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Upgraded Methodology for the Development of Early Diagnosis of Parkinson's Disease Based on Searching Blood Markers in Patients and Experimental Models

Alexander Kim[1] · Razina Nigmatullina[2] · Zuleikha Zalyalova[2,3] · Natalia Soshnikova[4] · Alexey Krasnov[4] · Nadezhda Vorobyeva[4] · Sofia Georgieva[4,5] · Vladimir Kudrin[6] · Viktor Narkevich[6] · Michael Ugrumov[1,7]

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Abstract

Numerous attempts to develop an early diagnosis of Parkinson's disease (PD) by searching biomarkers in biological fluids were unsuccessful. The drawback of this methodology is searching markers in patients at the clinical stage without guarantee that they are also characteristic of either preclinical stage or prodromal stage (preclinical–prodromal stage). We attempted to upgrade this methodology by selecting only markers that are found both in patients and in PD animal models. HPLC and RT-PCR were used to estimate the concentration of amino acids, catecholamines/metabolites in plasma and gene expression in lymphocytes in 36 untreated early-stage PD patients and 52 controls, and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice at modeling the clinical ("symptomatic") stage and preclinical–prodromal ("presymptomatic") stage of PD. It was shown that among 13 blood markers found in patients, 7 markers are characteristic of parkinsonian symptomatic mice and 3 markers of both symptomatic and presymptomatic mice. According to our suggestion, the detection of the same marker in patients and symptomatic animals indicates adequate reproduction of pathogenesis along the corresponding metabolic pathway, whereas the detection of the same marker in presymptomatic animals indicates its specificity for preclinical–prodromal stage. This means that the minority of markers found in patients—decreased concentration of L-3,4-dihydroxyphenylalanine (L-DOPA) and dihydroxyphenylacetic acid (DOPAC) and increased dopamine D3 receptor gene expression—are specific for preclinical–prodromal stage and are suitable for early diagnosis of PD. Thus, we upgraded a current methodology for development of early diagnosis of PD by searching blood markers not only in patients but also in parkinsonian animals.

Keywords Parkinson's disease · Early diagnosis · Animal model · Blood markers

Introduction

Parkinson's disease (PD), characterized by movement disorders, is among the most severe neurodegenerative diseases. Development of PD proceeds in three stages, preclinical, prodromal, and clinical, which are characterized by the onset of neurodegeneration in the absence of symptoms, the presence of non-motor symptoms, and the appearance of motor symptoms, respectively [1–3]. The diagnosis of PD at the clinical stage is largely based on the presence of bradykinesia and one other motor feature, either tremor or rigidity [2, 3]. Noteworthy is that motor symptoms first appear many years after the onset of neurodegeneration, at a loss of 70–80% dopamine in the striatum, and after the depletion of neuroplasticity, which explains the low efficiency of current therapy [4, 5]. This highlights the need to develop an early diagnostics of PD, long before the onset of motor symptoms, and to use the neuroprotective therapy for slowing down neurodegeneration [6–8].

✉ Michael Ugrumov
michael.ugrumov@mail.ru

1 Koltzov Institute of Developmental Biology, Russian Academy of Sciences, Moscow, Russia

2 Kazan State Medical University, Ministry of Health of the Russian Federation, Kazan, Russia

3 Kazan Hospital for War Veterans, Ministry of Health of the Republic of Tatarstan, Kazan, Russia

4 Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia

5 Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia

6 Zakusov Institute of Pharmacology, Moscow, Russia

7 National Research University Higher School of Economics, Moscow, Russia

PD is a systemic disease in which neurodegeneration begins in some brain areas and in the peripheral nervous systems earlier than in the nigrostriatal system [9, 10]. This leads not only to an initial impairment of some central and peripheral non-motor functions but also to changes in body fluids [11–13]. Although PD can be diagnosed with positron-emission tomography at the prodromal stage [14, 15], this technique, apparently, will never be used for preventive examination of population because of a high cost and low availability [16]. Therefore, current methodology for the development of early diagnosis of PD is mainly based on a retrospective evaluation of early non-motor symptoms in medical history and the search for changes in body fluids (CSF, blood) in untreated patients at the early clinical stage [17–19]. However, this methodology is questionable because (i) there is no evidence that the markers found in body fluids of PD patients at the clinical stage are also characteristic of either preclinical or prodromal stage, and (ii) all markers (non-motor symptoms, changes in biological fluids) found so far are unspecific [10, 13]. This explains why, despite the detection of dozens markers in untreated patients at an early clinical stage, the diagnostics of PD before the onset of motor disorders still is not available.

Proceeding from the above, the goal of this study was to upgrade the current methodology for the development of early diagnosis of PD—before the appearance of motor symptoms, by searching for markers in the blood, not only in untreated patients at the early clinical stage, but also in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice at the modeling of the presymptomatic stage and the symptomatic stage—before and after the onset of motor disorders, respectively. When searching for markers in biological fluids in PD patients at the clinical stage, we proceeded from the fact that it is impossible to distinguish potential markers of the preclinical stage and the prodromal stage [1, 20]. Therefore, these markers are designated in this paper as potential markers of the preclinical–prodromal stage. Specifically, we attempted to define changes in the plasma concentration of a number of amino acids, catecholamines, and metabolites, as well as in the expression of certain genes in lymphocytes. Catecholamines and selected amino acids are secreted by neurons that undergo degeneration at PD. In turn, lymphocytes sensitive to a number of physiologically active substances, including neurotransmitters [21–23], change the expression of certain genes when the environment (plasma) changes [23].

The objectives were (i) to identify the above markers in the blood of untreated patients at the early clinical stage, (ii) to identify the above markers in MPTP-treated mice at an early symptomatic stage and select only those markers that are characteristic of patients as evidence of adequate modeling of the pathogenesis of PD at least along this particular metabolic pathway, and (iii) to identify the above markers in MPTP-treated mice at the presymptomatic stage and select only those markers that are characteristic of patients and MPTP-treated mice at the symptomatic stage. In this paper, we present updated evidence on already known markers, as well as new markers, and believe that only those markers, which are found in PD patients, symptomatic and presymptomatic mice can be used to diagnose PD at preclinical–prodromal stage.

