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THE USE OF COMPLEX NETWORKS IN THE
INVESTIGATION OF PARKINSON'S DISEASE BY
THE ANALYSIS OF MIR-19B AND DRUG
REPURPOSING

ABSTRACT

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THE GENERAL PART

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's, with an annual global incidence of up to 35 new cases / 100,000 people. It is estimated that its overall burden will double by 2030. PD is a disease of the elderly (over 65 years), its prevalence increasing from 1% (65 years) to 3% in people over 80 years. The incidence of PD can be influenced by gender (more common in men), race (less common in African Americans), ethnicity (more common in Ashkenazi Jews, Alaska Natives), and environment (eg, pesticide exposure, trichlorethylene).

The diagnosis of PD is essentially clinical, based on non-motor symptoms (hyposmia, constipation, sleep disorders, cognitive decline, psychiatric disorders) and motor (stiffness, rest tremor, postural instability, bradykinesia). The diagnosis of PD is based on the evaluation of the clinical response to levodopa (rapid improvement of motor symptoms), to which are added a series of tests such as the evaluation of cardiac sympathetic denervation (by myocardial scintigraphy) and DaTSPECT. However, they cannot differentiate PD from Parkinson's syndromes that have both clinical and molecular similarities, for example in dopamine transport dysfunction.

The diagnostic criteria for Parkinson's disease allow the identification of clinically manifest disease, the symptoms appearing years after the beginning of the neurodegenerative process, when half of the dopaminergic neurons in the substantia nigra are already lost. Most patients with PD respond well to dopamine replacement therapy, with ON-OFF and dyskinesia occurring within 2 to 6 years of starting levodopa therapy.

There are no biochemical or molecular biomarkers for PD diagnosis, therapy monitoring or prognostic evaluation. Blood α -synuclein, saliva and cerebrospinal fluid (CSF) have been extensively researched over the past decade, but have been shown to lack specificity and sensitivity.

MicroRNAs are a species of small, non-coding endogenous RNAs involved in the post-transcriptional regulation of gene expression. A single microRNA modulates the stability of hundreds of mRNAs, while a mRNA can be the target by dozens of microRNAs, a biunivocal relationship that explains the ability of microRNAs to regulate the expression of almost half of the human transcriptome. Due to their remarkable stability in various biological fluids, microRNAs are ideal candidate biomarkers in a wide variety of pathologies, including neurodegenerative diseases.

The formation of mature microRNA is a complex process, very well controlled at each stage / level. MicroRNA biogenesis begins in the nucleus by transcription of the gene by an RNA polymerase II (in some particular cases, such as microRNA sequences located after the Alu transposable elements, by an RNA polymerase III). A primary microRNA (pri-miR) is thus formed which will also be processed in the nucleus by an enzyme called Drosha (RNAase III), resulting in a precursor microRNA (pre-miR). It will be exported by a protein called Exportin-5 through the nucleospores, into the cytoplasm, where another RNAase III enzyme, Dicer, will process it, resulting in a double-stranded microRNA. One of the two strands, called the guide strand, will be incorporated into the RISC complex (RNA Induced Silencing Complex) together with the AGO proteins, and the other strand, the passenger strand, will most likely be degraded. If microRNA biosynthesis follows these steps, it will be called canonical biogenesis. In the case of certain microRNAs (e.g. miR-451, miR-320, miR-484, etc.), the synthesis is independent of Dicer, Drosha or even AGO; in these cases it is a non-canonical biogenesis.

MicroRNA biosynthesis is subject to a strict regulatory process that can take place both in transcription and in processing. This regulation is specific and dependent on the age of the

organism, the type of cell or tissue and represents a very important aspect, because the alteration of microRNA expression can determine and accompany a very wide range of pathologies in humans.

The need to identify a biomarker (microRNA) in PD led to an impressive amount of data, characterized by a surprising lack of consistency, due to the different methods and analytical platforms used, the diversity of tissues evaluated (CSF, whole blood, plasma, serum) or the ethnicity and staging of the patients included in the study.

