4(40)	/
	stage 2.5	n(%)	4 (8.2)	-	2 (6.7)	-	2(20)	/
	stage 3	n(%)	2 (4.1)	-	3 (6.7)	-	0(0)	/
	stage 4	n(%)	-	-	-	-	0(0)	/
	stage 5	n(%)	-	-	-	-	0(0)	/
LID		n(%)	15 (30)	-	10 (32.3)	-	/	/

*Legend: cases: L-dopa treated PD patients; AAE: age at evaluation; AAO: age at onset; LID: Levodopa-induced dyskinesia; * age and sex matched controls for Not Treated IPD Patients;** age and sex matched controls for Drug-Naïve IPD Patients;*

3.2 RNA isolation and quality control

RNA was obtained from 2ml peripheral blood collected in Na_3-citrate buffered Venosafe®Plastic Tubes (VF-054SBCS07, Thermo) and processed within 6 hours after blood collection.

For PBMCs L-dopa treated PD patient's samples and matched controls:

RNA, including small RNAs was extracted from PBMCs after red blood cell lyses in whole blood: mix 2 ml of whole human blood added to 10 ml of RBC (red blood cell)-lyses buffer incubated for 15min on ice (to permit the lyses of erythrocytes), followed by centrifugation at 400 xg for 10 min at 4°C, in order to recover the leucocytes pellets; it was added 4 ml of RBC-lyses buffer to the pellet, followed by centrifugation at 400 xg for 10 min at 4°C, and completely removed and discarded the supernatant. RBC-lysis buffer in DEPC water (pH 7,4) contain 155 mM (8,29g /l solution) (Sigma a9434), MW= 53,49 of NH4Cl, 20 mM (1g/1l solution) (Sigma 60339), MW=100,12 of KHCO3, 0,1 mM (0,034 g or 200 μl of a 500mM ETDA sol/1l solution) (Sigma E5134), and MW= 372,24 of NaEDTA.

For PBMCs drug-naive PD patient's samples and matched controls samples:

RNA was extracted from buffy coats previously frozen in 10% dimethyl sulfoxide (DMSO; cat. no. D2650; Sigma-Aldrich, Milano, Italy) as mentioned above.

Total RNA was isolated using TRIzol reagent (cat. no. 15596-018; Life Technologies, Monza, Italy) according to the manufacturer's instructions. Samples were standardized controlled-rate -1°C/minute cell freezing in a -80°C freezer and cryo-preserved in liquid-nitrogen vapour.

RNA quality and quantity of the PBMCs samples was assessed with the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories s.r.l., Milano, Italy) using the StdSens Analysis Kit (Cat.no. 7007103; Bio-Rad Laboratories s.r.l., Milano, Italy). All samples showed an RNA Quality Indicator (RQI) ≥ 7.5.

Plasma from both L-dopa treated PD patients, drug-naïve PD patients, and matched controls was obtained centrifugating peripheral blood within 6 hours of blood collection in Na_3-citrate buffered Venosafe®Plastic Tubes (VF-054SBCS07,

Thermo) for 15 minutes at 1500 g at 4°C. Total RNA, including miRNAs, was extracted from aliquots of 200 µl plasma, using the mirVANA PARIS kit (AM1556, Ambion/Life Technologies) according to the manufacturer's instructions with few modifications. After the denaturation step, it was added 1.25 µl MS2 carrier (10 165 948 001 Roche; conc. 10 A260 units = 0.8 µg/ µl, stored at -20°C), and 3.5 µl (0.26 fmol/ µl) of each spike-in oligo, the cel-miR-39-3p (219610, Qiagen), cel-miR-238-3p and cel-miR-54-3p (Integrated DNA Technologies) to the 200 µl of starting plasma L-dopa treated PD, drug-naïve PD, and controls samples aliquots. After the Acid Phenol:Chloroform extraction, a second extraction step was performed adding 300 µl DEPC H_20 to the organic phase. The pooled fractions were further processed according to the protocol. We stored RNA in DEPC H_20 at -80°C.

The RNA yield from plasma was too low to be quantified by traditional methods.

3.3 Reverse Transcription and quantitative real time PCR

Reverse transcription (RT) and expression analysis (qRT-PCR) of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p was performed on blood samples from L-dopa treated PD patients, drug-naïve PD patients and matched controls, according to the following protocol. The RT reactions were performed on 7 ng of total RNA using a TaqMan miRNA Reverse Transcription Kit (Cat.no. 4366597; Life Technologies, Monza, Italy) and miRNA-specific stem-loop primers in a scaled down 10 µl reaction, according to the manufacturer's instructions. The thermal cycling parameters were 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. All RT-qPCR reactions were performed in triplicate on a 96CFX instrument (Bio-Rad Laboratories s.r.l., Milano, Italy) in scaled down 10 µl reaction volumes using 0.5 ng of RT product per reaction, Universal MasterMixII no UNG, and TaqMan® small RNA assays (Cat.no. 4440048 and, 4427975 Life Technologies, Monza, Italy), according to the manufacturer's instructions. The thermal cycling parameters were 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The relative amplification efficiencies range between 96% and 109%.

Reverse transcription and expression analysis of miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p, and miR-103a-3p was performed on plasma samples from L-dopa treated PD patients, drug-naïve PD patients and matched controls, according to the following protocol. The RT reactions were performed on 2.5 ul of total RNA using a TaqMan miRNA Reverse Transcription Kit (Cat.no. 4366597; Life Technologies, Monza, Italy) and miRNA-specific stem-loop primers in a scaled down 7.5 µl reaction, according to the manufacturer's instructions. The thermal cycling parameters were 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. All qRT-PCR reactions were performed in triplicate on the 96CFX instrument (Bio-Rad Laboratories s.r.l., Milano, Italy) in scaled down 10 µl reaction volumes using 2.5 µl of 1:15 diluted RT product per reaction, Universal MasterMixII no UNG, and TaqMan® small RNA assays (Cat.no. 4440048 and, 4427975 Life Technologies, Monza, Italy), according to the manufacturer's instructions.

All reactions were performed on hard shell PCR plates (Bio-Rad Laboratories Inc. Part.No HSP9645), sealed with adhesive Microseal 'B' Films (Bio-Rad Laboratories Inc. Part.No RSN 102595). One sample was used as an internal reference to monitor plate to plate variation, and a NTC (no template control) was included on each plate. The thermal cycling parameters were 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

PCR amplification efficiency for all target and reference genes was determined from the slope of the log-linear portion of the calibration curves.

3.4 Standard curve efficiency

A serial dilution of cDNA, reverse transcribed from total RNA extracted from pooled samples, was used to plot the standard curves for each gene assay. The relative expressions of miRNAs were normalized using RNU24 and Z30 as reference genes set for PBMCs samples, whereas for plasma samples were used spike-in oligos cel-miR-39-3p, cel-miR-54-3p and cel-miR-238-3p. It has been taken into account amplification efficiency arranges between 80% and 100% with a r^2 of 0.96 and 0.99.

3.5 qRT-PCR normalization and relative quantification of miRNAs

TaqMan Small RNA Assays are pre-formulated primer and probe sets designed to detect and quantify small RNAs, including mature microRNAs. During the annealing the TaqMan probe binds to the target sequence and when the quencher is near to the fluorescence dye, no signal is detectable. During the combined annealing/extension step of the amplification reaction, Taq polymerase degrades the probe that has annealed to the template. The reporter is released and separated from the quencher. The increase in fluorescence can be monitored following the threshold cycle (Ct) during a PCR reaction, which reflects the cycle number at which we have a detectable fluorescence. The fluorescence signal is proportional to the amplified DNA amount and it is measured at the end of each cycle.

The NormFinder (Andersen et al., 2004) and GeNorm (Vandesompele et al. 2002) algorithms, together with the comparative delta-Ct approach (Silver et al., 2006) were used to assess the variance in expression levels and to identify the most stable and reliable reference genes. The relative expression levels were assessed using the Bio-Rad CFX Manager v1.6/v3.1 (Bio-Rad Laboratories s.r.l., Milano, Italy) and qBasePlus (Biogazelle) software using RNU24 and Z30 as normalizers for the PBMCs L-dopa treated PD samples, drug-naïve PD samples and matched controls (chosen among RNU24, Z30, RNU6B and miR-103-3p), and cel-miR-39-3p, cel-miR-238-3p and cel-miR-54-3p for the plasma L-dopa treated PD samples, drug-naïve PD samples and matched controls (chosen among cel-miR-39-3p, cel-miR-238-3p, cel-miR-54-3p and miR-191-5p). The relative amplification efficiencies were ranged between 90% and 109%.

Minimum Information for Publication of Quantitative Real Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) were followed for description of samples, RNA extraction, RT-qPCR and data analysis.

TaqMan® MicroRNA assays. Gene name and relative TaqMan® MicroRNA assays, sequence.

miRNA	mirBase Accession/ NCBI Ref Sequence	Assay ID	Mature miRNAs Sequences
miR-29a-3p	MIMAT0000086	2112	UAGCACCAUCUGAAAUCGGUUA
miR-29b-3p	MIMAT0000105	413	UAGCACCAUUUGAAAUCAGUGUU
miR-30a-5p	MIMAT0000087	417	UGUAAACAUCCUCGACUGGAAG
miR-30b-5p	MIMAT0000420	602	UGUAAACAUCCUACACUCAGCU
miR-103a-3p	MIMAT0000101	439	AGCAGCAUUGUACAGGGCUAUGA
miR-191-5p	MIMAT0000465	2299	CAACGGAAUCCCAAAAGCAGCUG
cel-miR-39-3p	MIMAT0000010	200	UCACCGGGUGUAAAUCAGCUUG
cel-miR-54-3p	MIMAT0000025	1361	UACCCGUAAUCUUCAUAAUCCGAG
cel-miR-238-3p	MIMAT0000313	248	UUUGUACUCCGAUGCCAUUCAGA

3.6 Statistical Analysis

In order to determine the expression stability of the PBMCs (RNU24, Z30, RNU6B, miR-103a-3p) and plasma (Cel-miR-39-3p, Cel-miR-238-3p, Cel-miR-54-3p, miR-191-5p) candidate reference genes, statistical analyses of their expression levels across all samples were performed using three different methods: NormFinder and GeNorm algorithms, and comparative delta-Ct method. NormFinder computes a stability measure and the lowest value indicates the most stable gene expression. GeNorm compute M stability values, calculated assuming the specific efficiency for each gene assay where the lower M values indicate the most stable gene expression. Comparative delta-Ct compares relative expressions of pairs of genes within each sample set and ranks stability of reference genes according to the repeatability of the gene expression difference.

To determine if different reference gene sets could affect the direction of the target miR-29a-3p and miR-30b-5p, the distribution of the difference of the expression levels between cases and controls, within each matched pair and normalized with different combinations of reference genes, was checked using a Skewness-Kurtosis test for normality. A subset of the ladder of powers for variable transformation in case of departure from normality was evaluated. Relative expression of miR-29a-3p and miR-30b-5p was analyzed using a Wilcoxon

matched-pairs signed-ranks test. Although 15 tests for each microRNA were performed, no adjustment for multiple comparisons was done. This is because in practice the only test that would have been performed is the one based on data *a priori* normalized for the best reference gene combination. Spearman's rank correlation coefficients were computed on Ct reference gene data and on normalized miR-29a-3p and miR-30b-5p data. GeNorm analysis was performed using Biogazelle's QbasePLUS software (Bio-Rad Laboratories Inc.) and NormFinder analysis was performed using "NormFinder.xla", a Microsoft Excel-based Visual Basic application.

Relative expression of PBMCs miRNAs was assessed using the BIORAD CFX manager v1.6 and qBasePlus (Biogazelle) software. Basic descriptive statistics, as means and standard deviations, were generated to the distribution of the difference of expression levels showing no departure from normality with a Skewness-Kurtosis test. For each miRNA, the mean and the standard deviation (SD) of the differences of the relative expressions between each case and its matched control were used as input to calculate the appropriate sample size needed for a second experiment on untreated patients. Sample size was calculated in order to

achieve 80% power with alpha = 0.05, and was performed using the command sampsi implemented in Stata 12. All the analyses were performed using Stata 12 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

Relative expression of plasma miRNAs was assessed using the Biorad CFX manager v3.1 and qBasePlus (Biogazelle) software. It was checked the normality of the distribution of the differences of the relative expression levels within each matched set using a Skewness-Kurtosis test.