Materials and Methods

Subjects (Patients, Controls)

Blood samples, obtained from PD patients and control subjects at the Kazan Hospital of War Veterans (Kazan, Russia) with the assistance of the Republican Clinical Diagnostic Centre for Extrapyramidal Pathology and Botulinum Therapy (Kazan, Russia), were used for searching PD markers in the blood. All donors gave written informed consent, and the study procedure was approved by the Research Ethics Committee of the Republic of Tatarstan. Patients and control subjects were selected according to the criteria described in Table 1. PD was diagnosed according to the UK PD Society Brain Bank clinical diagnostic criteria [24] and the recent document "Clinical Diagnostic Criteria for Parkinson's Disease" of the Movement Disorder Society [2]. The clinical diagnosis of PD has centered on a definition of motor syndrome as bradykinesia, in combination with either rest tremor, rigidity, or both, and its unilateral manifestation [2]. The group of age-matching controls was cleared not to have neurological diseases under careful examination. Most important clinical characteristics of PD patients ($n = 36$) and control subjects ($n = 52$) are presented in Tables 1 and 2.

Blood Sampling and Preliminary Processing

Ten milliliters of blood was collected from the cubital vein of patients with PD and control subjects at 9.00 before morning meal. For HPLC assay of catecholamines and amino acids, blood samples were collected in plastic tubes with 0.1% EDTA (Sigma, USA) and 0.1% sodium metabisulfite (final concentrations) (Sigma), shaken and centrifuged for 20 min at 2000×*g* and 4 °C. The supernatant was frozen in liquid nitrogen and stored at − 70 °C. To assess gene expression in lymphocytes by real-time PCR, 1 blood volume was incubated in 5–10 volumes of lysis buffer (10 mM $NaHCO_3$, 80 mM NH_4Cl, 0.01% acetic acid, pH 7.2; Sigma) for 10 min at 20 °C. Then, lymphocytes were precipitated by centrifugation at 2000×*g* for 10 min at 4 °C. The pellet was suspended in 1 ml Trizol buffer (Invitrogen, USA), frozen in liquid nitrogen, and maintained at − 70 °C until RNA extraction.

Animals

We used 90 male mice C57BL/6 at the age 2–2.5 months (22–26 g), purchased in the "Pushchino" SPF animal facility (MO,

Table 1 Inclusion and exclusion criteria of patients with Parkinson's disease and control subjects

No.	Criteria	PD patients	Control
1.	Idiopathic Parkinson's disease	+	–
2.	Secondary parkinsonism	–	–
3.	Other extrapyramidal diseases	–	–
4.	Other neurological diseases	–	–
5.	Psychiatric disorders (dementia, etc.)	–	–
6.	Endocrine diseases	–	–
7.	Stroke and trauma over the past 2 years	–	–
8.	Somatic symptom disorders	–	–
9.	Neoplasms, including malignant tumors	–	–
10.	Specific antiparkinsonian therapy (levodopa, dopamine receptor agonists, MAO-A and B inhibitors, amantadine, etc.)	–	–
11.	Antagonists of dopamine receptors (metoclopramide, domperidone, cinnarizine, etc.)	–	–
12.	Sympatholytics (reserpine)	–	–
13.	Agonists and antagonists of serotonin and adenosine receptors	–	–
14.	Providing with informed consent	+	+

Russia). Animals were kept at 21–23 °C in a light–dark 12-h cycle at free access to food and tap water. PD at the clinical ("symptomatic") stage and preclinical–prodromal ("presymptomatic") stage ($n = 30$) was modeled in mice by subcutaneous administration of MPTP (Sigma) twice at the individual dose of 8 mg/kg ($n = 30$) or four times at the dose of 10 mg/kg ($n = 30$), respectively, with a 2-h interval between injections. According to our preliminary data, there are no differences in selected blood parameters in mice that received saline subcutaneously two, three, or four times with a 2-h interval between injections. Therefore, only mice receiving saline three times were used as a control ($n = 30$). No premature death of mice was observed in the experiments.

The animals were sacrificed 2 weeks following the injections of MPTP. At this time, the dopamine content decreases in the striatum of presymptomatic and symptomatic mice by 55 and 80%, respectively [25]. The blood of MPTP-treated and control mice was collected via decapitation under isoflurane anesthesia. Plasma was processed for HPLC assay of catecholamines and metabolites (8 animals per group) and amino acids (10 animals per group) and lymphocytes—for real-time PCR (12 animals per group) as described above. All experimental procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Institute of Developmental Biology RAS.

HPLC Assay of Catecholamines and Metabolites in Plasma

Catecholamines and metabolites were measured in plasma by HPLC with electrochemical detection. Frozen samples of plasma were thawed and added with internal standard 3,4-dihydroxybenzylamine in 0.1 N $HClO_4$ (Sigma) to a final concentration of 250 pmol/ml. After centrifugation at 20,000×*g* for 20 min at 4 °C, the supernatant was collected and norepinephrine, epinephrine, dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC with electrochemical detection.

Table 2 Characteristics of cohorts of patients with Parkinson's disease and control subjects used for blood collection and assay (plasma and cells)

Subjects used for specific blood assay		Cohort	Number	Mean age (years)	Sex (m/f)	Hoehn and Yahr scale	UPDRS score	Disease duration (years)
Blood assay	HPLC: catecholamines, metabolites	PD	20	59.2 ± 1.6	10/10	1.4 ± 0.1	16.6 ± 1.3	1.7 ± 0.3
		Control	23	60.7 ± 1.6	6/17	–	–	–
	HPLC: amino acids	PD	17	60.5 ± 2.2	8/9	1.4 ± 0.1	17.5 ± 2.1	1.5 ± 0.3
		Control	29	61.3 ± 1.3	7/22	–	–	–
	RT-PCR: gene expression in lymphocytes	PD	19	59.3 ± 1.7	9/10	1.6 ± 0.1	21.3 ± 2.0	2.2 ± 0.3
		Control	20	55.6 ± 1.9	3/17	–	–	–

f female, *HPLC* high-performance liquid chromatography, *m* male, *N* number of subjects, *PD* Parkinson's disease, *RT-PCR* real-time polymerase chain reaction, *UPDRS* unified Parkinson's disease rating scale

The analytes were separated on 100 × 4 mm column ReproSil-Pur C18, 3 μm (Dr. Maisch, Germany) at 30 °C. The mobile phase consisted of 0.1 M citrate–phosphate buffer (pH 2.3) with 0.3 mM sodium octanesulfonate, 0.1 mM EDTA, and 8% acetonitrile (all chemicals—Sigma). A flow rate of 0.8 ml/min was provided by a LC-20ADsp HPLC system (Shimadzu, Japan). Electrochemical detector DECADE II (Antec, the Netherlands) was equipped with a glass carbon flow cell and salt-bridge Ag/AgCl reference electrode with the potential set at + 0.85 V. The peaks of catecholamines and metabolites were determined from the elution time in a standard solution. Concentrations of analytes were estimated with the LabSolutions software (Shimadzu) as a ratio of the peak area of the internal standard and that of the sample.