Analyzing the literature, we identified 56 differentiated microRNAs expressed in the cerebrospinal fluid of patients with PD, 9 being common for at least 2 articles. Of these, only let-7g-3p (overexpressed), miR-184 (overexpressed) and miR-99a-5p (downregulated) had concordant expressions, all others having divergent expressions. In plasma, 44 microRNAs were identified, of which 14 were common to at least 2 studies. Three of them were identified as concordantly overexpressed (miR-124-3p, miR-132-3p, miR-433-3p), 8 were concordantly downregulated (miR-128-3p, miR-136-3p, miR-154-5p, miR-323a-3p, miR-382-5p, miR-409-3p, miR-410-3p, miR-485-5p). In the serum samples from patients with BP, 36 differentially expressed microRNAs were identified, of which 10 were common for at least 2 studies, all with concordant expressions: miR-24, miR-195 (upregulated), miR-141, miR-146b-5p, miR-193a-3p, miR-19b, miR-214, miR-29a, miR-29b, miR-29c (downregulated).

Very few studies meet the stringencies of a biomarker discovery study in terms of design, rigorous analysis, and extent of patient enrollment, which could explain why no microRNAs have entered clinical use so far. Therefore, a basic analytical method necessarily includes a screening step (usually next generation sequencing (NGS, microarray or RT-PCR array) followed by a validation step by another technology (e.g., qRT-PCR) and in an independent group of patients, in both stages, the use of strict criteria for the inclusion and exclusion of both patients and controls, as well as appropriate bioinformatics and statistical methods, are essential.

One of the bioinformatics methods used is the analysis of complex networks. The application of the theory of complex networks in biology has proven to be an important tool in understanding the regulatory mechanisms at the cellular and molecular level, being useful in deciphering the processes underlying the production of diseases. Because microRNAs participate in gene regulation, through direct interaction with mRNA, they are an important element in the functioning and stability of complex networks. The analysis of complex microRNA-mRNA networks is hampered by the fact that biogenesis, transport, interaction with mRNA and microRNA degradation can be regulated by the activity of other microRNAs, by the action of transcription factors, transporters. The stability of microRNA-mRNA networks is influenced by feedback and feed-forward mechanisms. Analysis of network stability dynamics can lead, for example, to understanding the clinical manifestations associated with altered microRNA expression. Disorder of microRNA expression can lead to many changes in gene expression: decreased expression of a microRNA leads to repression of target genes, and increased microRNA expression can lead to repression of target genes. In the case of several miRs, which are part of the same family, changing the expression of one of the microRNAs causes compensatory mechanisms from the other members of the family, a phenomenon that is missing in the case of isolated microRNAs. All these factors can lead to changes in the dynamics of the network and it is important to integrate all the factors to understand the proper functioning of the network and therefore the mechanisms underlying the associated pathologies.

THE SPECIFIC PART

PURPOSE

In the present study we aimed to identify a plasma microRNA (s) that can be used as biomarkers in Parkinson's disease, as well as to find by repositioning new antiparkinsonian drugs.

OBJECTIVES

1. Formation of the study group
2. Identification of microRNAs expressed in the plasmas of patients with Parkinson's
3. Validation of identified microRNAs
4. Identification of a microRNAs in response to LevoDopa therapy
5. Prediction of signaling pathways impacted by identified microRNAs
6. Transcriptional validation of the level of modification of some genes in the predicted signaling pathways
7. Formation of a complex network of drugs based on drug-receptor interaction
8. Validation of repositioned medicines
9. Identification of possible new antiparkinsonian drugs

MATERIAL AND METHODS

All patients in the screening and validation groups were recruited through the Neurology Clinic of the Timișoara County Emergency Hospital and signed an informed consent. The diagnosis of PD was made according to the clinical diagnostic criteria of the Parkinson's and Movement Disorder Society for Parkinson's Disease.

The screening group includes 10 patients and 10 controls, of which 6 males and 4 female in both cases. The validation group consists of 66 patients (35 men and 31 women) and 29 controls (14 men and 15 women).