For the plasma and PBMCs L-dopa treated PD samples and matched controls have been calculated the summary statistics as mean, standard deviation, median and interquartile range (IQR). Outliers have been detected, but since no technical reasons justifying their presence was identified, they were kept in the analysis. Data have been analyzed using a two-tailed paired t-test and a non-parametric

Wilcoxon matched-pairs signed-ranks test, when the differences of expressions within each set were not normally distributed. An exploratory subgroup analysis was also performed in males and in females groups separately.
For the plasma and blood drug-naïve PD patients and matched controls, the median and interquartile range was provided to summarize data. Data have been analyzed a non-parametric Wilcoxon matched-pairs signed-ranks test, applied due to the small sample size. Sample size needed to achieve a power of 80%, with alpha = 0.05, was calculated for the deregulated miRNAs, based on the effect sizes and the standard deviations observed for L-dopa treated patients using sampsi Stata command.

All the PBMCs analyses were performed using Stata 12 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP) and plasma analysis using Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

3.7 Bioinformatic analysis: target prediction and priorization

It was applied a computational approach for the identification and prioritization of candidate targets of miR-29a-3p, miR-30b-5p, and miR-103a-3p, and used multiple miRNA target resources for the identification of candidate targets. It was prioritized the candidates likely to be involved in PD based on a “guilt by association” principle by searching for links to PD-related genes and processes. Different scores were computed from the multiple types of evidence. In addition, were compared the ranked candidates to sets of PD-related genes and to results obtained with Endeavour, an established prioritization method.
Were collected experimentally validated miRNA targets from multiple sources, in particular, a manual literature search of reported target genes (labeled CBM) and the public databases TarBase (version 6.0) (Vergoulis et al., 2012), miRTarBase (retrieved on 2013-02-06) (Hsu et al., 2011), miRWalk (retrieved on 2013-02-05) (Dweep et al., 2011), and miRecords (retrieved on 2013-02-05) (Xiao et al., 2009). Were collected predicted miRNA targets from DIANA-microT-CDS (retrieved on

2012-01-18) (Reczko et al., 2012), miRmap (retrieved on 2013-02-04) (Vejnar et al., 2012), miRDB (version 4.0) (Wang X et al., 2008), TargetScan (retrieved on 2013-02-04) (Friedman et al., 2009) and miRanda (release 2010-08) (Betel et al., 2010).

Custom panels of PD-related processes and genes were compiled and applied for prioritization. GOPD is a panel of PD-related processes, AVPD is a panel of genes related to PD, and CPD is a list of candidate PD genes. AVPD and CPD are non-overlapping sets. Were compared the candidate miRNA targets to additional sets of PD-related genes, in particular sets of Parkin interactors from interaction screens (ParkinTAP, ParkinInt), or Parkin dependent ubiquitylation targets (ParkinUBQ). Given the known role of mitochondria function in PD (Arduino et al., 2011), we used also a set of mitochondrion related genes (Mito) to annotate the results. Were collected different scores from the multiple types of evidences. Several scores summarize the validated annotations (V_PMID) and predictions (PP and PF scores) from miRNA target resources. The GOSlim score allows the identification of candidate genes annotated to PD-related processes. BPscore quantifies the functional relationship to PD-related genes, PPI and Cpx summarize the number of protein interactions and complexes with PD-related proteins. Were used the InSet score to identify candidates in PD panel, and used the different scores to compute a combined score (CRscore) applied to rank the candidates.

3.8 Library preparation for NGS analysis

500ng/µl of total RNA was used to prepare the library according to the manufacturer's instructions (Nebnext Small Multiplex Library Preparation Prep Set for Illumina, NEB#E7300S/L, New England Biolab Inc.). The size-selection cleaning of the library was done first with a ratio 1:1.2X and 1:3.7X volume magnetic beads (AMPure XP Beads, Agencourt) and a second time, as suggested by the manufacturer, with a ratio 1:1.3X and 1:3.7X volume magnetic beads. The quality and quantity of the cleaned library were measured using KAPA Library Quantification Kit for Illumina Sequencing Platform (Resnova), according to the manufacturer's instructions on the 96CFX instrument (Bio-Rad Laboratories s.r.l., Milano, Italy). 12.5pM of library was prepared according to the manufacturer's

instruction of the Illumina TruSeq small RNA Library Prep kit and loaded on the Illumina MiSeq® System, NGS platform (Illumina), according to the manufacturer's instructions.

3.9 Bioinformatic's analysis workflow of the NGS data output

A multiple repetition of cutadapt was used to remove all adapters and technical sequence artefacts. The cleaned sequences were mapped to mature miRNA and snoRNA with bowtie2 in local mode (allowing for soft clipping of reads at the ends) and uniquely matching reads with no mismatches were selected. Differential expression analysis was done with DESeq2 and EdgeR.

4. RESULTS

4.1 Identification of a reliable set of endogenous reference genes for PBMCs miRNAs expression studies in Parkinson disease's

In order to identify a reliable set of endogenous reference genes to use on PBMCs samples for miRNA expression studies in Parkinson Disease's as normalizer, all the raw expression data were analyzed in a second moment using not only the NormFinder (Andersen et al., 2004) and the GeNorm (Vandesompele et al. 2002) algorithms, but also the comparative delta-Ct method (Silver et al., 2006).

GeNorm and Normfinder allow the ranking of candidate reference genes according to their stability and indicate the optimal number and combination of reference genes required for accurate normalization of gene expression. Comparative delta-Ct compares relative expressions of pairs of genes within each sample set and ranks stability of reference genes according to the repeatability of the gene expression difference. Samples were grouped to allow direct estimation of expression variation, ranking genes according to the similarity of their expression profiles by using a model-based approach. NormFinder takes into account inter- and intra-group variation for normalization factor calculations avoiding misinterpretation caused by artificial selection of co-regulated genes. GeNorm computes an M value describing the variation of a gene compared to all other candidate genes. Similarly to the NormFinder stability value, lower M values indicate stable gene expression. Specifically, stable reference genes are supposed to have an M value smaller than 1.5.

The results of the expression stability assessment of Z30, RNU24, RNU6B and miR-103a-3p among all analyzed samples are reported in Tables 1 and 2, showing the results of the comparative delta-Ct method and the comparison with the NormFinder and GeNorm results. Independently on the method used, the gene ranking remained unchanged, thus indicating that Z30 and RNU24 were the best reference genes to use as normalizer (Serafin et al., 2014). The combination of Z30 and RNU24 was selected as the best reference genes set among all the others to use in our study. This combination showed the smallest and therefore

best stability values among all the other combinations of reference genes, whereas the worst combination was attributed to RNU6B and miR-103a-3p.

Combination	Mean delta-Ct	SD	Mean SD
RNU24 vs Z30	−1.53	0.58	0.93
RNU24 vs RNU6b	−2.26	1.09	
RNU24 vs miR-103a-3p	−0.71	1.12	
Z30 vs RNU24	1.53	0.58	0.93
Z30 vs RNU6B	−0.73	0.99	
Z30 vs miR-103a-3p	0.82	1.23	
RNU6B vs Z30	0.73	0.99	1.25
RNU6B vs RNU24	2.26	1.09	
RNU6B vs miR-103a-3p	1.62	1.66	
miR-103a-3p vs Z30	−0.82	1.23	1.34
miR-103a-3p vs RNU24	0.71	1.12	
miR-103a-3p vs RNU6B	−1.62	1.66	

Table 1. Comparison of candidate reference genes expression stability using the comparative delta-Ct method. Mean delta-*Ct* = Ct target – Ct reference; *SD* = standard deviation of the mean; mean *SD* = mean of the calculated standard deviations (Serafin et al., 2014).

Gene	Comparative delta-Ct	NormFinder	GeNorm
RNU24	0.93	0.007	0.839
Z30	0.93	0.016	0.841
RNU6B	1.25	0.033	1.112
miR-103a-3p	1.34	0.018	1.310

Table 2. Comparison of the stability values estimated using the comparative delta-Ct, NormFinder and GeNorm algoritms. Comparative delta-*Ct* = mean of the standard deviations (SD); NormFinder = computes a stability measure and the lowest value indicate the most stable gene expression; GeNorm: M stability values, calculated assuming the specific efficiency for each gene assay, the lower M values indicate the most stable gene expression (Serafin et al., 2014).

4.2 Different combinations of reference genes set have a strong impact on the relative expression levels observed for both miR-29a-3p and miR-30b-5p

We tested the effect of using different combinations of reference genes on the relative expression values using miR-29a-3p and miR-30b-5p as targets, which were found to be deregulated in PD in previous studies (Margis et al., 2011;

Martins et al., 2011). As reported in Table 3, an increased relative expression of miR-29a-3p and miR-30b-5p in L-dopa treated PD patients in comparison to disease-free matched controls could be observed using the best reference gene set Z30/RNU24. The same trend could be observed using Z30, RNU24 and RNU6B as reference genes, either alone or in different combinations. The usage of miR-103a-3p alone as reference gene resulted in an opposite effect in target miRNAs expression direction and it was sufficient to completely reverse the direction of the expression difference between cases and controls of the investigated miRNAs (miR-29a-3p median difference = −0.58, IQR = −1.33; −0.05, p = 0.0003 and miR-30b-5p median difference = −1.16, IQR = −2.53; 0.23, p = 0.0005). The inclusion of miR-103a-3p in any other combination of reference genes systematically worsened the observed p values. This effect was clearly visible with the addition of miR-103a-3p to the set Z30 and RNU24 (Table 3), which had a strong impact on the results observed for both miR-29a-3p and miR-30b-5p, with the p value of miR-29a-3p changing from p = 0.0032 to p = 0.5473 and the p value of miR-30b-5p changing from p = 0.0009 to p = 0.3422, thus underling the importance and the complexity of the choice of reference genes set (Serafin et al., 2014). Indeed, the relative expression levels of miR-29a-3p and miR-30b-5p were not always well correlated with each other, but some differences could be observed, depending on the reference gene set used for normalization (Table 3).

Reference gene	miR-29a median of difference (IQR)	P value miR-29a	miR-30b median of difference (IQR)	P value miR-30b
Z30	0.76 (−0.23; 2.22)	0.0048	1.75 (0.17; 3.62)	0.0007
RNU24	0.39 (−0.08; 1.34)	0.0151	0.96 (−0.52; 3.39)	0.0075
RNU6B	0.98 (−0.17; 2.12)	0.0007	2.66 (0.2; 4.05)	0.0001
miR-103a	−0.58 (−1.33; −0.05)	0.0003	−1.16 (−2.53; 0.23)	0.0005
Z30- miR-103a	−0.02 (−0.65; 0.6)	0.7997	0.01 (−1.14; 1.32)	0.8221
Z30-RNU24*	0.68 (−0.17; 1.74)	0.0032	1.74 (0.05; 4.26)	0.0009
Z30-RNU24-miR-103a	0.18 (−0.48; 0.71)	0.5473	0.22 (−1.1; 1.63)	0.3422
Z30-RNU24-RNU6B-miR-103a	0.38 (−0.31; 1.23)	0.0511	1.05 (−0.31; 2.41)	0.0092
RNU24-RNU6B	0.85 (−0.02; 1.77)	0.0011	2.37 (0.2; 3.47)	0.0004
RNU24- miR-103a	−0.35 (−0.75; 0.15)	0.0645	−0.7 (−1.54; 1.13)	0.2372
RNU24-RNU6B-miR-103a	0.12 (−0.31; 0.91)	0.2097	0.61 (−0.44; 1.95)	0.0494
RNU24-RNU6B-Z30	0.74 (−0.08; 1.86)	0.0013	2.02 (0.1; 3.95)	0.0003
RNU6B- miR-103a	0.04 (−0.41; 0.66)	0.3962	0.51 (−0.4; 1.19)	0.0939
RNU6B-Z30	0.79 (−0.1; 1.98)	0.0011	2.44 (0.17; 3.8)	0.0003
RNU6B-Z30-miR-103a	0.31 (−0.36; 1.01)	0.0733	1.02 (−0.3; 1.99)	0.0119

*Best set of reference genes.