HPLC Assay of Amino Acids in Plasma

Inhibitory neurotransmitter amino acids—taurine, glycine, gamma-aminobutyric acid (GABA), and excitatory neurotransmitter amino acids—aspartate and glutamate were measured in plasma by HPLC with fluorescence detection according to modified protocol [26]. Frozen plasma samples were thawed and added with 0.1 M borate buffer (pH 9.5) and orthophthaldehyde in 1:1:0.4 ratio for amino acids derivatization for 20 min at room temperature. Amino acids were separated at a 250 × 4.6-mm column Hypersil ODS, 5 μm (Thermo, USA), at 25 °C with a flow rate of 1.5 ml/min. The mobile phase consisted of 0.06 M $NaH_2PO_4H_2O$, 0.0032 M Na_2HPO_4, 0.025 mM EDTA, and 1.24 mM CH_3OH, pH 5.6 (all chemicals—Sigma). The fluorescence was detected by the Agilent 1100 detector (Agilent, USA) with excitation/emission wavelengths of 230/392 nm. The peaks of derivatized amino acids were identified by the elution time in standard solution. Concentrations of analytes were estimated by the external standard method with the ChemStation software (Agilent).

Real-Time PCR in Lymphocytes

Real-time PCR was used to assess the expression of the gene encoding the D3 receptor to dopamine (DRD3) and the expression of the PHF10-P and PHF10-S—two alternative transcripts of the PHF10 gene-encoding plant homeodomain finger protein 10. Total RNA was isolated from the frozen samples with Trizol (Thermo) according to the supplier's protocol. Single-strand cDNA was synthesized from 3 μg total RNA using RevertAid Synthesis Kit (Thermo) according to the supplier's protocol. Gene expression was estimated by real-time PCR using the Eurogen qPCR Kit (Eurogen, Russia). The primers used are listed below:

DRD3 (human)	For_5′-TCAGCAAGACAGGATCTTGAGGAAGG; rev_5′-GAGAAGAAGGCAACCCAAATGGTGG
DRD3 (mice):	For_5′-CCGTGGGTGGTGTACTTGGAG; rev_5′-GATGGCACAGAGGTTCAGGATG
PHF10-P (human)	For_5′-CCAGGGAAGACAGAAATCAAAAGAC; rev_5′-CCATTGTCATATCCAGGCAAGAAGG
PHF10-P (mice)	For_5′-GGACACCTTCCACGGAAGACAG; rev_5′-CATATCCAAGCAAGAAGGGTGGC
PHF10-S (human)	For_5′-CCAGGGAAGACAGAAATCAAAAGAC; rev_5′-CAGGGGCTTTTTTCTTCTACCTTG
PHF10-S (mice)	For_5′-GGACACCTTCCACGGAAGACAG; rev_5′-CAGGGGCTTTTTTCTTCTACCTTG

For standardization of the reaction, the content of ENY2 mRNA detected with two primers (same for human and mice), for_5′-CCAGGGAAGACAGAAATCAAAAGAC and rev_5′-GGAGTGATTTCAGCCACCAAGTC, was estimated. The mRNA level was calculated using the $2^{-\Delta\Delta C(t)}$ method [27].

Statistical Analysis

HPLC and PCR data obtained in patients and mice are expressed as the mean ± standard error of the mean. Normality of the data was tested with Shapiro–Wilk criterion. For statistical comparison between the male/female data, PD patients/controls, and control/experimental mice, Student's t test or Mann–Whitney U test was used for parametric and non-parametric data, respectively. Correlation of HPLC and PCR data in PD patients with Hoehn and Yahr and UPDRS scale scores was assessed by the Pearson's (for parametric data) or Spearman's (for non-parametric data) tests. Moreover, an analysis of the receiver operator characteristic (ROC) was performed to assess the diagnostic efficiency of biomarkers found in PD patients. The optimal cutoff values of sensitivity and specificity were determined using the Youden index. In all analyses, all p values were two-tailed and $p < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism 6.0 (GraphPad Software, USA).

Results

Changes in the Blood in PD Patients Compared with Controls

The concentration of catecholamines (norepinephrine, epinephrine, and dopamine), as well as their metabolites (L-DOPA and DOPAC), was significantly lower in PD patients compared to the control group (Fig. 1a). In turn, the concentration of aspartate, GABA, glycine, and taurine in plasma in patients with PD was significantly higher than in control subjects (Fig. 1b). Conversely, the concentration of glutamate in plasma was lower in patients compared to the control subjects (Fig. 1b). The expression of genes of the DRD3 and PHF10-P and PHF10-S in lymphocytes decreased significantly in PD patients compared to the control (Fig. 1c). There were no gender differences observed when evaluating the blood markers, except for the expression of the DRD3 gene in PD patients group (Table 3). Nevertheless, DRD3 gene expression was decreased both in men and in women with PD in comparison to the control group.

ROC analysis was performed to assess the diagnostic accuracy of markers found in this study. The area under the ROC curve (AUC) for most markers was 0.8 or higher showing good ability to discriminate PD patients and controls (Fig. 2). However, analysis of the sensitivity and specificity values at the selected cutoff points showed that levels of epinephrine and dopamine are characterized by the high (> 75%) sensitivity, but low (< 60%) specificity (Fig. 2). Conversely, such markers as the levels of DOPAC and DRD3 expression have low (< 60%) sensitivity and very high (> 95%) specificity, whereas the majority of the rest is characterized by the sensitivity and specificity exceeding 75% (Fig. 2).

In addition, a correlation analysis was performed to determine if there is a correlation between blood markers and the severity of the disease in patients with PD. Two statistically significant correlations were found—between the concentration of L-DOPA in plasma and the Hoehn and Yahr scale and between the concentration of L-DOPA in plasma and the UPDRS scale (Table 4).

