Published in final edited form as:

Biochem Biophys Res Commun. 2009 November 13; 389(2): 321–327. doi:10.1016/j.bbrc.2009.08.150.

Abnormal serum concentrations of proteins in Parkinson's disease

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Abstract

Blood serum was used to identify protein biomarkers for diagnosis of Parkinson's disease (PD) using analytically validated quantitative 2D-gel electrophoresis, and single variable and multivariate statistics. Using banked samples from 3 first medical center, we identified 57 specific protein spot biomarkers with disease-specific abnormal levels in serum of patients with PD, Alzheimer's disease, amyotrophic lateral sclerosis and similar neurodegenerative conditions (337 samples), when compared to age-matched normal controls (132 samples). To further assess their clinical usefulness in Parkinson's disease, we obtained prospective newly drawn blood serum samples from a second (56 PD, 30 controls) and third (6 PD, 48 controls) medical center. The protein concentrations of the 57 biomarkers were assessed by 2D-gel electrophoresis. Stepwise linear discriminant analysis selected a combination of 21 of the 57 as optimal to distinguish PD patients from controls. When applied to the samples from the second site, the 21 proteins had sensitivity of 93.3% (52 of 56 PD correctly classified), specificity of 92.9% (28 of 30 controls correctly classified); 15 of 15 patients with mild, 28 of 30 with moderate to severe symptoms, and all of the 6 PD patients from the third site were correctly classified. Eleven of the 21 proteins showed statistically significant abnormal concentrations in patients with mild symptoms, and 14 in patients with moderate-severe symptoms. The protein identities reflect the heterogeneity of Parkinson's disease, and thus may provide the capability of monitoring the blood for a diverse range of PD pathophysiological mechanisms: cellular degeneration, oxidative stress, inflammation, and transport.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.150.

Keywords

Parkinson's; Neurodegenerative; Serum; Proteins; Biomarkers; Diagnosis

Introduction

Signs of parkinsonism - tremor, hypokinesia, rigidity, and postural instability - increase in prevalence with age to include 15% of people ages 65–74, 30% ages of 75–84, and over 50% in people over age 85 [1]. Although only 10% of people with parkinsonism receive a clinical diagnosis of Parkinson's disease (PD), the presence of parkinsonism carries about twice the risk of death [1,2]. Often people with parkinsonism do not seek medical attention. Physicians may not diagnose either parkinsonism or PD, particularly in the early stages. Accurate clinical diagnosis and staging remain challenging and misdiagnosis occurs in about 30% of patients, with early stages especially prone to misdiagnosis [3–5]. A clinical validation for PD can be time-consuming, with multiple tests including motor, olfactory, visual, and psychological assessments, imaging (MRI, PET), as well as biochemical testing of CSF, lung, liver, heart, and lymphocytes [6–8]. By the time symptoms of PD are manifest, substantial neurodegeneration has already occurred. Therefore an early diagnostic test that accurately detects PD is essential to evaluate and implement early intervention strategies. The identification of biochemical marker(s) from serum or other minimally invasive patient samples, which accurately and reproducibly discriminate between PD, healthy individuals, and those with other neurodegenerative diseases, have the potential to serve as extremely useful tools for the diagnosis of PD, the monitoring of disease progression, and the response to treatment.

Two-dimensional gel electrophoresis has been used in research laboratories for biomarker discovery for three decades [9–18]. In the past, this method has been considered highly specialized, labor intensive and non-reproducible. Recently with the advent of integrated supplies, robotics, and software, combined with bioinformatics, the progression of this proteomics technique in the direction of diagnostics has become feasible [19–25]. The utility of 2D-gel electrophoresis is based on its ability to detect changes in expression of intact proteins to separate and discriminate between specific intact protein isoforms that arise due to variations in amino acid sequence and/or post-synthetic protein modifications such as phosphorylation, ubiquitination, conjugation with ubiquitin-like proteins, acetylation, glycosylation, and proteolytic processing. These are critical features in cellular and physiological regulation [24].