Table 3. Changes in the results of the analyses depending on the choice of the reference genes. The median of difference is calculated as median (expression in cases – expression in controls) within each matched case–control set and it is indicated for a descriptive purpose. IQR = interquartile range. P values were computed using a Wilcoxon matched pairs signed-ranks test (Serafin et al., 2014).

4.3 First evidence of miR-103a-3p over-expression and deregulation of miR-29a-3p and miR-30b-5p in PBMCs samples from L-dopa treated PD patients

In order to identify miRNAs as potential diagnostic PBMCs biomarkers for PD was assessed the relative expression for the promising miRNAs, miR-29b-3p, miR-30b-5p, miR-29a-3p, and miR-30a-5p from L-dopa treated PD patients in comparison with unaffected matched controls. Based on the literature we selected small nucleolar/nuclear RNAs (RNU24, Z30, RNU6B) and miR-103a-3p as candidate normalizers. Expression levels of these candidate reference genes were analyzed by geNorm and NormFinder algorithms to select the best endogenous candidate controls. RNU24 and Z30 were selected as the best reference genes

set to use for normalization, whereas the miRNA-103a-3p, used by Martins and collaborators (Martins et al., 2011) as endogenous control, showed a high variance in expression. Moreover, miRNA-103a-3p is a member of the miR-15/107 group, involved in several physiological functions and pathologies, including neurodegenerative diseases. For these reasons, miR-103a-3p has been included in our study as a target rather than as a reference gene.

As reported (Table 4, Figure 1), we found a significant increase in relative expression of miR-29a-3p, miR-30b-5p in peripheral blood samples from L-dopa treated PD patients. This finding was unexpected, since in previous papers (Martins et al., 2011; Margis et al., 2011) was shown a decreased expression levels for miR-29a-3p and miR-30b-5p in PD patients. Furthermore, we demonstrated for the first time an over-expression of miR-103a-3p in PBMCs levels from L-dopa treated PD patients (Table 4; Figure 1).

miRNA **N pairs = 36**	Mean Difference within each pair (95% CI)	P-Value Paired t-test
miR-29a-3p	0.69 (0.23; 1.15)	**0.005**
miR-29b-3p	0.26 (-0.28; 0.79)	0.339
miR-30a-5p	0.12 (-0.14; 0.37)	0.364
miR-30b-5p	1.76 (0.68; 2.84)	**0.002**
miR-103-3p	1.80 (1.27; 2.33)	**<0.0001**

Table 4. Results of miRNA expression analysis on 36 PBMCssamples from L-dopa treated PD patients and unaffected matched controls (Serafin et al., 2015); CI= interval of confidence

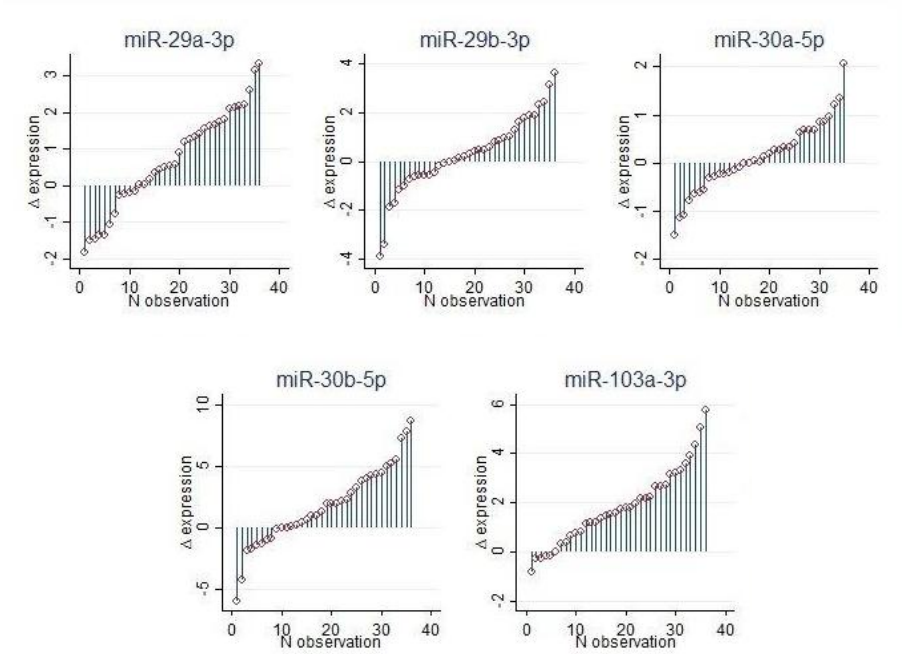


Figure 1. Pair plots of the expression analysis on 36 PBMCs samples from L-dopa treated PD patients and disease-free matched controls. The plots show the difference between cases and controls within each pair, hence accounting for matching. The horizontal zero line indicates no difference within the pair. Differences have been sorted in ascending order to allow a better visualization (Serafin et al., 2015).

4.4 Trend of miR-30a-5p over-expression in plasma samples from L-dopa treated PD patients

To assess whether plasma is a suitable alternative/additional easily accessible resource for PD miRNA biomarkers, we performed an exploratory expression analysis on 31 plasma samples from L-dopa treated PD patients, coming from the same individuals already analyzed in PBMCs, for miR-29a-3p, miR-29b-3p, miR-30a-5p, miR-30b-5p and miR-103a-3p.

The first step was to assess the protocol for the extraction of miRNAs from plasma L-dopa treated PD patients. For the expression analysis, a different normalization approach was selected, because of the absence in plasma of the small non-coding nuclear/nucleolar RNAs (RNU24 and Z30) used previously as reference genes in PBMCs expression analysis on L-dopa treated PD patients.

Based on the literature, we selected miR-191-5p (Cardo et al., 2013; Geekiyanage et al., 2012), and three exogenous C.elegans spike-in miRNAs (cel-miR-39-3p, cel-miR-54-3p and cel-miR-238-3p) (Kang et al., 2012) as candidate normalizers. Using GeNorm and NormFinder algoritms, we revealed a high expression variability of miR-191-5p in our data (data not shown) and we decided to use exclusively C.elegans spike-in miRNAs for normalization.
This synthetic miRNAs, named spike-in, are to be added into the plasma samples before the extraction step of miRNAs. In that way the expression analysis should be more sensitive because the same quantity of spike-in is added in equal measure to all the samples.

The exploratory analysis performed on 31 plasma samples from L-dopa treated PD patients and disease-free controls (matched 1:1 by sex and age) showed a borderline significant over-expression of miR-30a-5p (p=0.0745) (Table 6). Moreover, we demonstrated that miR-29b-5p, normally expressed in PBMCs, was not detectable in plasma samples from L-dopa treated PD patients, nor in the plasma of the controls.

4.5 Confirmation of the exploratory data obtained in the 31 plasma samples from L-dopa treated PD patients in an enlarged sample size

Due to the low abundance of miRNA levels in plasma compared to PBMCs, and in order to confirm the exploratory data obtained in the 31 L-dopa treated PD plasma samples, it has been enlarged the sample size from 31 to 50 of the plasma samples from L-dopa treated PD patients and unaffected matched controls.

The expression analysis showed a borderline significant over-expression for miR-30a-5p (p=0.054) whereas the other miRNAs were not deregulated (Table 5; Figure 2). These data confirmed the previously expression analysis on 31 plasma samples from L-dopa treated PD patients and matched unaffected controls where miR-30a-5p showed also an up-regulation trend (p= 0.0745).

Moreover, we confirmed that miR-29b-3p was not detectable in L-dopa treated PD plasma samples.

miRNA N pairs = 50	Mean Difference within each pair (95% CI)	P value paired t-test
miR-29a-3p	0.08 (-0.16; 0.31)	0.5118
miR-29b-3p	***Not found in plasma***	-
miR-30a-5p	0.17 (-0.003; 0.35)	***0.054***
miR-30b-5p	-0.04 (-0.23; 0.15)	0.6781
miR-103a-3p	-0.04 (-0.19; 0.11)	0.5927

Table 5. Expression of miRNA levels in 50 plasma samples from L-dopa treated PD patients and their matched controls; a two-tailed paired t-test was used to analyze data.

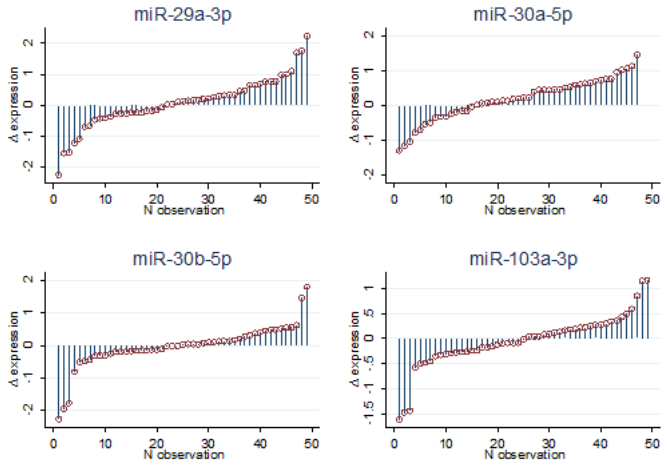


Figure 2. **Pair plots representing miRNA expression data in 50 matched plasma pairs.** Is shown the difference of miRNA expression (treated case – control) within each pair. The horizontal zero line indicates no difference in miRNA expression within the pair. For each miRNA, data have been sorted in ascending order for a better visualization.

4.6 Different miRNA expression profiles between PBMCs and plasma samples from L-dopa treated PD patients

The plasma and PBMCs samples derived from the same individuals, allowing a direct comparison of the expression profiles in PBMCs and plasma, revealing clear differences for all analyzed miRNAs (Table 6) in L-dopa treated PD patients and unaffcted matched controls.

We observed a significant up-regulation of miR-29a-3p, miR-30b-5p and miR-103a-3p in PBMCs samples from L-dopa treated PD patients, whereas no deregulation of these miRNAs were observed in plasma samples from L-dopa treated PD patients**,** where only miR-30a-5p showed a borderline significant over-expression (p=0.0745) (Table 6), thus indicating that different miRNA expression profiles can be observed in plasma and PBMCs samples from L-dopa treated PD patients and concluding that plasma is not an interchangeable source for miRNAs biomarkers.

miRNA N pairs = 31	**Median and IQR of the differences plasma**	**P-value Wilcoxon plasma**	**Mean Difference within each set (95% CI) PBMCs**	**P-value paired t-test PBMCs**	**Concordance PBMCs/plasma**
miR-29a-3p	0.14 (-0.37; 0.64)	0 .6807	0.52 (0.02; 1.01)	**0.0418**	Discordant
miR-29b-3p	Not found in plasma	**Not found in plasma**	0.26 (-0.34; 0.85)	0.3888	-
miR-30a-5p	0.22 (-0.19; 0.6)	**0.0745**	0.05 (-0.23; 0.32)	0.7361	Discordant
miR-30b-5p	-0.03 (-0.26; 0.31)	0.7989	1.59 (0.46; 2.72)	**0.0075**	Discordant
miR-103-3p	-0.1 (-0.3; 0.11)	0.217	1.61 (1.05; 2.17)	**<0.0001**	Discordant

Table 6. Comparison of miRNA expression profile in a dataset of 31 matched pairs for which both miRNA extracted from PBMCs and plasma. Due to the violation of paired t-test assumptions, a Wilcoxon matched-pairs signed-ranks test was applied. The median and the IQR (interquartile range) of the differences of miR expression within each set are provided for descriptive purpose.

4.7 Expression analysis on PBMCs and plasma samples from drug-naïve PD patients and unaffected matched controls.

Since all previously analyzed samples PD patients were undergoing L-dopa therapy, the observed difference in miRNA expression could be the consequence of the L-dopa treatment. So, in order to investigate if the observed difference in miRNA expression could be the consequence of L-dopa treatment rather than to the PD pathogenesis per se, we performed an expression analysis on PBMCs and plasma samples from drug-naïve PD patients and unaffected matched control.

4.7.1 MiRNAs expression analysis in PBMCs samples from drug-naïve PD patients and unaffected matched controls

The *ad hoc-power* calculation (the sample size needed to have the same difference in miRNAs expression between blood drug-naïve PD samples and controls) evidenced that only 6 pairs of PBMCs/controls samples pair from drug-naïve PD patients were needed to evaluate the expression of miR-103a-3p, and more then 23 for the others miRNAs (31 matched case-control pairs for miR-29a-3p, 294 pairs for miR-29b-3p, 299 pairs for miR-30a-5p, and 26 pairs for miR-30b-5p). In our study it was possible to enroll only 10 PBMCs samples from drug-naïve PD patients, with any possibility to enlarge the sample size.
Based on this and on the available number of drug-naïve PD patients, it was performed a first exploratory expression analysis only for miR-103a-3p, and no significant difference could be detected.