Changes in the Blood in MPTP-Treated Mice Compared with Controls

In view of the fact that gender differences in blood markers in patients with PD were practically not observed, on the one hand, and for men, there is approximately a 1.5-fold increased risk of PD, and on the other hand [2], only male mice were used for the search for blood markers. The concentration of norepinephrine and epinephrine was the same in plasma in MPTP-treated symptomatic and presymptomatic mice and in the control (Fig. 3a). The dopamine concentration in the control was the same as in symptomatic mice, but lower than in presymptomatic mice (Fig. 3a). The concentration of L-DOPA and DOPAC in

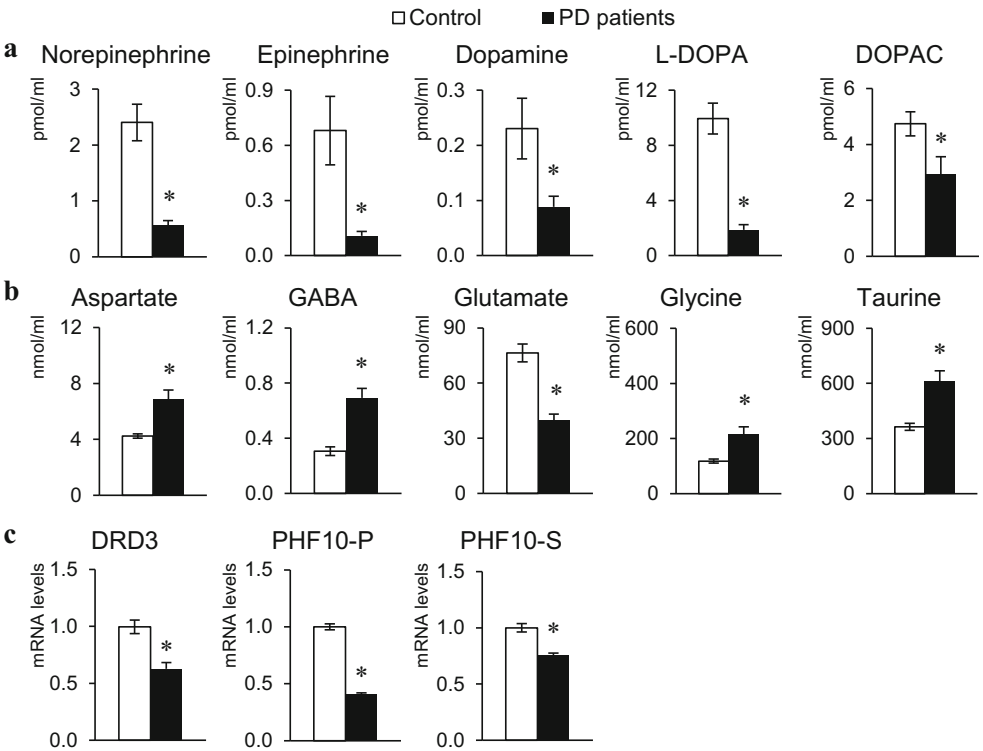

Fig. 1 The concentration of catecholamines and metabolites (**a**), amino acids (**b**) in plasma, as well as the expression levels of DRD3, PHF10-P, and PHF10-S genes in lymphocytes (**c**) in control subjects and PD patients. *$p < 0.05$

Table 3 Male and female data comparisons of the concentration of catecholamines and metabolites, amino acids in plasma, and gene expression levels in lymphocytes in control group and Parkinson's disease patients

Blood marker		Group					
		Control group			Parkinson's disease patients		
		Male	Female	*p* value	Male	Female	*p* value
pmol/ml	Norepinephrine	2.40 ± 0.76	2.41 ± 0.36	0.99	0.51 ± 0.08	0.64 ± 0.16	0.35
	Epinephrine	0.40 ± 0.13	0.79 ± 0.25	0.37	0.09 ± 0.03	0.13 ± 0.04	0.30
	Dopamine	0.20 ± 0.09	0.24 ± 0.07	0.77	0.09 ± 0.02	0.09 ± 0.03	0.78
	L-DOPA	8.31 ± 2.56	10.5 ± 1.24	0.40	1.57 ± 0.32	2.30 ± 0.62	0.24
	DOPAC	4.50 ± 1.08	4.83 ± 0.46	0.75	2.26 ± 0.86	3.75 ± 0.88	0.18
nmol/ml	Aspartate	4.35 ± 0.18	4.20 ± 0.20	0.68	6.97 ± 0.74	6.73 ± 1.19	0.87
	GABA	0.25 ± 0.05	0.32 ± 0.04	0.35	0.61 ± 0.08	0.75 ± 0.10	0.36
	Glutamate	76.5 ± 4.89	76.5 ± 6.33	1.00	36.5 ± 4.67	42.6 ± 5.09	0.39
	Glycine	93.2 ± 7.84	125.1 ± 9.1	0.11	198.8 ± 21	231.2 ± 48	0.57
	Taurine	345.0 ± 19	370.4 ± 25	0.58	567.7 ± 71	646.5 ± 94	0.53
Expression	DRD3	1.03 ± 0.20	1.00 ± 0.06	0.83	0.51 ± 0.07	0.73 ± 0.07	0.04*
	PHF10-P	0.96 ± 0.00	1.01 ± 0.03	0.50	0.40 ± 0.02	0.41 ± 0.01	0.63
	PHF10-S	1.01 ± 0.06	1.00 ± 0.04	0.95	0.76 ± 0.02	0.75 ± 0.03	0.77