Using the Prot Ex® 2D-gel electrophoresis proteomics platform [19–21] with retrospectively collected and stored patient samples, we identified a set of 57 serum protein biomarkers whose abnormal concentrations were associated with specific neurodegenerative disease processes from a first clinical site [22–25]. A statistical model was applied (multivariate linear discriminant bio-statistical analysis) to the concentrations of the biomarkers to determine a probability score that categorized the patient's disease status [23–25]. This approach was used with freshly drawn, prospective PD samples to investigate the

biomarker panel specifically in PD, and to optimize the biomarker set in samples collected and handled in a manner similar to a clinical diagnostic setting.

Subjects/materials and methods

Subjects.

Patients and age-matched controls were from three clinical sites: (1) Baylor College of Medicine, Houston, TX, USA; (2) University of Thessaly, Larissa, Greece; and (3) Banner Sun Health Research Institute, Sun City, AZ, USA. The numbers of patients and controls for retrospective and prospective samples are listed in Table 1. The study compared biomarker concentrations in serum samples of healthy participants and those with neurodegenerative diseases in the initial biomarker panel identification (site 1), and with PD in the extended investigation of the panel (sites 2 and 3). PD patients underwent clinical evaluation to provide clinical data, including the severity of PD symptoms, according to the Hoehn and Yahr Scale [2] and the Unified Parkinson's Disease Rating Scale (UPDRS) [26]. Inclusion and exclusion criteria for PD patients are listed in Table 2. Patient information provided included demographics and medical history. Evaluation of clinical signs of PD included rigidity (stiffness or inflexibility of limbs and joints), bradykinesia/akinesia (slowness of movement/absence of movement), tremor (involuntary, regular rhythmic shaking of the limb, the head, the mouth, the tongue or the entire body) and postural instability (coordination and impaired balance). Also evaluated were the history of past illness, patients' current health problems, and copies of conventional imaging (CAT, PET scans, MRI of brain, SPECT, etc.). All forms and copies of reports were identified by study number only in order to maintain confidentiality; a copy of the above mentioned medical information was sent to the testing site in accordance with Health Information Privacy concerns.

Sample collection, preparation and separation of serum proteins.

Blood samples were collected from patients by venipuncture using standard red cap glass clot tubes without accelerator or gel. Serum samples were prepared from the blood by centrifugation after standing at room temperature for 30–45 min. The serum supernatant was collected, aliquoted, placed on dry ice, shipped to the laboratory, and stored at -80°C until analyzed. Sample preparation and electrophoresis were performed essentially as described previously [19–22]. The first dimension electrophoresis (100 μg of serum proteins/gel) was on immobilized 11 cm IEF strips (Bio-Rad Laboratories, Hercules, CA), pH 5–8, and in the second dimension on pre-cast 8–16% acrylamide gradient CRITERION SDS–gels (Bio-Rad).

Fluorescent staining, digital image analysis and normalization.

The gels were stained (Lava Purple™, Fluorotechnics; SyproRuby™, Bio-Rad), fluorescent digital images captured (FLA 7000 Imager Fujifilm; FX Imager, Bio-Rad Laboratories), and protein spot detection and quantitation performed (PDQUEST™, Bio-Rad Laboratories). Spot quantities in parts per million (PPM) fluorescent pixel spot density were normalized to total gel density. Each serum sample was analyzed in duplicate or triplicate. Quantitation of individual spots was validated for linearity, dynamic range, limit of detection (LOD = 0.66

µg/ml of serum), limit of quantify ability (LOQ =6.6 µg/ml of serum), reproducibility, and robustness (CV = 20%, Supplemental Material Fig. 1A, B and Table 1).

Statistical analysis.