Although the sample size calculation demonstrated that there was not enough power to detect significant changes in expression for miR-29a-3p, miR-29b-3p, miR-30a-5p, and miR-30b-5p in 10 PBMCs samples from drug-naïve PD patients, we decided anyhow to evaluate their expression. It could be still argued that effect size estimated, used to calculate the sample size, came indeed from a different population, the group of PBMCs L-dopa treated PD patients. Hence, the

magnitude of the effects observed in the treated patients versus controls might not be totally comparable with the magnitude of the effects in the drug-naïve PD patients. From this different perspective, it becomes interesting to evaluate the expression of all miRNAs in the PBMCs drug-naïve PD group versus unaffected matched controls, even if the numbers were low and with no possibility to collect additional PBMCs samples from drug-naïve PD patients into the study.

The expression analysis indicated no significant differential expression, as shown in Table 7. Due to the very likely lack of power, these results are not conclusive since it cannot be ruled out that a small, but statistically and possibly clinically significant difference in miRNAs expression would be detected in a larger sample size.

miRNA N pairs=10	P-value Wilcoxon
miR-29a-3p	0.611*
miR-29b-3p	0.182*
miR-30a-5p	0.699*
miR-30b-5p	0.315*
miR-103-3p	0.785

Table 7. Results of miRNAs expression analysis in 10 PBMCs samples from drug-naïve PD patients and matched controls. Due to the small sample size a Wilcoxon matched-pairs signed-ranks test was applied. *underpowered sample.

4.7.2 MiRNAs expression analysis in plasma samples from drug-naïve PD patients and unaffected matched controls

The exploratory expression analysis was performed on 10 plasma samples from drug-naïve PD patients and their matched disease-free controls deriving from the same individuals of the previously analyzed 10 PBMCs samples from drug-naïve PD group.

The expression analysis has showed an interesting trend of higher expression of miR-30b-5p (p=0.0858) compared to healthy controls, whereas the trend of the

miR-30a-5p observed in the 50 plasma samples from L-dopa treated PD patients, has been lost (Table 8).

miRNA N pairs = 50	P value paired t-test	miRNA N pairs = 10	P-value Wilcoxon
miR-29a-3p	0.5118	**miR-29a-3p**	0.3139
miR-29b-3p	*Not found in plasma*	**miR-29b-3p**	*Not found in plasma*
miR-30a-5p	***0.054***	**miR-30a-5p**	0.2135
miR-30b-5p	0.6781	**miR-30b-5p**	***0.0858***
miR-103a-3p	0.5927	**miR-103-3p**	0.2135

Table 8. **Comparison of the expression analysis of miRNAs in 10 plasma samples from drug-naïve PD patients and 50 plasma samples from L-dopa treated PD patients and their matched controls.** Due to the small sample size a Wilcoxon matched-pairs signed-ranks test is applied for the 10 drug- naïve PD samples.

4.8 Preliminary statistical analysis suggested miRNAs over-expression trend according to the gender.

Very preliminary results of the subgroup analysis in PBMCs and plasma, stratifying the samples by the gender, suggest that there is a trend of differentially miRNA expression according to the gender. In particular, miR-29a-3p displays a borderline significance trend in females only, while there is a trend of significance for miR-30a-5p in males only (data not shown). However, due to the small gender groups available, these results have only an exploratory role and should be investigated in a larger sample set, allowing testing for the statistical interaction between gender and miRNAs expression.

4.9 Exploratory deep sequencing on PBMCs samples from L-dopa treated PD patients and unaffected matched controls.

In order to identify more PD miRNA candidates, we decided to perform miRNA profiling in PBMCs samples from L-dopa treated PD patients versus unaffected matched controls using a Next Generation Sequencing approach. The Illumina MiSeq NGS system was acquired as new platform.

The first step, needed to settle and standardized the NGS analysis methods, was to create cleaned libraries and to have an adequate number of total reads as sequencing output. The final idea was to compare the gold-standard qRT-PCR miRNAs results with the bioinformatics sequencing data analysis.

The experiment consisted in two critical steps: the library preparation for the sequencing data generation and the bioinformatics analysis of the NGS data output.
Regarding the library preparation, the critical issue was to obtain a final cleaned miRNAs library to load on the NGS system, starting from a low amount of total RNA.
Regarding the bioinformatics analysis, it was established an in-house bioinformatics analysis pipeline and it was compared to miRDeep2. MiRDeep2 is one of the most common analysis tool used for alignment and read count generation; it is based on the miRNA biogenesis model, the ability to predict a miRNA's existence by detection of mature miRNA or any one of its precursor or stem loop sequences. Moreover it is able to predict novel miRNAs based on the alignment of the putative miRNA to the genome (Metpally et al., 2013). Despite the differences in procedures of the in-house pipeline and miRDeep2, the results of both pipelines were quite similar. Differential expression analysis of miRNA read counts identified by miRDeep2 was performed using two different analysis packages designed to work with RNA based read count data, called DESeq2 and EdgeR (Metpally et al., 2013). These two different analysis packages are used normally to detect significant, differentially expressed miRNAs.

Despite the difference in procedures of the in-house pipeline and miRDeep2, the results of both pipelines were quite similar, namely no known small RNAs were found to be differentially expressed. Moreover, the bioinformatics sequencing analysis for both pipelines resulted in an opposite direction of miRNA expression in comparison to the qRT-PCR expression analysis (Figure 3). Expression analysis of miR-103a-3p in PBMCs samples from L-dopa treated PD patients and

unaffected matched controls, showed the highest overlapping rate between the sequencing and the expression results

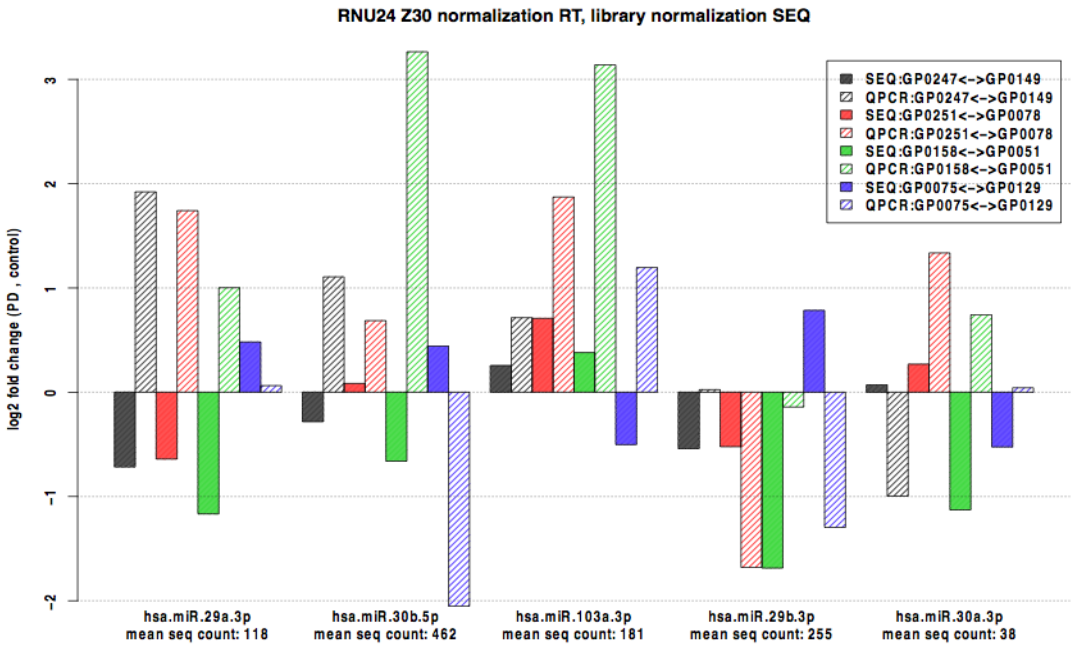


Figure 3. MiRNAS sequencing and qRT-PCR analysis results. Comparison of folds changes (FC) between sequence.

As consequence of these results, a saturation curve was done in order to estimate the number of reads needed to have depth enough to be able to identify miRNA deregulation and unfortunately the number of reads generated was not sufficient (Table 9). Further experiments will be carried out to expand the sample number and obtain around 5 million miRNA reads per sample. To conclude, despite the number of reads generated and the number of samples sequenced in this pilot phase, were not sufficient to generate sequencing results in a meaningful biological analysis and solid statistics.

MiSeq run	Number reads (milion)	% reads mapped to miRNA
1	13.5	17
2	15.8	35
3	1.74	10

Table 9. Number of total reads generated in three indipendent miRNA sequencing runs each for four samples.

4.10 Sophisticated *in silico* approach predicts reliable and promising putative target genes.

MiRNAs regulate simultaneously several different target genes, therefore even small differences in miRNA expression can potentially cause relevant biological effects (Ma et al., 2012). For this reason, in order to better understand the underlying biology and the role of the up-regulation of miR-29a-3p, miR-30b-5p, and 103a-3p in PBMCs samples from L-dopa treated PD patients and the up-regulation trend of miR-30a-5p in plasma samples from L-dopa treated PD patients, it was elaborated a more sophisticated *in silico* approach. We applied a computational approach for the identification and prioritization of candidate targets. Candidate targets were prioritized based on annotation and predictions from different miRNA target databases combined with different types of evidence relating candidates to PD genes and processes (Figure 4). For each deregulated miRNA, the results of the 20 top ranking candidates are reported in Table 10. Top ranking Endeavour prioritization candidates are also marked in the Table 10, providing additional evidence for relation with PD molecular mechanisms.

The *in silico* analysis revealed interesting putative target genes for miR-29a-3p, miR-30b-5p and miR103-3p, deregulated in PBMCs samples from L-dopa treated PD patients, related to PD and neurodegeneration, for example *LRRK2* for miRNAs-30b-5p and miR-103a-3p, *PARK7/DJ-1* for miRNA-29a-3p, and *Bcl-2* as common target for miRNAs-30b-5p, miR-29a-3p, and miR-103a-3p.

The first exploratory target predictions for miR-30a-5p, deregulated in plasma samples from drug-naïve PD patients, has highlighted interesting putative targets, such as Bcl-2 protein.