Naive plasma samples (and appropriate post-LevoDopa therapy) were provided by the Harvard NeuroDiscovery Center (Brigham & Women's Hospital). This is a group of 10 patients diagnosed with BP, from whom plasma samples were collected before and after treatment with LevoDopa; 7 men and 3 women with an average age of 67.2 years.

Exclusion criteria include cognitive impairment, inability to sign informed consent, an associated diagnosis of cancer and / or autoimmune disease, and a recent history of head or spinal cord injury.

To isolate and purify plasma RNA, we used the miRNeasy Serum / Plasma kit from Qiagen.

In order to identify microRNAs expressed differently in the screening group, we used the miScript® miRNA PCR Array kit from Qiagen.

For the validation and naive group we used a two-step qPCR technique, reverse-transcription and Real-Time PCR are performed in two separate steps, using specific TaqMan assays.

LUHMES human dopaminergic neuron cell precursors were used to differentiate dopaminergic neuronal cells, which were exposed for 24 hours to three different concentrations of LevoDopa (10 μ M, 20 μ M and 50 μ M).

We isolated and purified the microRNA and mRNA using the mirVana miRNA Isolation kit. Subsequently, we also used a two-step qPCR technique using specific TaqMan assays (Thermo Fisher Scientific).

All statistical calculations were performed using Prism 8 for MacOS, version 8.3.0.

RESULTS

In the screening step we evaluated a group of 10 patients diagnosed with Parkinson's disease vs. 10 control patients. We used a PCR Array technique and evaluated 1008 possible microRNA targets, of which only 15 were expressed differently in patients' plasmas vs. controls and showed corrected p values below the threshold of 0.05 with changes in expression level ranging from 1.5 to 5.1.

The validation step included a group of 66 patients diagnosed with BP and 29 controls. Of the microRNAs studied, 5 had a statistically significantly modified expression, namely: miR-16, miR-19a, miR-19b, miR-195, miR-92a. The expressions were increased, confirming the result obtained in the screening group. We analyzed a possible link between the Ct values of these microRNAs and the age of the patients, respectively the Y-H stage of the disease, but no correlation was identified. ROC analysis performed for the 5 microRNAs shows that miR-19a, miR-19b, miR-195 have the largest areas under the curve (AUC), having the ability to distinguish Parkinson's patients from controls, instead miR-16 and miR-92a shows more modest results.

We performed stratification by sex for the 5 microRNAs and of these, only miR-19a, miR-19b, miR-195 have a statistically significant increased expression in both men and women. There were no significant differences between the miR-19a, miR-19b and miR-195 plasma levels of control men compared to control women. miR-19a and miR-19b have significantly lower plasma values in male patients with BP compared to women with BP.

Because the patients in the validation group were being treated with LevoDopa, we wondered if there was a correlation between the treatment given and the level of microRNA expression. In this regard, we used a third batch in which we evaluated the expression miR-19a, miR-19b, miR-195 in plasma from patients before and after treatment with LevoDopa, and the results indicate that a single microRNA, namely miR-19b showed statistically significantly increased expression.

To further demonstrate the direct effect of LevoDopa in modulating miR-195 / miR-19a / miR-19b expression, we monitored the response of dopaminergic neurons derived from LUHMES progenitor cells at different concentrations of LevoDopa. Dopaminergic neurons were exposed to three different concentrations of LevoDopa (10uM, 20uM and 50uM) for 24 hours, then washed with 1x PBS, collected and archived at -80 * C. Following the analysis, the results show a significant increase in miR-19b expression correlated with an increase in the dose administered. In contrast, the expression miR-19a and miR-195 remained unchanged or decreased.

To obtain a perspective on the biological role of miR-19b we tried to identify its possible targets by combining several analytical techniques: prediction of targets using mirWalk algorithms, study of complex networks, and interrogation of transcripts published in GEO (Gene Expression Omnibus).

Using the study of complex networks we tried to identify the signaling pathways targeted by miR-19b. The target genes (considering the interaction with the 3'UTR, 5'UTR regions and the CDS coding sequence) for miR-19b were predicted using the miRWalk 3.0 algorithm, with a $p \leq 0.05$ (adjusted Bonferroni) as limit. Then, using the Force Atlas 2 algorithm, a network of miR-19b target genes resulted. The genes were grouped topologically into 35 communities. The first 11 centrally clustered communities are the most numerous. DAVID analysis of genes in each community resulted in different signaling pathways, some known to be associated with Parkinson's disease, others not.