Statistical significance of differences in individual biomarker blood serum concentrations (as Fold of Standard Normal Mean Concentration) between patient and controls was determined by non-parametric Dot Box and Whiskers (medians) and parametric Receiver Operator Characteristics analysis using “Analyze-it” in Microsoft XL. Analysis of joint performance of groups of biomarkers was by multivariate linear discriminant analysis using SAS® statistical software.

Results

Selection of serum proteins related to neurodegenerative disease in retrospective patient samples

Retrospective banked serum samples from patients with Alzheimer’s disease (AD), PD, AD/PD-like and mixed disorders, amyotrophic lateral sclerosis (ALS), ALS-like and age-matched normal controls (Table 1) were analyzed by 2D-gel electrophoresis, fluorescent staining, quantitative digital image analysis, and individual and multivariate biostatistics [22–25]. Fifty-seven protein biomarker spots were selected by exhaustive and painstaking comparisons of quantitative 2D-gel images and individual protein concentration statistics (PPM pixel spot densities), which exhibited reproducible statistically significant abnormal, disease-specific serum concentrations, differences between the following patient groups: AD vs. PD vs. ALS; AD vs. AD-like; PD vs. PD-like; ALS vs. ALS-like disorders; and familial vs. sporadic ALS. Multivariate discriminate analyses, in independent training and test sets, with combinations of sub-sets of the 57 biomarkers showed sensitivities and specificities of 85–95% [23–25]. The proteins were characterized by in-gel trypsin digestion of protein spots, peptide LC-MS/MS, spot molecular weights, isoelectric points, and Edman degradation where appropriate, to identify the protein molecular entities (Supplemental Material Fig. 2 and Table 2). The identified biomarker proteins clustered by function into four groups: I, cellular degeneration related; II, haptoglobin proteins; III, inflammatory proteins; and IV, albumin proteins (Supplemental Material Fig. 2 and Table 2).

Verification of clinical usefulness in prospective freshly drawn samples from PD patients

A two site prospective clinical validation trial was conducted using freshly drawn samples from the University of Thessaly (56 PD patients, 30 age-matched normal controls) and Banner Sun Health Research Institute (6 PD patients and 48 age-matched normal controls). Age-matched control samples from the two sites ($n = 78$) were subjected to duplicate or triplicate 2D-gel runs ($n = 174$, Supplemental Material Table 3A). Quantitative analysis performed on the 57 protein biomarkers followed by statistical analysis of individual biomarker proteins of this control group were used to calculate the standard normal control mean values for each biomarker (Supplemental Material Table 3B). These were employed as constants by which all the spot PPM density values were divided to convert each data point from PPM spot density to Fold of Standard Normal Mean Concentration (FSN) for each biomarker.

The serum concentration data (as FSN) of the 57 protein biomarkers were subjected to linear discriminant analysis (Table 3A). A subgroup of the 57, namely 21 biomarkers, was selected by stepwise linear discriminant analysis, based on their complimentary contributions to the overall diagnostic classification of control vs. Parkinson's disease with the samples from Thessaly (Tables 3B, 4 and 5). The identified biomarker proteins in this subgroup contained representatives of the four functional groups (Table 4). When the resulting discriminant function was used (Tables 3, 5 and 6 and Supplemental Material Table 4), 28 of 30 controls from Thessaly scored as controls (specificity = 93.3%), and 52 of 56 PD patients from Thessaly scored as PD (sensitivity = 92.9%). The six patients from Sun City that were not used in the generation of the discriminant function were all correctly classified. Of the PD patients for which symptom severity was measured, 15 of 15 patients with mild PD (Hoehn and Yahr Scale = 1–2; UPDRS = 13.7 ± 4.9 SD), scored as PD (sensitivity 100%, Table 6), and 28 of 31 patients with moderate to severe PD (Hoehn and Yahr Scale = 2.5–5; UPDRS = 26.6 ± 9.1 SD) scored as PD (sensitivity 90.3%, Table 6). When the posterior probabilities of Parkinson's disease (PD-P) from discriminant analysis were compared by Dot, Box, and Whiskers graphs (Fig. 1A), and Receiver Operator Characteristics (ROC) plots (Fig. 1B), both PD groups showed marked separation from the controls (Figs. 1 and 2). Of the 21 selected proteins, 11 showed individual statistically significant abnormal concentrations in the patients with mild, and 14 with moderate-severe PD symptoms (Fig. 2 and Table 7). Use of the concentrations of all 57 of the biomarkers in linear discriminant analysis provided a modest increase in sensitivity for PD diagnosis beyond using the 21 biomarker set (Table 3: A, 96.4% vs. B, 92.9%).