Table 10. MicroRNA target prioritization (Serafin et al., 2015). **(A)** miR-29a-3p target prioritization; **(B)** miR-103a-3p target prioritization; **(C)** miR-30b-5p target prioritization; **(D)** miR-30a-5p target prioritization

(A) miR-29a-3p target prioritization

Rank	GeneID	HGNC Symbol	Rank miR-29a-3p	Rank miR-30b-5p	V_PMID	#PF	#PP	InSet_AVPD	GOSlim	BPscore	PPI	Cpx	Mito	ParkinTAP	Endeavour100
1	1281	*COL3A1*			3	3	3								
2	3479	*IGF1*	232		1	3	3		●	●					
3	26959	*HBP1*			1	3	3								
4	2200	*FBN1*		399	2	2	3					●			
5	7837	*PXDN*	240		1	3	3		●	●					
6	207	*AKT1*	6		1				●	●	●	●			17
7	54454	*ATAD2B*	1592			3	3								
8	1287	*COL4A5*				3	3			●					
9	2006	*ELN*			1	2	3			●			●		
10	11315	*PARK7*					1	●	●		●	●	●		1
11	91404	*SESTD1*				3	2			●					
12	2861	*GPR37*				3	2			●	●	●			9
13	596	*BCL2*	32	17	2				●	●	●		●		
14	6018	*RLF*				3	3				●	●			
15	51050	*PI15*				3	3								
16	998	*CDC42*		159	2	1	2		●		●	●			
17	55048	*VPS37C*				3	3			●		●			
18	84206	*MEX3B*	7			2	3			●	●				
19	2597	*GAPDH*	28		1				●		●	●		●	8
20	5728	*PTEN*	851	190	2	2	3			●	●				14

Rank: candidate rank according to CRscore. Rank miR-30b-5p, Rank miR-103a-3p = Rank in miR-30b-5p and miR-103a-3p target lists respectively. V_PMID = Number of distinct PubMed identifiers reporting candidate as miRNA target in validated target resources. #PF, #PP = PF scores above cutoff according to target prediction databases with full and incomplete identifiers respectively. InSet_AVPD = Candidates included in panel set AVPD are marked with . GOSlim = Candidate genes are marked if their annotation matches GO terms in GOPD panel or any of their children. BPscore = Candidate gene is marked if functionally related (BPScore >= 0.8) to genes in AVPD or CPD sets. PPI, Cpx = Candidate gene is marked if their encoded protein is reported

to interact with proteins from AVPD or CPD sets or form a complex with AVPD or CPD proteins. Mitochondrion, ParkinTAP = Candidate gene is marked if included in Mito set with Mitochondrion related proteins and in set ParkinTAP obtained from a Parkin interaction screen, respectively. Endeavour100 = Candidate rank within Endeavour top 100. *COL3A1*= collagen, type III, alpha 1; *IGF1*= insulin-like growth factor 1; *HBP1*= HMG-box transcription factor 1; *FBN1* = fibrillin 1; PXDN= peroxidasin homolog (Drosophila); *AKT1* = v-akt murine thymoma viral oncogene homolog 1; *ATAD2B* = ATPase family, AAA domain containing 2B; *COL4A5* = collagen, type IV, alpha 5; *ELN*= elastin; *PARK7*= parkinson protein 7; *SESTD1* = SEC14 and spectrin domains 1; *GPR37* = G protein-coupled receptor 37; *BCL2* = Bcell CLL/lymphoma 2; *RLF* = rearranged L-myc fusion; *PI15* = peptidase inhibitor 15; *CDC42* = cell division cycle 42; *VPS37C* = vacuolar protein sorting 37 homolog C (S. cerevisiae); *MEX3B* = mex-3 RNA binding family member B; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *PTEN*= phosphatase and tensin homolog (Serafin et al., 2015).

(B) miR-103a-3p target prioritization

Rank	GeneID	HGNC Symbol	Rank miR-29a-3p	Rank miR-30b-5p	V_PMID	#PF	#PP	InSet_AVPD	GOSlim	BPscore	PPI	Cpx	Mito	ParkinTAP	Endeavour100
1	23405	*DICER1*	150	761	2	3	3								
2	4763	*NF1*		854	1	3	3		●						26
3	9683	*N4BP1*	692		1	3	3			●					
4	2247	*FGF2*		451	1	3	2			●					
5	63982	*ANO3*				3	3								
6	1983	*EIF5*				3	3			●	●	●			1
7	8313	*AXIN2*			1	3	3			●					
8	3267	*AGFG1*			1	3	2					●			
9	7533	*YWHAH*			1	2	2		●		●	●			72
10	11244	*ZHX1*			1	3	2								
11	93	*ACVR2B*			1	2	2			●					
12	8573	*CASK*				3	1			●	●				13
13	9441	*MED26*	1050			3	3								
14	120892	*LRRK2*		16			1	●	●		●		●		3
15	25777	*SUN2*			1	2	3					●			
16	23710	*GABARAPL1*			1				●		●	●			
17	596	*BCL2*	13	32	1				●	●	●		●		
18	81565	*NDEL1*		283	1	3	3			●					
19	1021	*CDK6*	26		2	2	1			●	●				60
20	3181	*HNRNPA2B1*		191	1	2	2					●		●	

See column description in Table 10 (A). *DICER1* = dicer 1, ribonuclease type III; *NF1* = neurofibromin 1; *N4BP1* = NEDD4 binding protein 1; *FGF2* = fibroblast growth factor 2 (basic); *ANO3* = anoctamin 3; *EIF5* = eukaryotic translation initiation factor 5; *AXIN2* = axin 2; *AGFG1*= ArfGAP with FG repeats 1; *YWHAH* = tyrosine 3- monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide; *ZHX1* = zinc fingers and homeoboxes 1; *ACVR2B* = activin A receptor, type IIB; *CASK* = calcium/calmodulin-dependent

serine protein kinase (MAGUK family); *MED26* = mediator complex subunit 26; *LRRK2*: leucine-rich repeat kinase 2; *SUN2* = Sad1 and UNC84 domain containing 2; *GABARAPL1* = GABA(A) receptorassociated protein like 1; *BCL2* = B-cell CLL/lymphoma 2; *NDEL1*= nudE neurodevelopment protein 1-like 1; *CDK6* = cyclin-dependent kinase 6, *HNRNPA2B1* = heterogeneous nuclear ribonucleoprotein A2/B1 (Serafin et al., 2015).

(C) miR-30b-3p target prioritization

Rank	GeneID	HGNC Symbol	Rank miR-29a-3p	Rank miR-103a-3p	V_PMID	#PF	#PP	InSet_AVPD	GOSlim	BPscore	PPI	Cpx	Mito	ParkinUBQ	ParkinInt	Endeavour100
1	6622	*SNCA*			1			●	●	●	●		●			1
2	860	*RUNX2*	1560		2	3	3									
3	4734	*NEDD4*			1	2	2			●	●	●				
4	27327	*TNRC6A*		1849		3	3									
5	255520	*ELMOD2*			1	2	3			●				●		
6	207	*AKT1*	6		1				●	●	●	●				24
7	84206	*MEX3B*	18			3	3			●	●					
8	27252	*KLHL20*				3	3					●				
9	166968	*MIER3*			1	3	2									
10	8726	*EED*	1789		1	3	3									
11	4907	*NT5E*			1	3	3									
12	84002	*B3GNT5*	1380		1	3	3									
13	57551	*TAOK1*		820	1	3	2			●						
14	9140	*ATG12*			1	3	2		●							
15	51809	*GALNT7*		425	2	2	3									
16	120892	*LRRK2*		14		1	1	●	●		●		●			3
17	57763	*ANKRA2*				3	3									
18	5305	*PIP4K2A*			1	2	2			●						
19	8878	*SQSTM1*			1				●	●	●	●		●	●	12
20	92140	*MTDH*				3	2		●							

See column description in Table 10 (A) ; Rank miR-29a-3p, Rank miR-103a-3p = Rank in miR-29a-3p and miR-103a-3p target lists respectively. ParkinUBQ = Candidates marked with are included in set ParkinUBQ consisting of Parkin dependent ubiquitylation targets. ParkinInt = Candidates marked with are included in set ParkinInt obtained from a Parkin interaction screen. *SNCA* = synuclein, alpha (non A4 component of amyloid precursor); *RUNX2* = runt-related transcription factor 2; *NEDD4* = neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase; *TNRC6A* = trinucleotide repeat containing 6A; *ELMOD2* = ELMO/CED-12 domain containing 2; *AKT1* = v-akt murine thymoma viral oncogene homolog 1; *MEX3B* = mex-3 RNA binding family member B; *KLHL20* = kelch-like family member 20; *MIER3* = mesoderm

induction early response 1, family member 3; EED = embryonic ectoderm development; *NT5E* = 5'-nucleotidase, ecto (CD73); *B3GNT5* = UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5; *TAOK1* = TAO kinase 1; *ATG12* = autophagy related 12; *GALNT7* = UDP-N-acetylalpha- D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc- T7); *LRRK2* = leucine-rich repeat kinase 2; *ANKRA2*= ankyrin repeat, family A (RFXANK-like) 2; *PIP4K2A* = phosphatidylinositol-5-phosphate 4-kinase, type II, alpha; *SQSTM1* = sequestosome 1; *MTDH* = metadherin (Serafin et al., 2015).

(D) miR-30a-5p target prioritization

Rank	GeneID	HGNC Symbol	mTB	TB	DIA	PD	GO	PPI	Cpx	Reac	FSBP	FSMF
1	207	AKT1		1			8	5	1		8	2
2	1956	EGFR	W	5				4		1	5	3
3	25	ABL1	F	1	0.97		5	4			4	4
4	120892	LRRK2			0.86	Val	12	4	2		3	4
5	5594	MAPK1	W	3				2	2	1	5	2
6	351	APP		1			2	7	2	1	1	2
7	11315	PARK7		1		Val	15	3	2		2	6
8	10273	STUB1	W	2				5	4	1	1	2
9	581	BAX	W	6			18	2			1	1
10	57099	AVEN	F	2	0.97						9	22
11	8301	PICALM	W	7	0.99		1	1	1	2	2	1
12	598	BCL2L1	W	4			9	3			2	5
13	1499	CTNNB1	W	6			1	2			5	1
14	596	BCL2			0.77		17	2			5	
15	3320	HSP90AA1		1			3	7	4			1
16	8678	BECN1	F	2	0.86		9	1			2	
17	26986	PABPC1	W	2				2	4	1	1	2
18	7157	TP53		2			14	2	1		2	
19	27303	RBMS3	W	5							9	
20	7534	YWHAZ	W	7	0.83		1	3	3			3

HGNC: HUGO Gene Nomenclature Committee gene symbol; GeneID: Entrez Gene identifier; mTB: miRTarBase database v4.5. W: weak funtional interaction (e.g. proteomics study), F: functional interaction (e.g. luciferase reporter assay, qRT-PCR, Western blot); TB: TarBase database v7. Number of gene records in miRNA results. DIA: DIANA microT database v5 miTG score; PD: Overlap with PD gene panel. Val: ValidatedPD; GO: Overlap with PD GO panel. Number of GO terms annotated to miRBA target gene that are descending from a panel term; PPI, Cpx, Reac: Number of proteins from PD gene panel co-annotated with a direct PPI in iRefIndex v14 (PPI), in the same protein complex in iRefIndex v14, in the same reaction step in Reactome v52. FSBP, FSMF: Number of proteins from PD gene panel that have similar Gene Ontology annotations using GO release 2015.07 and biological process (FSBP) or molecular function (FSMF) ontology.

AKT1 [PDB:P31749], EGFR [PDB:P00533], ABL1 [PDB:P00519], LRRK2 [PDB:Q17RV3], MAPK1 [PDB:P28482], APP [PDB:P05067], PARK7 (DJ-1) [PDB:Q99497], STUB1 [PDB: Q9UNE7], BAX [PDB: Q07812], AVEN [PDB: Q9NQS1], PICALM [PDB: Q13492], CTNNB1 [PDB: P35222], BCL2L1 [PDB: Q07817], BCL2 [PDB: P10415], HSP90AA1 [PDB: P07900], BECN1 [PDB: Q14457], PABPC1 [PDB: P11940], TP53 [PDB:P04637], RBMS3 [PDB: Q6XE24], YWHAZ [PDB: P63104].

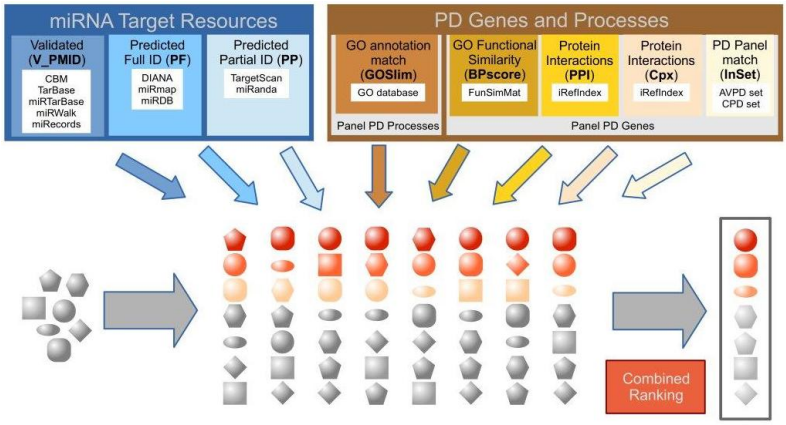


Figure 4. Prioritization of miRNA target candidates. Prioritization of miRNA target candidates based on annotation from multiple miRNA target resources and different types of evidence relating them to PD genes and processes. Candidate genes are represented by different geometric shapes, they are scored according to the different types of evidence (center), which are used to compute a combined rank (right) (Serafin et al., 2015).