Data is displayed as mean ± standard error of the mean

*$p < 0.05$

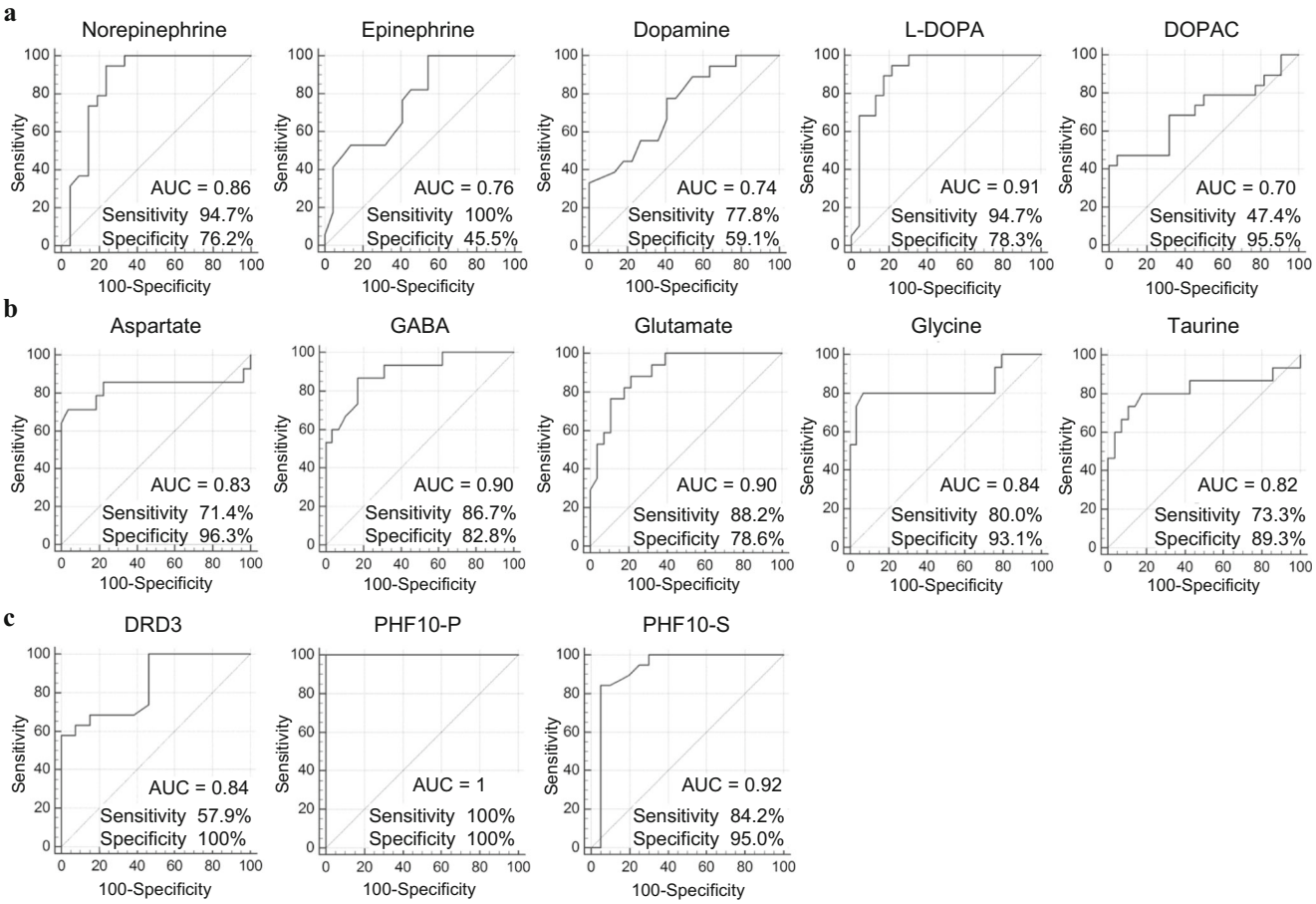

Fig. 2 ROC curves of biomarkers for discriminating PD patients from control group: catecholamines and metabolites in plasma (**a**), amino acids in plasma (**b**), and gene expression levels in lymphocytes (**c**). Area under the curve (AUC) and the percentage of sensitivity and specificity at cutoff points are indicated in each plot

Table 4 Correlation analysis between the blood markers and the Hoehn and Yahr and UPDRS scores

Parameter 2	Parameter 1			
	Hoehn and Yahr scale		UPDRS scale	
	Correlation coefficient, *r*	*p* value	Correlation coefficient, *r*	*p* value
Norepinephrine	0.14	0.56	0.12	0.63
Epinephrine	0.33	0.20	0.31	0.23
Dopamine	0.00	1.00	− 0.31	0.21
L-DOPA	0.49	0.03*	0.51	0.03*
DOPAC	0.38	0.11	0.44	0.06
Aspartate	0.40	0.16	0.31	0.33
GABA	0.19	0.50	0.36	0.23
Glutamate	− 0.13	0.62	0.06	0.84
Glycine	0.10	0.71	− 0.17	0.58
Taurine	0.16	0.56	0.17	0.57
DRD3	− 0.18	0.47	− 0.31	0.21
PHF10-P	0.19	0.44	0.27	0.29
PHF10-S	− 0.02	0.92	0.03	0.90

*$p < 0.05$

presymptomatic and symptomatic mice was lower than in the control, and only the DOPAC concentration was higher in presymptomatic mice than in symptomatic mice (Fig. 3a).

The concentration of aspartate and taurine in control mice was the same as in presymptomatic mice, but lower than in symptomatic mice (Fig. 3b). Conversely, the concentration of GABA in control mice was the same as in symptomatic mice but higher than in presymptomatic mice (Fig. 3b). The concentration of glutamate and glycine in plasma did not change in mice in both models of PD (Fig. 3b).

Gene expression of DRD3, PHF10-P, and PHF10-S in presymptomatic mice was the same as in the control, although there was a tendency ($p = 0.11$) to increase the DRD3 gene expression (Fig. 3c). In symptomatic mice, the expression of all studied genes was higher than in the control and presymptomatic mice (Fig. 3c).