Discussion

Parkinson's disease (PD) is a progressive multisystem neurodegenerative disorder, an α -synucleinopathy, in which dopaminergic cell death exceeds a critical threshold [27,28], and olfactory, autonomic, gastrointestinal dysfunction, dementia, depression, and sleep disorder sometimes appear prior to motor manifestations [29–30]. PD has a long pre symptomatic phase where dopamine homeostasis compensates for dopaminergic neuronal loss. Breakdown of dopamine homeostasis results in alteration of basal ganglia output structures, and emergence of symptoms [31,32]. PD is associated with multiple transmitter dysfunctions in dopamine, GABA, glutamate, acetylcholine, and enkephalin systems in the CNS [29,33], in multiple brain regions, and peripheral tissues [34,35]. Familial PD is associated with mutations in the *α -synuclein* [6–8,36], *parkin* [37,38], *UCHL-1* [39], *DJ-1* [40], *PINK1* [41,42], *LRKK2* [43,44] Omi/Htra2 [45] and FBXO7 [46] genes. Differences in transcription and translation of non-mutant forms of these genes, post-synthetic processing of their products, and additional molecular pathways, including mitochondrial function, protein turnover, oxidative stress, and inflammation, underlie similar manifestations of sporadic PD [47–51].

Multiple blood tests have been evaluated for PD diagnosis, including mitochondrial complex I, markers of oxidative stress, and dopamine metabolism [52–53], but have not been robust. Expression of individual genes has been assessed in peripheral blood. Proteasome activity related to caspase-3 activation was shown to be decreased in PD but not in AD patients [54–55]. Molecular signatures of transcripts and protein levels in peripheral blood may serve as

biomarkers for Huntington disease [56], AD, and ALS [22–25,57–63]. Proteomic profiling in AD CSF and blood by mass spectrometry of small peptides, derived from biomarker proteins by proteolysis, can discriminate between AD individuals and normal controls [54,55,64], but provide limited information about the proteins and pathophysiological processes involved [21].

We have demonstrated that rigorous 2D-gel technology coupled with image analysis can identify a collection of 57 serum proteins that were abnormally expressed in neurodegenerative diseases when compared to controls in banked, retrospectively analyzed samples. Twenty-one of these proteins optimally classified PD and normal samples in prospectively collected samples when a multivariate discriminant analysis was applied to this set. When the same discriminant function was applied to the 2D-gel data from a small test set of PD samples from an independent site that were not used for the determination of the function, it correctly identified all six of them. The identities of the 21 proteins are consistent with mechanisms related to neuronal degeneration that are understood to be active in PD (Fig. 2 and Table 4). The 36 remaining proteins of the 57 protein group still displayed substantial, statistically significant abnormal concentrations in PD patient sera. Like the 21 proteins, the 36 proteins are represented in the same four functional groups, and so add to the understanding of PD mechanisms (Supplemental Material Table 2 and Figs. 3–6). For some proteins, only specific isoforms or variants were among the 21, whereas the others were among the 36 not selected, for instance Apolipoprotein E3 selected, E4 not selected; Haptoglobin HP-1c selected, HP-1a, b, d, and e, not selected; Immunoglobulin kl selected, κ c not selected; and albumin PRO2044–1 selected, PRO2044–11 not selected (Supplemental Material Figs. 3–6).