Another approach was to analyze published transcriptomic data from two experiments on a mouse model with progressive loss of striatal dopaminergic neurons induced by 6-hydroxydopamine (6-HODA). These mice were given two concentrations of LevoDopa: one lower and the other higher. We performed this analysis on this data set because 1) there are no human transcriptomics data to track the expression level of disordered genes depending on the level of LevoDopa and 2) mature miR-19b is extremely well preserved in evolution in vertebrates. Geo2R analysis (FDR < 0.05 , with Benjamin-Hochberg adjustment) of the murine model of hemiparkinsonian involvement under LevoDopa therapy identified 5722 (high LevoDopa regimen) and 2999 (low LevoDopa regimen) differentially expressed unique genes (GED), of which 651 (11.38%) and 378 (12.61%), respectively, are possible 5'-UTR / CDS / 3'-UTR targets of miR-19b (according to the miRwalk3.0 algorithm). KEGG ontological analysis (FDR < 0.05) of miR-19b targets using the DAVID 6.8 platform identified Ubiquitin-mediated proteolysis (UDP) as the target of miR-19b expression disorder following LevoDopa supplementation.

Next, we asked whether changes in the UDP genes identified in the murine model are found in human transcriptomic data stored in GEO. In this regard, we analyzed 22 sets of transcriptomic data (Geo2R analysis, FDR < 0.05) tracking the expression levels for the 16 UDP target genes of miR-19b.

All data sets are consistent in decreasing the expression ANAPC1 (in 72.7% of data sets), UBE2D3 (68.2%), CUL2, CUL3, and KEAP1 (63.6%), while FBXW8 and UBE2J2 are downregulated only in 22.7% of the analyzed data sets.

From these possibly targeted miR-19b genes, we selected 5 of them (CUL2, CUL3, UBE2D3, WWP1, ANAPC1) that had the lowest expressions and analyzed them at the transcriptomic level in dopaminergic neuronal cells exposed to different concentrations of levodopa. We obtained low expressions for CUL2, CUL3 and UBE.

Using complex network analysis techniques, we set out to try to reposition some drugs in Parkinson's disease. The network formed contains 1008 nodes / drugs that were analyzed and grouped into 26 topological communities labeled according to the dominant pharmacological property. Of the total nodes in the network, 59.52% are medicines whose property corresponds to that of the corresponding community confirmed by the new version of DrugBank 5.1.4, 26.98% are medicines confirmed by the literature and 13.49% are unconfirmed medicines, possible repositioning

Community 20 corresponds to Antidepressant and Antiparkinsonian drugs and includes 21 drugs, most of which are confirmed (57.14% by DrugBank and 14.29% by literature), and 28.57% are possible repositioning, of which I mention Quinidine, Propafenone, Chichocaine, MDMA and Aprindina. It should be mentioned that Propafenone and Quinidine are proposed for repositioning because they have the highest b / d values.

DISCUSSION

In the literature there is a lack of consensus regarding changes in the expression of circulating microRNAs, depending on their origin: plasma, serum or mononuclear peripheral cells from Parkinson's patients. These differences reflect not only the tissue source, the size of the analyzed batches, the experimental design (from RNA isolation to the validation method), but also (as our data show) the level of individual responses to different doses of levodopa.

Most studies that investigated serum microRNA levels (including exosomes), peripheral mononuclear cells, and CSF showed a decrease in miR-19b levels in patients with PD compared to control groups. In contrast, plasma microRNA analysis demonstrated elevated levels of miR-19b in patients with Parkinson's versus healthy controls, but also versus multisystem atrophy. Interestingly, the overall analysis of plasma exosomes did not identify differences in miR-19b expression, which may lead to the exclusion of exosomes as factors contributing to miR-19b associated changes in PD.