5. DISCUSSION

MicroRNAs are small non-coding RNAs of 20-22 nucleotides involved in transcriptional and post-transcriptional regulation of gene expression. MiRNAs function via base-pairing with complementary sequences within target mRNA molecules, usually resulting in gene silencing via translational repression or target degradation. MiRNAs are involved in several physiological functions, and several studies have demonstrated abnormal expression levels of miRNAs in different pathologies, including Parkinson's disease. Biomarkers from easy accessible resources, such as peripheral blood, plasma, serum, urine, saliva, and cerebrospinal fluid could be used to detect and monitor the disease much earlier, even before symptoms appear. For this study we decided to consider peripheral blood and plasma as potential diagnostic sources as they are readily obtainable and reflect dynamically a system-wide biology.

The work of the present study revealed for the first time an over-expression of miR-103a-3p and an up-regulation of miR-30b-5p and miR-29a-3p in PBMCs samples from L-dopa treated PD patients. On the contrary was observed a trend of higher expression of miR-30a-5p in plasma samples from L-dopa treated PD patients and of miR-30b-5p in plasma samples from drug-naïve PD group. Moreover, using bioinformatics tools, candidate target genes for each blood and plasma deregulated miRNA have been identified and prioritized.

5.1 Importance of the miRNAs expression data normalization approach

The importance of the miRNAs expression data normalization is currently broadly recognized. The normalization process consist in the comparison of the expression levels of the investigated miRNAs (case-control groups) with the expression of one or more endogenous reference gene/s in order to obtain a relative quantification of the investigated miRNAs (Wotschofsky et al., 2011). The expression of an ideal reference gene should not vary in the tissues or cells under investigation, in response to treatment or in presence of pathology (Sanders et al., 2012). It is recognized that combining different reference genes for normalization can avoid misleading estimation of the expression analysis data, which could arise

if only a single or improper reference genes is used (Vandesompele et al., 2002) and for that reason it is important to use at least 2 reference genes.
The factors that can affect the efficiency of endogenous reference genes chosen as normalizers, could be technical factors including variability during the sample collection and processing, as well the presence in body fluids of various factors affecting miRNAs purification and qRT-PCR analysis. At the moment different normalization approaches are described in literature, but at the same time no single standard normalization method is commonly accepted.
Indeed, it has been shown in several publications that traditional reference genes used in qRT-PCR studies do not always show a stable expression pattern, because the same gene revealed as almost invariant for certain tissues or cell types, could present highly variable expression levels in other tissues or experimental conditions (Margis et al., 2011; Burgos et al., 2014). Thus, suitable control genes are extremely specific for particular sample sets and experimental models, being a crucial component in assessing gene expression patterns with confidence.

To date, has not been defined an established and standardized reliable reference gene set for miRNA profiling analysis by qRT-PCR in peripheral blood samples from PD patients. We performed a pilot study using GeNorm and NormFinder algorithms to identify, among several candidate reference genes (RNU24, Z30, RNU6B and miR-103a-3p), a reliable set of normalizers for expression analysis in PBMCs samples from L-dopa treated PD patients. The selection of the candidate endogenous reference genes was based on the literature (Margis et al., 2011; Martins et al., 2011) and we identified RNU24 and Z30 as the best stably endogenous reference genes set to use in PBMCs L-dopa treated PD expression study. Although Martins and collaborators (Martins et al., PLOS one, 2011) used miR-103a-3p in their study as reference gene, in our dataset miR-103a-3p alone or in combination with the other reference genes reverses the expression directions of miR-29a-3p and miR-30b-5p. Our results are consistent with the available literature evidence, which may have suggested *a priori*, the unsuitability of miR-103a-3p as reference gene in a PD study, as miRNA-103a-3p is a member

of the miR-15/107 group, involved in several physiological functions and pathologies, including neurodegenerative diseases. Indeed, miR-103a-3p is involved in PD targeting human CDK5R1 (Cyclin-dependent kinase 5, regulatory subunit 1), (Moncini et al., 2011) and the requirement of CDK5 is the major signal in neuronal death pathways in vivo (Smith PD et al., 2003), and it is involved in other neurodegenerative diseases, such as Alzheimer disease (Wang G et al., 2008).

For the expression analysis in plasma, we selected from the literature miR-191-5p as putative candidate endogenous reference gene (Cardo et al., 2012; Geekiyanage et al., 2011) and three exogenous oligos spike-in miRNAs (cel-miR-39-3p, cel-miR-54-3p and cel-miR-238-3p) (Kang et al., 2012). As miR-191-5p, used by Cardo and collaborators as endogenous control, showed in our data high expression variability, we excluded it as reference gene and decided to normalize our expression data exclusively against the three spike-in miRNAs. Because of the lack of established reference genes (Kang et al., 2012), even if oligos spike-in are almost exclusively used to monitor RNA purification and retrotranscription efficiencies, is not rare to find them in several miRNAs expression studies in serum or plasma as normalizers (Mitchell et al., 2008; Brase et al., 2011).

Our study demonstrated the complexity of the choice of a suitable reference gene set. The differences in relative expression of the selected microRNAs between PBMCs samples from L-dopa treated PD patients and unaffected controls were profoundly affected by the choice of reference gene set, and in some cases can completely reverse the direction of miRNAs expression. The results highlight the importance of a careful choice of a stable reference genes set, which should be evaluated depending on the specific on-going study, not relying solely on published data.

5.2 Over-expression of miR-29a-3p and miR-30b-5p in PBMC samples from L-dopa treated PD patients

The expression analysis on PBMCs samples from L-dopa treated PD patients and unaffected matched controls, showed an over-expression of miR-29a-3p and miR-30b-5p. These finding was unexpected, since in previous papers miR-29a-3p and miR-30b-5p were showed down-regulated by Margis (Margis et al., 2011) and Martins (Martins et al., 2011) studies, respectively. This discordant result can be explained by the use of PD sample sets with different size and clinical features, and by differences in the pharmacological treatment. Moreover, the discordant results could be explained by distinct experimental conditions, including RNA purification, expression analysis methods, and the selection of reference genes used as normalizer, because a misleading estimation of the expression analysis data can arise if only a single or improper reference genes are used (Vandesompele et al., 2002).

For our qRT-PCR analyses we used a matched case-control experimental design on 36 PBMCs samples from L-dopa treated PD patients and unaffected matched controls, whereas Margis (Margis et al., 2011) and Martins (Martins et al., 2011) followed an unmatched study design.

Margis and collaborators analyzed extremely small sample sets. They showed a significant down-regulation of miR-29a-3p in PBMCs samples from 8 drug-naïve PD patients by qRT-PCR and only a trend to lower expression in 4 L-dopa/Carbidoba treated PD patients, whereas no difference in expression was observed for the 7 early onset PD patients. Furthermore, no data are available regarding the reference genes used in their qRT-PCR study, and the selection of the normalizer could be one of the reasons of the different direction in miRNAs expression.

Martins and collaborators profiled by microarray assays 19 PD patients and 13 controls and they found a down-regulation of miR-30b-5p in PD patients. To validate their microarray results, they performed an expression analysis on a subset of five miRNAs, unfortunately not including miR-30b-5p. The five deregulated miRNAs were validated by Martins and collaborators using miR-103a-

3p as normalizer. In our study miR-103-3p has been identified as unsuitable reference gene as normalizer, as discussed above.

5.3 Target prediction priorization

Our study demonstrated for the first time an over-expression of miR-103a-3p and a deregulation of miR-30b-5p and miR-29a-3p in PBMCs samples from L-dopa treated PD patients and unaffected controls, and on the contrary it has been found a trend of higher expression of miR-30a-5p in the plasma samples from L-dopa treated PD patients. In order to better address further analyses and to understand the underlying biology and the role of the observed miRNAs up-regulation, we performed an *in silico* analysis of the three deregulated PBMCs miRNAs in L-dopa treated PD patients and an exploratory target prediction for the potentially up-regulated plasma miRNA in L-dopa treated PD patients. Even if we observed only a trend towards significance for up-regulation of miR-30a-5p in plasma samples from L-dopa treated PD patients, investigating its potential biological context was anyway interesting even becuase it was found up-regulated in serum post-mortem samples from PD patients by Burgos and collaborators (Burgos et al., 2014).

The *in silico* analysis, used to prioritize putative targets of the deregulated miRNAs, has identified putative candidate target genes, including genes related to neurodegeneration and PD. In this chapter we want to focus on *Bcl-2*, protein involved in apoptosis and mithocondrial dysfunction, as common predicted target for all up-regulated miRNAs (miR-30b-5p, miR-29a-3p, miR-103a-3p in PBMCs, and miR-30a-5p in plasma) and the putative involvement of miR-103a-3p in the insulin resistance process, even if its precise role remains still to be defined.

5.3.1 Bcl-2 as putative common target for all up-regulated miRNAs in PBMCs and plasma samples from L-dopa treated PD patients

The *in silico* analyses of all over-expressed microRNAs suggest as common target, the anti-apoptotic protein Bcl-2, belonging to a family involved in the

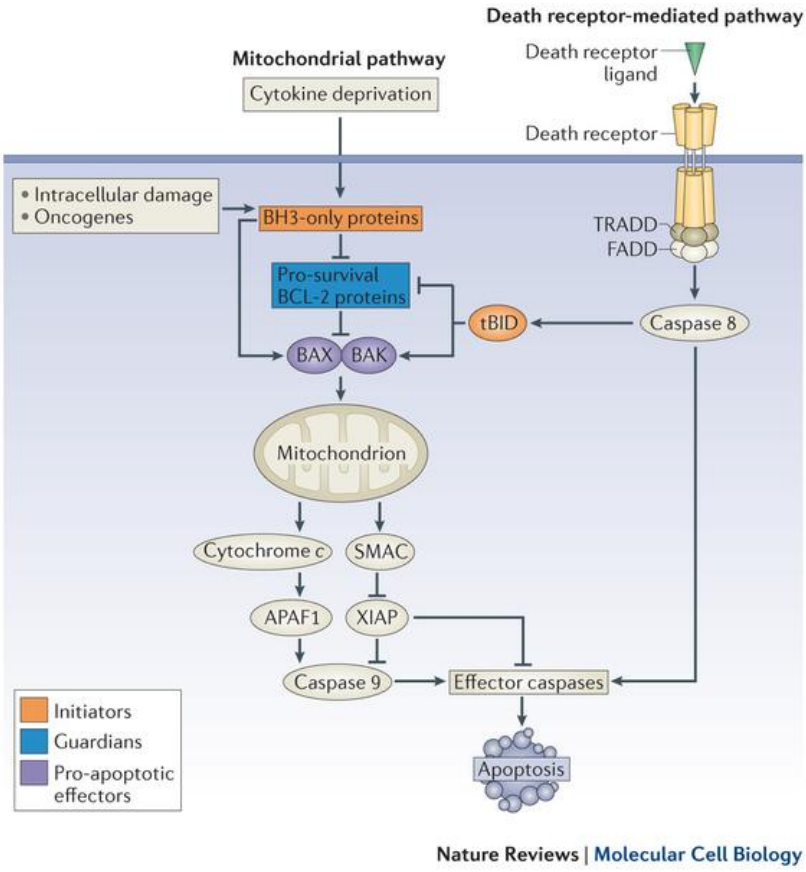
regulation of mitochondrial dynamics and apoptosis. To date, the knowledge related to the function of Bcl-2 family proteins, supports the idea of their involvement in neuronal death in different models of PD, albeit it has not yet been clarified in detail the role and mechanism of action of each of them.

On a physiological level in cells there must always be a homeostatic balance between proliferation and apoptosis, because excessive cell death can lead to neurodegenerative disease and on the contrary, excessive cell survival function could lead to autoimmune diseases or cancer. Indeed, the ratio between these two conditions helps to determine in part, the susceptibility of cells to a death promoting signal. The apoptotic process can occur through two different activation pathways: the extrinsic pathway mediated by the activation of pro-caspases and cell death receptors, and the intrinsic or mitochondrial pathway, regulated by the Bcl-2 family (Brunelle and Letai, 2009), focus of our discussion.