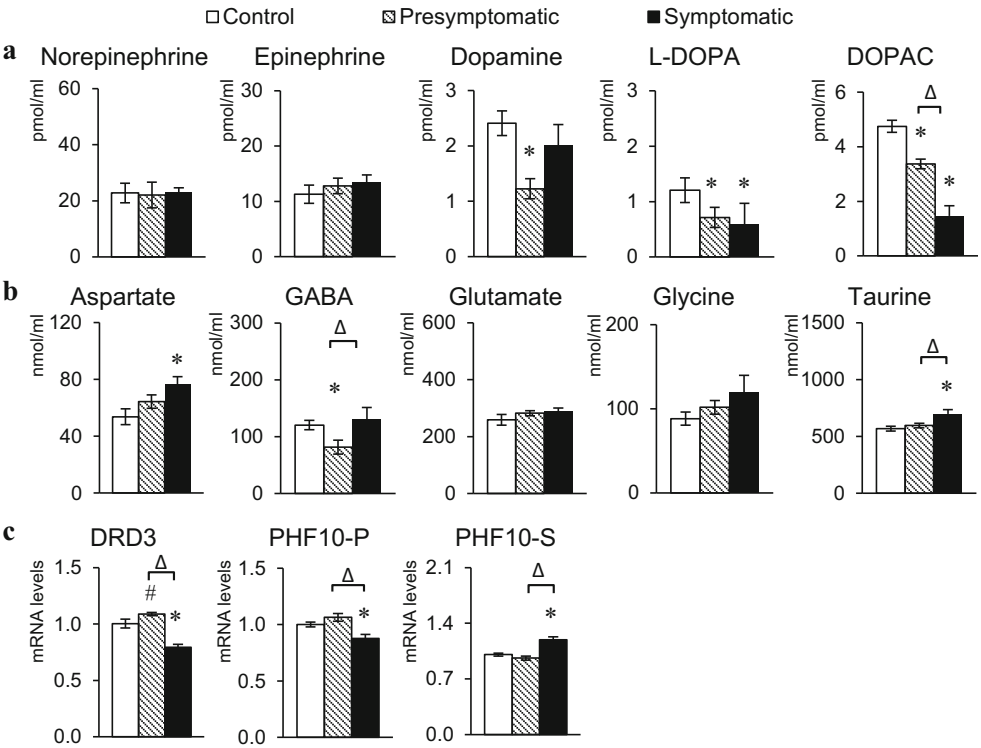

Fig. 3 The concentration of catecholamines and metabolites (**a**), amino acids (**b**) in plasma, as well as the expression levels of DRD3, PHF10-P, and PHF10-S genes in lymphocytes (**c**) in the control, presymptomatic, and symptomatic parkinsonian mice. *$p < 0.05$, #$p < 0.15$ compared to the control; Δ$p < 0.05$ between selected groups

Discussion

Basic Approaches to the Search for Peripheral Biomarkers in PD Patients

Although a search for biomarkers in body fluids in patients is a highlight of current methodology for the development of early diagnosis of PD, literature data are rather contradictory [28]. More reproducible data were obtained when detecting markers in the CSF than in blood that is apparently due to the lack of a barrier between the brain and cerebral ventricles for brain-derived substances [3, 28, 29]. Nevertheless, it is unlikely that the CSF will be used for preventive examination of population, since CSF sampling in humans is relatively expensive and invasive technique with a risk of complications [30]. Blood is more promising for this purpose because it is easily accessible and contains markers delivered from peripheral organs and the brain with a damaged blood–brain barrier in neurodegenerative diseases [31, 32]. In addition to the change in plasma, much attention is paid in literature to changes in blood cells, in particular lymphocytes [33, 34] and erythrocytes [35–38], which are also considered markers of PD.

Proceeding from the above, we searched for markers in the blood of untreated PD patients at an early clinical stage, assessing changes in the concentration of plasma components involved in the pathogenesis of Parkinson's disease (catecholamines/metabolites, amino acids–neurotransmitters) and gene expression in lymphocytes. A number of authors attempted to detect the changes in the concentration of some of these substances in the blood in PD patients, but their results are contradictory (Table 5). The efficacy of the markers found in this study for the separation of the control group and PD patients at the clinical stage was assessed using ROC analysis. Moreover, markers with high AUC and specificity values may in the future be used as ancillary indicators for the diagnosis of PD at the clinical stage [2].

Catecholamines and Metabolites in Plasma of Patients as Markers of PD

When measuring catecholamines and metabolites in the blood in PD patients with HPLC, we found a decrease in the concentration of norepinephrine, epinephrine, dopamine, L-DOPA, and DOPAC. The accuracy of the HPLC assay in our study was indirectly confirmed by the fact that the concentration of catecholamines and metabolites in the blood in the control group (see "Results") is similar to that found earlier in healthy people [39]. Noteworthy is that such marker as the decrease in the concentration of norepinephrine and L-DOPA in the blood has especially high AUC values exceeding 0.85, as well as high sensitivity and specificity at selected cutoff points.

Noteworthy is that previous data on the content of catecholamines and metabolites in the blood in PD patients are inconsistent (Table 5), showing (i) no change compared to the control [40–42], (ii) an increase in the content of some catecholamines and metabolites [43–45], and (iii) a tendency to their decrease [42], although the authors did not pay attention to the last interesting fact. Indeed, according to Goldstein et al. [42], although the average concentration of norepinephrine reduced by 27% and L-DOPA by 41% in the blood of PD patients, there was no statistically significant difference, which is probably due to the insufficient number of patients.

In addition to plasma, PD markers have also been found in CSF as changes in the content of physiologically active substances, mainly neurotransmitters and metabolites (Table 5). However, the data on the content of catecholamines and metabolites in CSF, as in plasma, are controversial. Some authors failed to recognize changes in the content of catecholamines and metabolites, whereas others showed their decrease (Table 5) [40, 42, 43, 46, 47]. Some authors observed the same changes of catecholamines and metabolites in CSF, as we found in plasma, a decrease in the concentration of norepinephrine [40, 46], epinephrine [40], dopamine [47], L-DOPA [47], and DOPAC [40, 42] (Table 5). Despite unidirectional changes in catecholamines and metabolites in CSF and plasma, the sensitivity of the diagnosis appears to be higher when assessing CSF. This conclusion follows from Goldstein et al. observations [42] of a significant decrease in L-DOPA and DOPAC in CSF without changes or only a tendency to a decrease in plasma. Nevertheless, blood markers are most probably to be used in the future for early diagnosis of PD due to the low cost and safety.

Amino Acids in the Blood of Patients as Markers of PD

We measured in the blood amino acids that in the brain plays the role of inhibitory (GABA, glycine, taurine) or excitatory (aspartate, glutamate) neurotransmitters involved in PD pathogenesis [48–50]. The concentration of most amino acids, aspartate, GABA, glycine, and taurine increased in patients with PD (Table 5).

Still, literature data on the content of amino acids in plasma in PD patients are contradictory. Some authors found an increase or a tendency to increase the concentration of aspartate, glycine, and taurine [51–53] which agrees with our data. Others either did not detect changes in the concentration of these amino acids and GABA or showed their decrease (Table 5) [52, 54–59].

In contrast to the above amino acids, we observed a decrease in the content of glutamate in plasma in PD patients. This is consistent with some studies [57, 58] but contradicts the others that showed the increase [51] or no change [52–54] in circulating glutamate (Table 5). According to the ROC analysis, the change in the concentration of amino acids in the blood observed in this study shows a high AUC value exceeding 0.8. This makes promising further use of these markers as auxiliary criteria for the diagnosis of PD at the clinical stage.