Conclusion

The present study demonstrates the usefulness of a 2D-gel generated protein biomarker panel to distinguish Parkinson's disease patients from age-matched normal controls in blood serum, independent of the severity of PD symptoms. The make-up of the panel is consistent with the heterogeneity of PD pathophysiological mechanisms, which are understood to include cellular degeneration, oxidative stress, inflammation, and transport dysfunction. Further clinical validation trials are underway; these have been designed to include disease controls as well as drug naive patients to investigate the potential of this panel for early detection and monitoring of drug response in PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Posterior probability of membership in diagnosis vs. severity of PD symptoms; linear discriminant analysis; 21 biomarkers; (A) Box and Whiskers plot; (B) Receiver Operator Characteristics (ROC).

Fig. 2.

Abnormal protein concentrations (median of fold of standard mean concentrations) of the 21 individual biomarkers (ID's Table 4); control, mild PD, moderate-severe PD.

Table 1

Retrospective and prospective patients and controls.

Blood serum sample type	Number of patients ^e	Disease status
Retrospective stored samples ^c	115	Alzheimer's disease (AD)
	29	Parkinson's disease (PD)
	24	AD/PD-like and mixed ^a
	75	AD/PD age-matched normal controls
	136	Amyotrophic lateral sclerosis (ALS)
	33	ALS-like ^b
	57	ALS age-matched normal controls
Prospective newly drawn samples ^d	62	PD
	78	AD/PD age-matched normal controls

^aAD/PD-like disorders including frontotemporal dementia; Lewy body dementia; vascular (multi-infarct) dementia; alcohol related dementia; semantic dementia; stroke (CVA); post-irradiation encephalopathy and seizures; vascular (multiinfarct) parkinsonism; multiple system atrophy; essential tremor; corticalbasal ganglionic degeneration; and mixed disorders including Alzheimer's disease combined with vascular (multi-infarct) dementia; Alzheimer's disease combined with Lewy body dementia; Parkinson's disease combined with Lewy body dementia; Alzheimer's and Parkinson's disease combined with Lewy body dementia; frontotemporal dementia combined with chronic inflammatory demyelinating polyneuropathy; and thalamic CVA combined with HX of lung CA.

^bNon-ALS disorders of motor neurons, muscles, nerves, and spinal cord.

^cFrom Houston, TX, USA.

^dFrom Thessaly, Greece and Sun City, AZ, USA.

^eThis study was approved by the Institutional Review Boards of Baylor College of Medicine and the Banner Sun Health Research Institute and the Ethics Committee of the University of Thessaly, with written informed consent. Subjects were evaluated by neurologists Katerina Markopoulou, MD, The University of Thessaly, Greece; Marwan Sabbagh, MD and Holly Shill, MD, Banner Sun Health Research Institute, Sun City, AZ, USA, and Stanley H. Appel, MD, Baylor College of Medicine, Houston TX, USA.

Table 2

Inclusion and exclusion criteria for Parkinson's disease (PD) patients and controls for prospective clinical validation.

Inclusions	Exclusions
Male or female >50years old	Secondary parkinsonism (non-PD)
Fully understood informed consent	Vascular parkinsonism
Controls	Encephalitis
Healthy	Exposure to neuroleptics
No family history of	Dementia (MMSE < 25)
Parkinson's	Gaze palsy
Disease subjects	Amyotrophy
At least three signs of PD	Cerebellar signs
Resting tremor	Symptomatic orthostatic hypotension (mean arterial pressure drop > 20 mm Hg, recumbent to standing)
Bradykinesia	
Rigidity	Unstable medical condition
Postural instability	History of alcohol or substance abuse
Responsive to levodopa or dopamine agonists	Major depression (Hamilton score > 19). History of malignant melanoma

Table 3

Linear discriminant analysis of patient and control data from prospective studies.