The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 family member's activity. Bcl-2 protein is an integral membrane protein with an anti-apoptotic function, belonging to the family of proteins Bcl-2, localized in the outer mitochondrial membrane (MOM) and its function is to regulate the permeability of the MOM during the apoptosis (Brunelle and Letai, 2009). Normally Bcl-2 anti-apoptotic family members, including the Bcl-2 protein, modulate the pro-apoptotic Bax and Bak proteins by suppressing their activity, through inhibition of BH3-only proteins (Brunelle and Letai, 2009). We hypothesized that the over-expressed miRNAs could repress the expression of Bcl-2 anti-apoptotic proteins, resulting in an uncontrolled activation and homo-oligomerization of Bax and Bak. The consequent homo-oligomerization of the pro-apoptotic proteins Bax and Bak, results in the formation of pores (MAC, mitochondrial apoptosis-induced channel) at the level of the MOM. The formation of these pores, increases the permeability of the mitochondrion, and reduces in part the ability of the cell to control the passage of water and ions, resulting in the release into the cytosol of pro-apoptotic factors including cytochrome c and SMAC (Second Mithocondria-derived Activator of Caspases) (Lindsay et al., 2010). SMAC binds to inhibitor of apoptosis (IAPS) inhibiting its function, while the

cytochrome c binds to Apaf1 and ATP generating a protein complex, called apoptosome, which aggregates and activates pro-caspase 9, inducing apoptosis (Lindsay et al., 2010).

The hypothesis to be confirmed through target functional analysis is that the over-expressed miRNAs inhibit the activity of the protein Bcl-2 by eliminating its anti-apoptotic activity in response to many varieties of insult and failing in preventing the activation and homo-oligomerization of both Bax and Bak.



Mitochondrial and death receptor-mediated pathway to apoptosis. (Czabotar et al., 2014).

Understanding the mechanism of action of microRNAs linked to Bcl-2 family proteins in PD, could clarify the intracellular mechanisms leading to neuronal death and may also provide a rationale in using them as biomarkers.

Interesting, Rasagiline is a selective and irreversible inhibitor of MOA-B (Monoamine oxidase B), and can be used as monotherapy or as adjunctive

therapy to L-dopa treatment in patients with fluctuations end-dose interval of time between the administrations of L-dopa (Weinreb et al., 2006). Fluctuations happen when the effects of the L-dopa treatment are exhausted and the symptoms recur. These symptoms are related to a reduction of the effects of L-dopa and the patient suddenly changes from the state "on", in which he is able to move, to the "off", state of immobility. Weinreb and colleagues (Weinreb et al., 2006) showed a potential neuroprotective effect of Rasagiline, up-regulating the expression of anti-apoptotic proteins, such as Bcl-2, Bcl-xL and Bcl-w.

5.3.2 Insulin resistance and over-expression of miR-103a-3p

Several recent discoveries have highlighted common cellular pathways that potentially relate neurodegenerative processes with abnormal mitochondrial function and abnormal glucose metabolism.
MiR-103a-3p has documented relationships to insulin resistance, a process possibly linked also to neurodegeneration in PD, whereas miR-30a-5p plays a central role in the mitochondrial fission process and indirectly to the insulin secretion.

In a recent study (Trajkovski et al., 2011) it was shown in mouse model as miR-103 is related to insulin-metabolism. The connection between miR-103 and insulin is relevant, since insulin is known to regulate dopamine release. Moreover in a recent study a correlation between PD and a hyperglycaemic status was shown in rats (Herrera et al., 2010).
Neuropathological studies of patients with Parkinson’s disease have shown that insulin receptors are densely represented on the dopaminergic neurons of the substantia nigra pars compacta, and loss of insulin receptor immunoreactivity and messenger RNA in the substantia nigra pars compacta of patients with Parkinson’s disease coincides with loss of tyrosine hydroxylase messenger RNA (the rate-limiting enzyme in dopamine synthesis) (Aviles-Olmos et al., 2013).

Animal and *in vitro* studies have suggested a role for insulin in regulation of brain dopaminergic activity featuring a reciprocal regulation between the two chemicals (Craft and Watson, 2004).

Pancreas secretes two important hormones involved in regulation of glucose metabolism, lipids and proteins. First, glucagon which acts to increase the glucose in blood through degradation of glycogen into glucose and it is release in blood. Second, insulin that acts to decrease the blood glucose through its uptake by adipocytes and muscles and to increase the activity of glycogen synthase in the liver. The plasma glucose concentration reflects the balance between the absorption from the gastrointestinal tract, the usage at tissue level and the endogenous production. The hormone insulin and several insulin-like growth factors mainly control the glucose homeostasis.

The insulin resistance condition results in an uncontrolled release of glucose in the blood and a reduced response of cellular insulin receptors to insulin. Pancreas tries to compensate the reduced sensitivity of cells to insulin by increasing its synthesis and its release.

The first evidence of a relationship between PD and insulin resistance is linked to the early 70s. Van Woert and Mueller (Van Woert and Mueller, 1971) showed that drug-naïve PD patients presented reduced insulin-mediated glucose uptake, and Boyd (Boyd et al. 1971) identified an inhibition of early insulin secretion and long-term hyperinsulinemia and hyperglycemia after glucose loading. In addition, L-dopa used since the 60s in the common PD treatment, can induce hyperglycemia and hyperinsulinemia (Aviles-Olmo et al., 2013). Different studies have reported high rates of glucose intolerance in PD patients and the effective hyperglycemic response to L-dopa and dopamine (Smith Y et al., 2012). They showed that L-dopa treatment coupled with Carbidopa diminishes glycogen concentration, glycogen synthase activity, and insulin-stimulated glucose transport, suggesting that PD patients may have unrecognized insulin resistance.

Although to date has not yet been demonstrated a direct *in vivo* regulatory action of miRNAs on insulin sensitivity, Trajkovski and colleagues (Trajkovski et al., 2011) have shown that miR-103 and miR-107 are up-regulated in obese mice,

showing evidence of invovment of miRNAs in the regulation of insulin and in the process of insulin resistance. They have demonstrated that miR-103 and miR-107 target caveolin-1, a critical regulator of the insulin receptor.

Caveolin-1 is the main protein component of the caveolae, invaginations of the plasma membrane abundant in the insulin-dependent tissues, mainly adipose cells and muscle, and activates insulin signaling. The down-regulation of caveolin-1 suppresses the expression of insulin receptor alpha and the insulin receptor substrate 1. Although the role of caveolin-1 is not still fully clear, its action may stabilize the insulin receptor at caveolae level. Caveolin-1 is inhibited as a result of high levels of expression of miR-103 and miR-107 in the adipocytes, destabilizing insulin receptors and inhibiting insulin-stimulated glucose uptake (Traijkovski et al., 2011), acting as negative regulators of insulin sensitivity. The mechanism of action proposed suggests that miR-103 targets caveolin-1 reducing the number of insulin receptors in the caveolae and resulting in decreased downstream insulin signaling (Traijkovski et al., 2011).

Our speculation is that, miR-103a-3p is over-expressed as consequence of L-dopa treatment in a negative feedback still unclear acting on caveolin-1 receptor, imparing insulin secretion and contributing to the insulin resistance. Obviously this idea has to be corroborated on the basis of further investigation with the aim to validate the predicted targets for miR-103a-3p in cell models.

Interestingly, not only miR-103a-3p but also miR-30a-5p seems to be indirectly related to the insulin metabolism. Indeed, p53 has been identified as putative target gene for miR-30a-5p and validated by Li and colleagues (Li et al., 2010).

There are evidences suggesting a regulation of mitochondrial fission by miR-30a-5p through inhibition of p53, which causes a down-regulation in Drp1 levels (Dynamin-Related Protein-1) (Tanaka et al., 2008). Drp1 is a GTPase that causes scission of the mitochondrial outer membrane, resulting in mitochondrial fragmentation. A down-regulation of Drp1 causes both fission and autophagy inhibition (Li et al., 2010). The result of the inhibition of the fission process and a decreased mitochondrial autophagy is an accumulation of oxidized mitochondrial

proteins, reduced respiration and impaired insulin secretion (Twig et al., 2008). Therefore, in accordance to this evidence the up-regulation of miR-30a-5p in plasma drug-naïve PD samples might carry to over-regulation of p53 and Drp1, resulting in hyper secretion of insulin.

The findings provided by our study and the data available in the current literature reveal the complexity of the biological context in which the deregulated miRNAs could act. This is not surprising taking into consideration that in general miRNAs exert their function by regulating several target genes, potentially involved in more than one single pathway. The hypotheses presented here require absolutely further investigations, but they highlight the impact that could have a confirmation of the deregulated miRNAs in PD regarding apoptosis and insulin resistance.
Despite the preliminary character of the study, the results provide a rationale for validating the miRNAs diagnostic potential.

5.4 The difference in miRNAs expression could be the consequence of L-dopa treatment rather than to the PD pathogenesis per se?

Our study demonstrated for the first time an over-expression of miR-103a-3p, and showed a deregulation of miR-30b-5p, and miR-29a-3p in PBMCs samples from L-dopa treated PD patients, whereas no significant differential expression was detected in the PBMCs samples from drug-naïve PD patients sample set (Serafin et al., 2015).
On the contrary, it has been found a trend of higher expression of miR-30b-5p in the plasma samples from drug-naïve PD patients and a trend of miR-30a-5p in the plasma samples from L-dopa treated PD patients. We can argue that the over-expression trend of miR-30b-5p decrease until disappear in concomitance with the L-dopa drug treatment, and in parallel will appear an over-expression of miR-30a-5p. Moreover, miR-29b-3p, normally expressed in PBMCs, was not detected in plasma samples from L-dopa treated PD patients.

The comparison between expressions profiles in PBMCs (Serafin et al., 2015) and plasma revealed clear differences for all analyzed miRNAs in L-dopa treated and drug-naïve PD/control pairs, indicating that different miRNA expression profiles are present in plasma and PBMCs samples from L-dopa treated PD patients and that L-dopa treatment might have an impact on the expression levels of miRNAs.

In accordance to the PBMCs expression data, it can be hypothesized that the deregulation of the investigated miRNAs is due to the progression of the disease, resulting in miRNAs release from tissue into the circulation.
On the other hand, in accordance to the plasma expression data, it cannot be excluded that the observed over-expression of miRNAs in plasma samples from L-dopa treated PD patients could be the consequence of the L-dopa treatment.

Only further investigations could confirm our preliminary data and validate the role of the deregulated miRNAs as putative biomarkers for PD.

5.5 Plasma and blood as sources for PD biomarker

The identification of specific biomarkers at early stages of the disease could lead to early patient's treatment, and during the progression of the disease a useful biomarker could help to monitor the secondary side effects in response to the L-dopa treatment. A biomarker should meet requirements such as easy accessibility, high specificity and sensitivity, low costs and standard applicability in diagnostic laboratories. Biomarkers from easily accessible sources, such as peripheral blood, plasma/serum, saliva, urine, and cerebrospinal fluid could be used to detect and monitor PD in early stages, even before symptoms appear.

For this study we decided to consider peripheral blood and plasma as potential diagnostic sources as they are readily obtainable and reflect dynamically a system-wide biology. Liew and colleagues (Liew et al., 2006) have compared genes expressed both in circulating blood cells and in different nine human tissues, finding approximately 80% of co-expressed genes and revealing that expression of 81.9% blood genes were shared by brain. This data suggest that

blood could act as sentinel of the disease, through an aberrant gene expression, reflecting changes within the cells occurring in association with injury or disease and as consequence of continuous interaction between blood and other body tissues.

At the moment the precise mechanism of transport of brain tissue-specific miRNAs through the blood brain barrier (BBB) into the body circulation is still unknown. The BBB acts as checkpoint specialized to regulate the transport of nutrients and macromolecules and block the entry of viruses and bacteria (Cheng et al., 2013). However, some protein complexes can cross the BBB, suggesting that some tissue-specific disease biomarkers can be detected in body fluids.
Circulating miRNAs are released in two different manners, the first one as result of cell lyses or apoptosis by passive leakage in pathological conditions, such as tissue damage, metastasis or inflammation. The other process, which still is not completely clear, is an active transport from the cells based on microvesicle-free miRNAs, associated with various multiprotein, such as Ago2 or lipoprotein complexes (HDL) or mediated by microvesicles (MVs) (e.g. microparticles and exosomes) or apoptotic bodies (ABs) (Grasso et al., 2014).
As the cell-free miRNAs in body fluids are transported by exosomes or microvesicles, the suggested pathway indicates the transcytosis of the extracellular vesicles as the main actor to carry miRNAs from brain to body fluids. In that way the vesicles such as exosomes can cross the BBB endothelial cells by receptor-mediated endocytosis and release the exosomal contents into circulation. Montecalvo and colleagues (Montecalvo et al., 2012) showed that exosomes, carrying miRNAs, were secreted by dendtritic cells and that they could fuse with the target cell releasing their content. An effective trace of this mechanism is resulting by different studies showing that high abundant brain miRNAs were present in body fluids, such as plasma (Haqqani et al., 2013).