Table 5 Markers of Parkinson's disease, found in body fluids in this and previous studies

This study					Previous studies					
Blood markers		Presymptomatic mice	Symptomatic mice	PD patients	PD patients					
					Plasma			CSF		
Catecholamines and metabolites in plasma	Norepinephrine	=	=	↓	↓ [42]$^{\#}$	= [40, 41]	↑ [43–45]	↓[40, 42, 46]	= [43, 47]	
	Epinephrine	=	=	↓	↓[43]$^{\#}$	= [40, 41]		↓[40]		↑[43]
	Dopamine	↓	=	↓		= [40–42]		↓[42]$^{\#}$, [47]	= [40]	
	L-DOPA	↓	↓	↓	↓[42]$^{\#}$	= [40, 43]		↓[40]$^{\#}$, [42, 47]	= [43]	
	DOPAC	↓	↓	↓		= [40, 42, 43]	↑[44]	↓[40, 42]	= [43]	
Amino acids in plasma	Aspartate	=	↑	↑	↓[52, 57, 58]		↑[51]	↓[58]		↑[49]$^{\#}$
	GABA	↓	=	↑	↓[58]	= [52, 55, 57]		↓[49]	= [55]	↑[52]
	Glutamate	=	=	↓	↓[57, 58]	= [52–54]	↑[51]	↓[53]	= [52]	↑[49, 54]$^{\#}$
	Glycine	=	=	↑	↓[58]	= [54, 57]	↑[51, 52], [53]$^{\#}$		= [52–54]	↑[49]
	Taurine	=	↑	↑	↓[57, 59]	= [56]	↑[51]$^{\#}$	↓[56]		↑[49]
Gene expression in lymphocytes	DRD3	↑$^{\#}$	↓	↓	↓[60]					
	PHF10-P	=	↓$^{\#}$	↓						
	PHF10-S	=	↑	↓						

↑ increased compared to the control, = no difference compared to the control, ↓ decreased compared to the control

$^{\#}$ Tendency

Gene Expressions in Blood Cells in Patients as Markers of PD

We evaluated the expression of some genes in lymphocytes, which could potentially be changed at PD as a result of changes in plasma, an extracellular environment for lymphocytes. Given that lymphocytes express a dopaminergic phenotype [21–23], central and peripheral catecholaminergic neurons, on the one hand, and lymphocytes on the other, should be functionally linked by humoral regulation [23]. This means that the degeneration of catecholaminergic neurons and the associated deficiency of catecholamines should lead to a change in the functional state of lymphocytes sensitive to dopamine. Based on the foregoing, we evaluated the expression of DRD3 in lymphocytes in PD patients. Our data agree with previous studies, showing a decrease in gene expression and the membrane density of DRD3 in lymphocytes at PD [23, 60]. Conversely, gene expression of D1 and D2 receptors in lymphocytes increased at PD [33], whereas gene expression of the D5 receptor remained unchanged [60].

In addition to the DRD3 gene, we evaluated the expression of the PHF10-P and PHF10-S genes, which are alternative transcripts of the gene encoding the PHF10 subunit of the SWI/SNF chromatin remodeling complex of higher eukaryotes [61]. PHF10 is expressed in the brain and in lymphocytes and was shown to be essential for the development of neuroblasts [62]. The mechanism of alternative splicing is highly important for development, functioning and reparation of the nervous system in pathology. Several studies have shown that neurodegenerative diseases are associated with changes in the level of alternative splicing [63]. Furthermore, specific splice variants were found in the brain and lymphocytes in PD patients [64–67].

According to our data (see "Results"), the expression of the PHF10-P and PHF10-S genes decreased in lymphocytes at PD (Table 5), which is considered as a diagnostic marker. The changes in expression of genes in lymphocytes detected in this study are characterized by very high diagnostic efficacy with AUC exceeding 0.8 or even reaching 1 for the PHF10-P gene. The same markers show very high specificity, reaching 95–100%, which makes them extremely promising for further use for differential diagnosis of PD at the clinical stage.

Upgraded Methodology for the Development of Early Diagnosis of PD

The main drawback of current methodology for the development of the early diagnostics of PD is the search for markers in body fluids in untreated patients at an early clinical stage, that is, after the appearance of motor symptoms. However, there is no evidence that these markers are also characteristic of patients at the preclinical–prodromal stage. The weakness of this methodology is also the fact that each so far detected marker is non-specific, since it is characteristic not only of PD but also of some other diseases [13, 42]. It means that the development of the early diagnosis of PD is currently based on the search for a set of non-specific markers (diagnostic kit or panel), and therefore, the validity of the diagnostic panel is questionable. Furthermore, if only single markers found at the clinical stage are also characteristic of the preclinical–prodromal stage, their contribution to early diagnosis of PD will be "diluted" by more numerous non-specific markers.

There are several approaches to somehow improve the methodology for the development of the early diagnosis of PD based on the search for markers in the blood in untreated PD patients at an early clinical stage. The first one is based on excluding from the diagnostic panel those markers, which are characteristic not only of PD, but also of some other brain diseases. In this case, patients with diseases such as Alzheimer's disease are used as additional controls [44, 47]. The second approach is based on the identification of markers in the CSF and in the blood of the same patients [42]. The third approach is a combination of searching for markers in the blood of untreated patients at an early clinical stage with a retrospective analysis of early non-motor manifestations of PD (medical history), mostly olfactory deterioration, constipation, and sleep behavior disorder, including rapid eye movement [1]. Nevertheless, all these approaches did not lead to the development of an early diagnosis of PD, which is recommended for clinical use [41].

In this study, we have used a fundamentally novel methodology for the development of early diagnosis of PD. It is based on searching blood markers, not only in untreated patients at an early clinical stage, but also in MPTP-treated mice at modeling PD before the onset of motor disorders (preclinical–prodromal stage) and just after the appearance of motor disorders (early clinical stage) (Fig. 4). Both original models of PD were reproduced in mice using different regimes of systemic treatment with MPTP [25]. Our comprehensive studies of MPTP-treated mice—from the expression of specific genes to motor behavior [25, 68–70]—have shown that they reproduce many aspects of the PD pathogenesis and are similar to models developed in monkeys [71–74]. It is of particular importance that the MPTP models in mice reproduce the systemic character of the PD pathogenesis [75], which manifests in the impairment of the functioning of internal organs and the manifestation of peripheral markers.

Comparative Analysis in Changes of Catecholamines and Amino Acids in Plasma of PD Patients and Parkinsonian Animals

When comparing changes in the plasma concentration of catecholamines and metabolites in PD patients and symptomatic and presymptomatic mice (Table 5), it is evident that the concentration of L-DOPA and DOPAC decreased in all groups,

suggesting that these changes may serve as markers for early diagnosis of PD (Fig. 4). In the case of DOPAC, this assumption is supported by a previous observation of a decrease in the DOPAC concentration in plasma and CSF in MPTP-treated monkeys [75, 76]. As for L-DOPA, we found a correlation between its content in the blood and the degree of progression of the disease, which may also be characteristic of the preclinical–prodromal stage of PD.