From diagnosis	Classified into diagnosis		
	Control specificity	PD sensitivity	Total
<i>A. 57 Biomarkers^b</i>			
Control	28	2	30
	93.3%	6.7%	100
PD	2	54	56
	3.6%	96.4%	100
<i>B. 21 Biomarkers^a</i>			
Control	28	2	30
	93.3%	6.7%	100
PD	4	52	56
	7.1%	92.9%	100
<i>C. Additional patients</i>			
PD	0	6	6
	0%	100%	100

Additional patients: six serum samples from PD patients from the US correctly classified as PD obtained by the same discriminant function that was used in B, i.e., 21 biomarkers and trained by the database of patients and controls from Greece.

^aSee Tables 4 and 5 for protein identities and discriminant statistics of the 21 biomarkers selected by stepwise discriminant analysis.

^bSee Supplemental Material Table 2 for protein identities of the 57 biomarkers

Table 4

The 21 biomarkers of PD vs. control selected by stepwise discriminant analysis.^b

Protein spot	Protein ID	Function group ^a
1. N5514 Alb mutant R218H-I	Chain A albumin mutant R218H protein	IV
2. N5123 HP-2A	Haptoglobin HP-2a protein	II
3. N5515 X1	X1 protein	V
4. N1416 Factor I	Complement factor I protein	III
5. N3314 Apo E3	Apolipoprotein E3 protein	I
6. N3307 TT"D"	Transthyretin "Dimer" protein	I
7. N7007 NUP 188	Nucleoporin NUP 188 protein	I
8. N2407 HP-1c	Haptoglobin HP-1 protein	II
9. N2511 Alb PRO2441	Albumin protein PRO2044	IV
10. N6306 PDLaH	Acidic Histone H2A protein (PD/LBD)	I
11. N2502 Apo A-IV	Apolipoprotein A-IV protein	I
12. N3007 TT HYPE	Transthyretin HYPE protein	I
13. N7304 C4by	Complement C4b gamma chain protein	III
14. N4420 Alb mutant R218H-II	Chain A albumin mutant R218H protein	IV
15. N8301 Fidgitin I	Fidgitin protein I	I
16. N6224 Igκ	Immunoglobulin kappa light chain protein	III
17. N4411 factor H/Hs	Complement factor H/Hs protein	III
18. N6214 Fidgitin II	Fidgitin protein II	I
19. N3417 Alb PRO2675	Albumin protein PRO2675 protein	IV
20. N4130 X2	X2 protein	V
21. N4402 HP-RP	Haptoglobin related protein	II

^a*Functional Groups.* I: Cell degeneration biomarkers: amyloid, oxidative stress, apoptosis, DNA repair. II: Haptoglobin proteins: oxidative stress. III: Inflammatory proteins: Innate and autoimmune. IV: albumin proteins: Transport. V: Unknown function.

^bFor a complete listing of the 57 biomarkers see Supplemental Material Table 2.

Table 5

Summary statistics for 21 biomarkers used for their selection by stepwise linear discriminant analysis as the optimal complementary biomarker set required to distinguish between prospective newly drawn serum samples from 56 PD patients and 30 age-matched normal controls from Thessaly Greece.