Regarding miR-19a and miR-195 expressions, the studies show increased expressions for miR-19a in the cerebrospinal fluid, and for miR-195 also an overexpression in serum. These results are consistent with our data.

It is also worth noting that, with very few exceptions, naturally enriched microRNAs in the brain have elevated plasma levels in patients with PD, suggesting that the tissue source of microRNA is a major factor in influencing plasma levels in pathological conditions. The scenarios that may explain the increased level of microRNA are: increased transcription by various factors that may act at the level of gene promoters, which will translate into an increased level of pri-miR; increased microRNA stability, which is not necessarily accompanied by increased pri-miR, but could be associated with increased levels of proteins that help maintain stability, an example being AGO; decreased microRNA degradation may be related to decreased levels of long noncoding RNAs that have the ability to interact with microRNAs. Identifying the mechanisms that lead to increased microRNA levels requires additional complex experiments.

Studies investigating the response of microRNA to LevoDopa therapy in Parkinson's disease have used a targeted approach to microRNA expression in peripheral blood cells. However, the results show the same lack of disconcerting consensus: Alieva et al. described a significant increase in miR-7, miR-9-3p, miR-9-5p, miR-129 and miR-132, Serafin et al. showed overexpression of miR-103a, miR-30b and miR-29a, while Caggiu et al. found an increase in miR-155 and a decrease in miR-146 in peripheral mononuclear cells in Parkinson's patients treated with LevoDopa. Notably, with the exception of miR-146, all microRNAs analyzed have increased expression, including microRNAs found in the brain, namely miR-7 and miR-132.

We did not find any correlation between any of the five plasma microRNAs and the age or H&Y stage of patients with BP. Male gender has been highlighted as one of the risk factors for the development of BP. Interestingly, the sex-dependent differential expression of miR-16 in plasma is lost in patients with BP, while miR-19a and miR-19b remain sex-dependent. It should also be noted that miR-19a and miR-19b are considered microRNAs "specific to men", while miR-195 is rather specific to women.

A large amount of evidence has shown that Dopamine is a neuronal toxicant and can cause neuronal cell death, a dose-dependent, time-dependent effect associated with altered proteasome activity mediated by oxidative stress.

The proteasome is essential for neuronal survival, and the reduction in its activity has been correlated with aging and the onset of neurodegenerative diseases. The accumulation of protein aggregates in stressed neurons is normally counteracted by ubiquitin-dependent proteolysis (UDP), whose overloading leads to an increase in oxidative stress, a reduction in mitochondrial bioenergetics and neuronal death. Oxidative stress affects ubiquitination-dependent proteolysis by oxidizing UDP components and, specifically, by inhibiting the activity of the 26 proteasome subunit, S-glutathylation and S-nitrosylation of ubiquitin ligands. Notably, α -synuclein can be eliminated independently of ubiquitination, by direct endo-proteolytic activity of the 20S proteasome. To complicate the problem, α -synuclein aggregates resulting from UDP alteration further affect proteasome activity in a biunivocal relationship that leads to neuronal death.

Analysis of transcriptomic data from PD shows a lack of consensus regarding the level of differentially expressed genes in all tissues; however, despite the intra- and inter-experimental heterogeneity of the analyzed cell populations, concordance improves when transcriptomic analysis focuses on modified signaling pathways. Proteasome activity has been reported to be altered in transcriptomic studies in various regions of the brain (including the extra-nigral level), blood, or CSF, indicating a systemic change in this pathway in PD. Our data suggest that this change may actually reflect the response of different tissues to LevoDopa therapy by modulating the level of microRNA expression.

The relationship between microRNA and UDP has been explored in multiple experimental and clinical contexts *in vivo* and *ex vivo*, including the heart, osteosarcoma cells, and neurodegenerative diseases. Various components of the UDP system have been described as microRNA targets, including: UE2A, UBE2B, UBE2D3 and UBCH10 isoforms targeted by miR-7, miR-455-5p, miR-21-5p and miR-631, respectively. Parkin-associated E3-ubiquitin ligase BP is targeted by miR-181a in senescent muscles. MiR-103a-3p levels are elevated in an *in vitro* and *in vivo* MPTP model of Parkinson's disease, miR-146a is overexpressed in a rotenone-induced neurodegeneration pattern, and miR-218 levels are elevated in HEK293 cells. Our data suggest that LevoDopa alters UDP activity in dopaminergic neurons by altering the activity of the E2 Ubiquitin / E2 Ubiquitin-conjugating enzyme, the E3 Ubiquitin ligase system, and the Cullin-2, -3, and -7 complexes, mediated by miR-19b-mediated growth.