Although the dopamine concentration in plasma in presymptomatic mice and in PD patients decreased significantly, the use of dopamine as a marker of the preclinical–prodromal stage is questionable, since there is no change in the dopamine concentration in symptomatic mice (Table 5). As to the concentration of norepinephrine and epinephrine, measured in this study, it decreased in patients but did not change in symptomatic and presymptomatic mice (Table 5). However, in MPTP-treated monkeys, in contrast to MPTP-treated mice, the concentration of circulating norepinephrine and epinephrine decreases, as in PD patients [77]. It means that the PD modeling in monkeys is more adequate then in mice along these metabolic pathways, and therefore, a decrease of norepinephrine and epinephrine in plasma can still be considered as a potential marker of PD at the preclinical–prodromal stage.

Among studied amino acids, the concentration of aspartate and taurine was changed (increased) in plasma in both patients and symptomatic mice (Table 5), which indicates the adequacy of modeling of the PD pathogenesis along these metabolic pathways. However, these amino acids apparently cannot serve as markers for early diagnosis of PD, since their concentration in presymptomatic mice remained unchanged.

The GABA level in the plasma rises, and glutamate and glycine decrease in PD patients, but not in symptomatic mice, showing that MPTP modeling is not adequate to the pathogenesis of PD. According to the ROC analysis, all three amino acids are characterized by a high AUC, reaching 0.8 and good values of sensitivity and specificity. Although there is no direct relationship between the diagnostic efficacy of markers at the clinical stage and the clinical–prodromal stage, it is reasonable to assess the blood content of GABA, glutamate, and glycine in other PD models, which, perhaps, better reproduce the changes in the metabolism of these amino acids.

Comparative Analysis of Gene Expression in Lymphocytes in PD Patients and Parkinsonian Animals

We found a decrease in the expression of the DRD3 gene both in untreated patients at the early clinical stage and in symptomatic mice (Table 5), which indicates the adequacy of modeling to the PD pathogenesis along this metabolic pathway. Moreover, there is a tendency ($p = 0.11$) to increase the expression of this gene in presymptomatic mice that allows us to consider it as a potential marker for early diagnosis of PD (Fig. 4), which is indirectly supported by the observation of a high AUC (0.84) and 100% specificity. However, in this case, we first met a situation when the change in the level of the presumed marker in patients and in symptomatic mice (decrease) is opposite to that in presymptomatic mice (increase) (Table 5). This fact was predictable since we have shown earlier that the genes of most important proteins of the vesicular cycle were unidirectionally changed in PD patients and in MPTP-treated symptomatic mice, whereas in some cases, there was a reverse in presymptomatic mice [69].

Unlike the expression of DRD3 receptor gene, the expression of genes of PHF10-P and PHF10-S cannot be considered as markers of PD at the preclinical–prodromal stage. Indeed, although the expression of the PHF10-P gene was reduced in patients, and the same tendency was observed in symptomatic mice ($p = 0.1$), this parameter did not change in presymptomatic mice. Despite AUC is 1 and sensitivity and specificity are 100%, we can assume that our MPTP model correctly reproduces the PD pathogenesis with respect to this marker and, therefore, the absence of changes in the presymptomatic mice suggests that this marker is a characteristic of the clinical stage, but not of preclinical–prodromal stage.

As for PHF10-S gene expression, it decreased in patients and increased in symptomatic mice, whereas remained unchanged in presymptomatic mice (Table 5). Given the high AUC = 0.92, it is

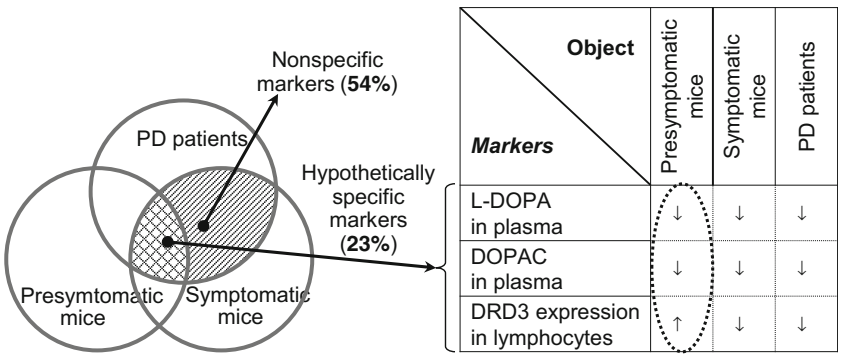

Fig. 4 Schematic representation of the upgraded methodology for the development of early diagnostics of PD based on searching blood markers in PD patients and MPTP-treated mice at modeling the clinical ("symptomatic") stage and preclinical–prodromal ("presymptomatic") stage of PD. Upwards arrow, increased compared to the control; downwards arrow, decreased compared to the control

desirable to test this marker in other PD models, which are more adequate in this aspect to the PD pathogenesis.

Thus, we upgraded a current methodology for development of early diagnosis of PD by searching blood markers not only in PD patients but also in parkinsonian animals, which allowed us among all markers found in patients at the clinical stage to identify about 20% of markers that could be used for early diagnosis of PD at preclinical or prodromal stage (Fig. 4).

Conclusion and Prospect

The current methodology for the development of early diagnosis of PD by searching for blood markers in untreated patients at an early clinical stage was upgraded in this study by using only those markers, which are also characteristic of MPTP-treated mice at modeling clinical (symptomatic) stage and preclinical–prodromal (presymptomatic) stage of PD. It is believed that the detection of the same marker in patients and symptomatic animals indicates correct reproduction of the PD pathogenesis, whereas the detection of this marker in presymptomatic animals suggests its specificity for preclinical–prodromal stage. Further studies should expand the list of these markers that will make possible to turn to clinical trials in subjects without motor disorders, but possessing the blood markers of preclinical–prodromal stage (risk group). The validity of the novel methodology can be proved by showing with a positron-emission tomography of the failure of the nigrostriatal dopaminergic system in subjects in a risk group.

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Compliance with Ethical Standards

All patients gave written informed consent, and the study procedure was approved by the Research Ethics Committee of the Republic of Tatarstan. All the animal studies were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Institute of Developmental Biology RAS.

Competing Interests The authors declare that they have no competing interests.

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