A: Summary statistics					B: Biomarker protein identities		
Step	Label	Partial R^2	F-value	Pr > F	Wilks' lambda	Pr < lambda	Average squared canonical correlation
1	N5514	0.1019	26	<.0001	0.8981	<.0001	0.1019
2	N5123	0.0594	14.39	0.0002	0.8447	<.0001	0.1553
3	N5515	0.0592	14.3	0.0002	0.7947	<.0001	0.2053
4	N1416	0.0538	12.86	0.0004	0.7519	<.0001	0.2481
5	N3314	0.0432	10.16	0.0016	0.7194	<.0001	0.2806
6	N3307	0.0435	10.18	0.0016	0.6881	<.0001	0.3119
7	N7007	0.0444	10.37	0.0015	0.6576	<.0001	0.3424
8	N2407	0.0382	8.82	0.0033	0.6324	<.0001	0.3676
9	N2511	0.052	12.13	0.0006	0.5995	<.0001	0.4005
10	N6306	0.0316	7.18	0.0079	0.5806	<.0001	0.4194
11	N2502	0.0261	5.87	0.0163	0.5654	<.0001	0.4346
12	N3007	0.0291	6.54	0.0113	0.549	<.0001	0.451
13	N7304	0.0248	5.52	0.0196	0.5353	<.0001	0.4647
14	N4420	0.0233	5.14	0.0243	0.5229	<.0001	0.4771
15	N8301	0.0179	3.93	0.0488	0.5135	<.0001	0.4865
16	N6224	0.0159	3.45	0.0646	0.5054	<.0001	0.4946
17	N4411	0.0173	3.75	0.0543	0.4966	<.0001	0.5034
18	N6214	0.0147	3.17	0.0764	0.4893	<.0001	0.5107
19	N3417	0.0115	2.46	0.1186	0.4837	<.0001	0.5163
20	N4130	0.0149	3.17	0.0766	0.4765	<.0001	0.5235
21	N4402	0.0134	2.83	0.0937	0.4701	<.0001	0.5299

Table 6

Classification of patients into diagnosis vs. severity of PD symptoms; Sensitivity and specificity of diagnosis by linear discriminant analysis with the 21 biomarkers.

From diagnosis	Classified into diagnosis				Severity of PD symptoms ^b
	PD	Control	Sensitivity	Specificity (%)	Hoehn and Yahr Scale
PD	15	15	0	100 ^c	Mild (HY 1–2) ^a
PD	31	28	3	90.3 ^c	Moderate-severe HY (2.5–5) ^a
Control	30	2	28	93.3 ^c	Control

^aHY 1–2: UPDRS 13.7 ± 4.89; HY 2.5–5: UPDRS 26.6 ± 9.07 (avg. ± SD).

^bAll patients under treatment with levodopa or other dopamine agonists.

^cSensitivity and specificity by Receiver Operator Characteristics (ROC) of posterior probability of membership in diagnosis: >0.3707 = PD.

Receiver Operator Characteristics (ROC) of fold of standard mean concentration values per patient vs. Severity of PD symptoms; 16 of the 21 biomarkers; greater or less than control, area under ROC curve (AUC), significance (ROC-P).

Table 7

Biomarkers	Mild = HY 1-2			Moderate to severe = HY 2.5-5		
	HY 1-2 ><Ctrl	AUC	SE	HY 2.5-5 ><Ctrl	AUC	SE
N5514	<Ctrl	0.70	0.049	<Ctrl	0.68	0.042
N5123	>Ctrl	0.68	0.052	>Ctrl	0.74	0.039
N5515				<Ctrl	0.65	0.046
N1416	>Ctrl	0.63	0.054	>Ctrl	0.58	0.056
N3314	<Ctrl	0.64	0.055	<Ctrl	0.66	0.043
N2407	>Ctrl	0.67	0.042	>Ctrl	0.63	0.055
N2511	<Ctrl	0.66	0.057	<Ctrl	0.65	0.044
N6306	<Ctrl	0.62	0.058	<Ctrl	0.60	0.044
N3007	>Ctrl	0.64	0.056			
N4420	<Ctrl	0.65	0.057			
N8301				<Ctrl	0.60	0.045
N6224	<Ctrl	0.62	0.054	<Ctrl	0.60	0.045
N4411				>Ctrl	0.62	0.044
N3417	<Ctrl	0.68	0.054	<Ctrl	0.66	0.043
N4130				<Ctrl	0.58	0.045
N4402				<Ctrl	0.63	0.044
The 21 combined	PD- <i>P</i> >0.3707	0.99	0.009	PD- <i>P</i> >0.3707	0.97	0.025