Plasma samples showed a completely different miRNAs expression profile in comparison to peripheral blood samples, as discussed above. Thus, indicating that PBMCs and plasma are not interchangeable sources for miRNA biomarkers for

PD. The explanation of this discordance between blood and plasma miRNAs expression profile can be attributed to the different cell sources used for isolating miRNAs.

Blood and plasma are both classifiable as optimal biomarkers sources, but each of them presents different advantages and disadvantages.

It is widely believed that miRNAs are present mainly in plasma associated to vesicles, including exosomes, protein complexes (including non-vesicular lipoprotein complexes, non-vesicular RNA-binding proteins), and miRNA processing proteins, which may explain the different abundance levels of miRNAs, in fact, miRNAs isolated from PBMCs are more abundant, in comparison with miRNAs isolated from plasma.

PBMCs share more than 80% miRNA of the transcriptome with brain (Liew et al., 2006), but being living cells the miRNA expression can change after the sampling. This problem does not exist in plasma, once plasma has been isolated (after centrifugation of whole blood and separation of plasma), miRNA expression does not change anymore. This feature represents an important advantage in terms of biomarker measurements and laboratory procedures.

An advantage to assess expression profiles using miRNAs isolated from plasma is the possibility to normalize variations during the recovery and amplification steps, using exogenous miRNAs as reference genes added to the plasma before the RNA extraction. Conversely, an advantage to use PBMCs for the miRNAs expression profiling is that they are therefore L-dopa responsive cells expressing, similarly to dopaminergic neurons, dopamine receptors and transporters. Moreover, miRNAs are much more abundant in PBMCs than in plasma.

The other variables which can affect the miRNAs expression profile are due to variation and/or problems linked to the normal laboratory practice, such as the plasma/blood collection and processing. Plasma is the cell-free supernatant phase obtained after blood sample centrifugation. During this phase it is strictly recommended the use of suitable anticoagulants. Indeed, heparin inhibits the downstream PCR or sequencing reactions, whereas EDTA and citrate are acceptable anticoagulants. Moreover, during the aspiration and separation

procedures of the plasma there is a risk of contamination by cells from the buffy coat.

It is always highly recommended to match cases and controls specimens in respect of many variables, which have not yet been carefully evaluated and that may be able to affect the study results, such as duration of freeze-storage of the sample, diurnal variation in miRNA levels (e.g. fasting vs non-fasting state), and white blood cell count (Kroh et al., 2010). It is known that some blood components could vary according to the fasting, but it is not clear if this status might be able to affect miRNAs levels in blood. MicroRNAs can be transported in the blood carried out by lipoproteins or vesicles, which may be affected by an individual's food intake (Moldovan et al., 2014) such as HDL particles whose concentration in the blood can vary during the day. In addition there may be improper recovery of miRNAs because of extraction methods influencing the expression profile.
In conclusion, it is evident that, even being both peripheral blood and plasma good resources for biomarkers, it is important to carefully choose the tissue to analyse based on the specific on-going study and on the laboratory practice.

5.6 Deep sequencing analysis: new insights.

The Illumina MiSeq NGS platform was acquired as new platform, and the first approach in order to settle and standardized the NGS analysis methods, was comparing the gold-standard qRT-PCR miRNAs results previously done, with the bioinformatics sequencing data analysis.

Despite the number of reads generated and the number of samples sequenced in this pilot phase, the performed saturation curve revealed that the number of reads needed to have enough coverage depth to identify miRNAs deregulation for our experiment was not sufficient. Also compared to other studies the number of total reads generated in our pilot experiments needed for an accurate miRNA expression analysis was low. For example, Dhahbi and collegues (Dhahbi et al., 2011) obtained 9.1 to 11.4 million reads per sample, the group of Hu (Hu et al., 2011) obtained 7.6 million reads per sample, and Morin (Morin et al., 2008)

obtained 6 million reads per sample.

Regarding the sequencing and real time technologies, the data resulted in contrasting opposite miRNAs expression profiles. At this stage we were in for an interesting question, namely whether the NGS and qRT-PCR data could be directly compared. Unfortunately, there is no gold standard technique for measuring miRNA expression. Currently oligonucleotide microarray and quantitative real-time reverse-transcription PCR are two of the most common methods for evaluating known miRNAs.

The analysis of the current literature stresses the need to compare the different expression results coming from the different platforms used for the expression analysis, such as microarray, NGS and qRT-PCR in terms of reproducibility, sensitivity, accuracy, and specificity.

Some research groups found a good correlation between microarray, NGS and qRT-PCR for selected miRNAs, while others revealed conflicting data.

Mestdagh and collaborators (Mestdagh et al., 2014) focused properly on that point. They selected a total of 66 miRNAs, found to be differentially expressed by at least one of the 12 most common commercially platforms. A comparison of small RNA sequencing, qRT-PCR and microarray, revealed that only two miRNAs (3%) were differentially expressed by all the platforms. Only 48% of the miRNAs were concordant for half of the analysed platforms. They pointed out the necessity to choose the appropriate analysis platform, because the choice could impact on the expression results. qRT-PCR platform remains the “gold standard” in terms of sensitivity and accuracy, analyzing samples with a low input RNA, having an overall better score, whereas sequencing platforms lose sensitivity for low-input-amount RNAs differences (Mestdagh et al., 2014). Metpally and collaborators put in evidence that not only the choice of the platform depending on the study design is critical but also the selection of the analysis software packages used for data analysis (Metpally et al., 2013). They compared three common analysis tools used for alignment and read count generation named miRExpress, miRNAKey, and miRDeep2. Their study showed that miRDeep2 software has detected and aligned more miRNAs than miRExpressor and miRNAKey, and that miRExpress and miRNAKey have performed more similarly.

Another point to take in consideration and that can affect the sequencing analysis are the periodical changes in the miRBase database, used to design the arrays and qRT-PCR probes and to map the sequencing reads to known mature miRNAs. Indeed, the number of human miRNAs represented on an array depended mainly on the miRBase version at the time of array design. Moreover, the probe and primer design (qRT-PCR) can heavily influence the expression results. In conclusion, it is necessary to re-annotate microarray and qPCR probes prior to go on with the bioinformatics analysis (Git et al., 2010).

Although qRT-PCR is considered the gold-standard and is used widely as a validation method for miRNA NGS and microarray data, there are various conflicting opinions regarding the reliability of qRT-PCR as validation method for NGS. Some evidences suggest the inadequacy of qRT-PCR as validation method for miRNA microarray (Git et al., 2010; Koshiol et al., 2010) and NGS data (Metpally et. al., 2013; Git et al., 2010). qRT-PCR do not detect small but significant variations of miRNAs sequences (Metpally et. al., 2013), probably because of the logarithmic nature of qRT-PCR, which may make difficult to detect modest changes in molecule number. It seems necessary to find some alternatives to qRT-PCR for microarray and sequencing validation data.

To conclude, our analyses revealed an over-expression of miR-103-3p, miR29a-3p, and miR-30b-5p in peripheral blood, and a trend of over-expression of miR-30a-5p found in plasma of L-dopa treated PD patients. These miRNAs could act as diagnostic biomarkers specific for Parkinson's disease or they could be a challenging resource as biomarkers monitoring the secondary side effects related to the drug therapy. Because of the side effects in L-dopa treatment it would be important to be able to monitor its levels in blood, so that an individual personalized therapy could be developed, and could be possible predict and avoid significant motor fluctuations. Indeed, each patient requires a different dosage of drug and the use of miRNAs as biomarker could represent an important variable for therapy improvement.

6. CONCLUSION

Our study identified a set of reliable endogenous reference genes (RNU24 and Z30) to use in miRNAs expression studies on PBMcs samples in Parkinson's disease.

We highlighted for the first time an over-expression of miR-103a-3p in PBMCs samples from L-dopa treated PD patients and unaffected matched controls and showed a deregulation of miR-29a-3p and miR-30b-5p from L-dopa treated PD patients and unaffected matched controls. No differences in miRNAs expression levels in PBMCs samples from drug-naïve PD patients were found. Further analysis in plasma samples from L-dopa treated PD patients indicated a trend of over-expression for miR-30a-5p and a trend of over-expression for miR-30b-5p in plasma samples from drug-naïve PD patients. Moreover, miR-29b-3p, previously found expressed in peripheral blood samples, is lost in plasma samples. This findings suggested that different miRNA expression profiles between PBMCs and plasma existed. Further analyses in patients before and after the beginning of the pharmacological therapy will be needed to define the involvement of L-dopa treatment in the observed miRNAs over-expression.

Finally, the elaboration of a more sophisticated *in silico* approach generated a reliable and promising target prediction.

Despite the preliminary character of our study, the results provide rationale to perform additional experiments to clarify the role of the identified miRNAs in the pathogenesis of PD, to clarify their role in apoptosis and insulin resistance, to add further evidence in L-dopa role and to further investigate miRNAs diagnostic potential.

Our study do not allow the conclusion that the identified candidate miRNAs are ready to be used as diagnostic markers, but contributes to identify a PD specific miRNAs signature. Our study suggests that microRNAs, both from peripheral blood and plasma, can act as diagnostic biomarkers specific for Parkinson’s disease or they could be a challenging resource as prognostic biomarkers

monitoring the disease progression and the secondary side effects, which could appear during the drug therapy.

CONCLUDING REMARKS

The value of miRNAs as diagnostic and prognostic biomarkers and therapeutic targets is now widely recognized. Within 10 years more than 1500 human miRNAs have been discovered and show a potential utility in clinical application. The study of small non-coding RNAs, and in particular the class of miRNAs, has had a great impact and its widespread interest in the understanding of their role in gene regulation is becoming fundamental to understand their physiological and pathological role.

The use of miRNAs as diagnostic or prognostic biomarkers for PD is not so far to become real. Indeed, Petillo and collaborators published a validation study, at the beginning of 2015, for a panel of four plasma-based circulating miRNA biomarkers for PD (miR-1826, miR-450b-3p, miR-505, and miR-626), as molecular biomarkers and qRT-PCR as potential diagnostic assay.

7. PERSPECTIVES

The results of this study throw the basis for future analysis in order to validate the deregulated miRNAs as biomarkers, to clarify the involvement of L-dopa in the miRNAs expression and to investigate some interesting putative miRNAs target genes.

To confirm and validate the role of the deregulated miRNAs as diagnostic biomarkers for PD it will be necessary enlarge the PD and drug-naïve PD patients group under investigation and perform additional independent expression profiling studies by comparing L-dopa treated PD and drug-naïve PD patients groups. Furthermore, it will be possible to confirm the trend of miRNA expression according to the gender, after a subgroup analysis stratifying the samples by gender.

To evaluate the impact of L-dopa treatment on the levels of miRNAs expression, and confirm the role of deregulated miRNAs as biomarkers for monitoring the response to the treatment, further investigations are needed. An interesting approach might be the development of a longitudinal study (pharmacokinetic study), able to monitor and evaluate the expression levels of the miRNAs at different time-points before and during drug administration, and the use of *in vitro* or *ex-vivo* PBMCs dopaminergic models for exploring the miRNA expression levels at different time-points of L-dopa dosage.

In order to clarify the specific role of the deregulated miRNAs in the pathology of PD and to confirm the proposed hypotheses on a possible link of miRNAs to insulin resistance and apoptosis process and PD, it is necessary to validate some predicted targets in cell models through a luciferase assay and by measuring protein levels after over-expression and/or suppression of the analyzed miRNAs. In this way, it will be possible to better define the role of miR-103a-3p, miR-29a-3p, miR-30b-5p, and miR-30a-5p in the molecular mechanism of the pathogenesis and progression of Parkinson’s disease.

Moreover, an additional interesting approach, that is developing now, could be the possibility to identified miRNA-mRNA interactions or the parallel expression analysis of miRNAs and proteins of same samples from L-dopa and/or drug-naïve PD patients. Indeed, miRNAs regulate protein levels and therefore the combined analysis of miRNAs/protein profiles might better reflect the transcriptional program of normal and PD cells.

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