Although we show that dopaminergic neurons respond to LevoDopa by increasing the level of mature miR-19b, the exact source of plasma miR-19b in patients treated with LevoDopa is far from clear. However, subject to validation in larger and more heterogeneous groups, we believe that miR-19b could be used as a response biomarker in LevoDopa therapy, opening a new path in clinical research. Another open question concerns the role of this circulating miR in the progression of PD under substitution therapy. In this regard, buffering the increase in miR-19b expression could prove to be an effective way to prevent or at least reduce the neurotoxicity associated with LevoDopa.

MiR-19b targets detected by complex network analysis

Signaling pathways resulting from the analysis of the network of possible target genes of miR-19b have been searched in the literature to correlate with Parkinson's disease.

From community 1 pathways Focal adhesion, Phosphatidylinositol signaling system. Chemokine signaling pathways have been reported by other groups as being associated with Parkinson's disease, and in community 2 only the cell cycle signaling pathway.

Shehadeh et al. shows that Serine / Arginine Repetitive Matrix 2 (SRRM2), which is a component of the spliceosome, is increased in the blood of patients with BP, so in community 3 the only signaling pathway that is associated with BP is the Spliceosome pathway.

In community 4, of the four signaling pathways identified, only 2 were associated with Parkinson's disease, namely Metabolic pathways (also found in community 9) and Valine, leucine and isoleucine degradation found in the literature as Amino acid metabolism.

Community 6 is represented only by Ubiquitin mediated proteolysis, this being one of the most frequently cited pathways in the literature as being associated with BP.

Complex network analysis in drug repurposing

The network contains a large number of confirmed drugs with specific properties in the communities they belong to (57.14%); in addition, another 14.29% of drugs have been validated by new literature studies, so there is an increased likelihood that those proposed for repositioning will have new properties.

If we analyze the communities separately, the number 16 that contains antidepressants and CNS stimulants (a community close to the one that contains antiparkinsonian drugs) contains all drugs confirmed 92.31% by DrugBank and 7.69% by the literature which is encouraging for the validation of drugs in the community 20 antidepressant and antiparkinsonian drugs. Other examples of communities where all drugs are validated are: 2 antihypertensives, 14 anticholinergics, 26 hypnotics and sedatives. In addition, of the 14 drugs proposed for repositioning, azelaic acid and meprobamate were studied by molecular docking methods that show the possibility of repositioning in anticancer and antifungal.

Repositioning in Parkinson's has also been proposed in the literature, and an interesting example is that of Ayoub et al., Who propose an antidiabetic agent as a possible reuse. Omarigliptin is the first in the class of gliptinins that has the ability to cross the blood-brain barrier with possible neuroprotective action.

There is also a study in the literature linking Parkinson's to quinidine, but in this case the drug is used in combination with dextromethorphan to treat levodopa-induced dyskinesia in Parkinson's patients.

So, our repositioning proposals as antidepressant / antiparkinsonian agents are quinidine and propafenone, two antiarrhythmic drugs.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

We tried to identify microRNAs associated with Parkinson's disease because they have a number of advantages (stability in biological fluids and can be quantified using fairly fast methods) over protein biomarkers. In addition, the determination of alpha-synuclein proposed for the diagnosis of Parkinson's is made from cerebrospinal fluid, and its collection requires specialized personnel and a dedicated hospital environment.

Unfortunately, regarding the expression of microRNA in Parkinson's disease, the literature presents divergent data, due to the influence of the source tissue, the circulating

environment (serum, plasma or mononuclear peripheral cells). Some of these data are consistent with our results, but others are not.

Two-step analysis (screening and validation) of plasma microRNAs as possible biomarkers in Parkinson's disease led to the identification of only 5 microRNAs (miR-16, miR-19a, miR-19b, miR-195, miR-92a) whose expression is statistically significant increase. None of these correlate with the age or Y-H stage of patients.

Of the five microRNAs, only miR-19a, miR-19b, miR-195 remain significantly altered in both men and women. ROC analysis shows that all three microRNAs, miR-19a, miR-19b, miR-195 have the ability to differentiate Parkinson's patients from controls, having the largest areas under the curve, compared to miR-16 and miR-92a of the whose results are more modest.

Comparing our results with those in the literature, a concordance can be observed for miR-19a and miR-195, but this is not maintained in the case of miR-19b. In the literature, both miR-19a (cerebrospinal fluid) and miR-195 (serum) have increased BP expressions, whereas for miR-19b the data are contradictory. Most have low expressions in cerebrospinal fluid, serum, mononuclear peripheral cells, while Burgos et al. identified elevated expressions for miR-19b in cerebrospinal fluid, and Uwatoko and colleagues show increased plasma expression in patients with PD.

Evaluation of the plasma expression of miR-19a, miR-19b, miR-195 in samples from patients before and after treatment with levodopa shows that only miR-19b showed statistically significant increased expression.

Thus, we propose miR-19a and miR-195 as having a possible role in PD biology, and miR-19b could be considered as a response to levodopa therapy.

To the best of our knowledge, except for Margis' study of cumulative whole blood samples from only 4 patients before and after treatment, we performed the first study that individually investigated plasma from the same patients before and after levodopa treatment. All other data regarding the microRNA response to levodopa therapy are performed on different patient groups: Serafin et al. (miR-103a, miR-30b and miR-29a), Alieva et al. (miR-7, miR-9-3p, miR-9-5p, miR-129 and miR-132), Caggiu et al. (miR-155 and miR-146). In these studies, with the exception of miR-146, all microRNAs analyzed had increased expression.

To demonstrate the possible role of levodopa in modulating the expression of miR-19b, miR-195, miR-19a, we studied the response of dopaminergic neurons in culture at different concentrations of levodopa. We found a gradual decrease in miR-19a and miR-195 expression with increasing levodopa concentration, while miR-19b had a significant increase in expression depending on the increase of the administered dose, this aspect being consistent and confirming the results from the naive group.

In order to obtain a perspective on the biological role of miR-19b we made the prediction of its possible targets (miRWalk analysis); these were studied with the help of complex networks and were clustered in several communities, each corresponding to one or more signaling pathways associated or not with PD. But unfortunately in this case the results were not encouraging, except for the UDP pathway, a pathway containing genes validated in PD by other authors.

Another approach to revealing the biological role of miR-19b in Parkinson's was to identify the target genes of miR-19b (miRWalk analysis) from the set of genes expressed in an experimental model of parkinsonian mice treated with levodopa. Prediction of signaling pathways indicated as the main UDP pathway, with 16 target genes, 2 of which were validated as miR-19 targets, namely UBE2D3 and TRIM37.

This transcriptomic analysis was validated in LevoDopa-treated cells, where we found significantly lower expressions for UBE2D3, CUL2, CUL3. It is plausible that LevoDopa-induced

miR-19b is a new modulator of the UDP pathway by altering the activity of the E2 Ubiquitin / E2 Ubiquitin-conjugating enzyme, the E3 Ubiquitin ligase system, and the Cullin-2, -3, and -7 complexes.

Another goal was to reposition some drugs in BP using a complex network-based algorithm. We obtained Quinidine and Propofol (two antiarrhythmics) as possible useful drugs in Parkinson's therapy. This is a pure *in silico* study that, although with strong arguments in favor of repositioning, needs experimental validation. To our knowledge, this is the first time that these drugs are proposed as antiparkinsonian.

In conclusion, we propose miR-19a, miR-195 as being involved in the biology of Parkinson's disease, while miR-19b could serve as a response biomarker in LevoDopa therapy, opening a new path in clinical research.