Causal Connection Between Serum Levodopa Metabolic Profile and Medication in Parkinson's Disease

Akiko Chukyo

Juntendo University School of Medicine

Motoki Fujimaki

Juntendo University School of Medicine

Naoko Kaga

Juntendo University School of Medicine

Hikari Taka

Juntendo University School of Medicine

Yuanzhe Li

Juntendo University School of Medicine

Shin-Ichi Ueno

Juntendo University School of Medicine

Haruka Takeshige-Amano

Juntendo University School of Medicine

Manabu Funayama

Juntendo University School of Medicine

Tomohiro Ishimaru

Juntendo University School of Medicine

Kengo Miyamoto

Juntendo University School of Medicine

Hirofumi Teranishi

Juntendo University School of Medicine

Taku Hatano

Juntendo University School of Medicine

Kei-Ichi Ishikawa

Juntendo University School of Medicine

Yutaka Oji

Juntendo University School of Medicine

Akio Mori

Juntendo University School of Medicine

Taiji Tsunemi

Juntendo University School of Medicine

Yuta Ishiguro

Juntendo University School of Medicine

Kensuke Daida

Juntendo University School of Medicine

Yoko Imamichi

Juntendo University School of Medicine

Yukiko Sasazawa

Juntendo University School of Medicine

Tetsushi Kataura

Juntendo University School of Medicine

Mitsuhiro Kitagawa

Juntendo University School of Medicine

Noriko Nishikawa

Juntendo University School of Medicine

Masaya Imoto

Juntendo University School of Medicine

Takashi Ueno

Juntendo University School of Medicine

Osamu Komori

Seikei University

Yoshiki Miura

Juntendo University School of Medicine

Wado Akamatsu

Juntendo University School of Medicine

Nobutaka Hattori

Juntendo University School of Medicine

Shinji Saiki (✉ ssaiki@juntendo.ac.jp)

Juntendo University School of Medicine

Research Article

Keywords: Parkinson's disease, levodopa, metabolite, catechol-O-methyltransferase, monoamine oxidase B

Posted Date: April 13th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-388258/v1>

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Abstract

No methods to assess efficacy of levodopa-associated therapy by blood sampling in Parkinson's disease (PD) have been established. In this study, we investigated levodopa associated metabolites to characterize their associations with medication and clinical symptoms in PD patients. Comprehensive metabolome analysis using plasma from PD and controls was performed in two independent cohorts (PD: 109, controls: 32; PD: 145, controls: 45). In another validation cohort [251 PD patients (16 *de novo*, 17 receiving only dopamine-receptor agonists, 218 receiving levodopa/benserazide or levodopa/carbidopa with/without other parkinsonian drugs) and 40 age-matched controls], serum levels of levodopa and its six metabolites were examined by liquid chromatography-mass spectrometry. The association of each metabolite with clinical parameters, medication, and enzymic genotypes was investigated. Significant increases in 3-methoxytyrosine and homovanillic acid were observed in PD patients administered levodopa/benserazide or levodopa/carbidopa. Serum levels of levodopa and five of its metabolites were significantly increased in PD patients administered levodopa and were related to the levodopa or entacapone dose but not to disease severity. Levodopa levels were more effectively preserved in PD patients given levodopa/benserazide than in those given levodopa/carbidopa, especially when taken with entacapone. Each dopamine or 3-methoxytyramine level was efficiently expressed with a numerical model using levodopa, entacapone, and selegiline doses as variables, indicative of its application for drug efficacy monitoring. Benserazide (25 mg) blocked AADC and preserved levodopa levels more effectively than carbidopa (10 mg), and entacapone provided a concomitant effect on levodopa level preservation. The drug efficacy of levodopa-associated medication could be monitored by dopamine or 3-methoxytyramine levels.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. It is characterized by motor symptoms of akinesia, tremor and rigidity, which respond to levodopa, a precursor of dopamine.^{1,2} Remarkable effects of oral levodopa administration were first described for PD by George Cotzias in 1967, and levodopa with peripheral aromatic amino acid decarboxylase inhibitors (AADC-Is) was subsequently established as a standard therapy.^{3–5} Although various routes of levodopa administration are available, oral administration of levodopa is the central pharmacological therapy against PD.^{6,7}

Levodopa-associated metabolites including 3,4-dihydroxyphenylacetaldehyde are toxic to nigral dopaminergic neurons and glia.⁸ Levodopa is actively absorbed in the duodenum and proximal jejunum and is then metabolized to dopamine by AADC in intestinal epithelial cells or to 3-methoxytyrosine by catechol-O-methyltransferase (COMT) in the liver, muscles, kidneys, and red blood cells (Supplementary Fig. 1).⁹ Because 10% of levodopa actually reaches the brain,¹⁰ the residual levodopa around 90% are degraded by both AADC and COMT during the systemic circulation. Thus, precise monitoring of the systemic levodopa metabolism enables us to perceive its internal alteration, leading to an appropriate adjustment of antiparkinsonian drugs. However, levodopa pharmacokinetics in PD have been investigated since 1975,^{11,12} comprehensive metabolic profiles of levodopa have not been investigated in *de novo* PD or in pharmacologically-treated PD patients. Additionally, associations of common AADC single nucleotide variants with levodopa metabolism have not been reported. Finally, integrated effects of COMT inhibitors and/or monoamine oxidase inhibitors (MAOB-Is) along with levodopa/AADC-Is on levodopa metabolism in PD have never been investigated. Here, we comprehensively analyzed levodopa metabolism using liquid chromatography-mass spectrometry^{13–17} and associated metabolite levels with medications and clinical features.

Results

Participants

Comprehensive metabolome data obtained from previously reported double independent cohorts were reanalyzed.¹⁴ Levels of two metabolites downstream of levodopa [3-methoxytyrosine and HVA] were significantly higher in PD patients treated with levodopa/AADC-Is ($p < 0.0001$) than in HCs (Supplementary Table 1). The levels of both metabolites are influenced by levodopa/AADC-I and COMT-I,^{10,18} and we were unable to estimate medication efficacy using non-consecutive metabolite levels in the same metabolic pathway. To determine the precise efficacy of PD medications, we used an LC-MS/MS system to measure seven consecutive levodopa-associated metabolites in serum simultaneously. We recruited 40 HCs and 251 PD patients. Based on their medication characteristics (Supplementary Table 2), we subdivided the 251 PD patients into three groups [*de novo* PD, PD patients receiving only DAs, and PD patients receiving levodopa/benserazide (L/B) or levodopa/carbidopa (L/C) with/without other antiparkinsonian medications (DAs, entacapone, selegiline, amantadine, zonisamide, droxidopa), hereafter described as "PD with L/B or L/C"]. We analyzed the level of each levodopa-associated metabolite in each group and compared the results with the HC group using Steel's test (Table 1). No significant differences were detected in mean ages at blood sampling or in the sex ratio between the HC and *de novo* PD, PD with only DA or PD with L/B or L/C groups. Disease duration was significantly longer in the PD with L/B or L/C group than in other PD groups. On average, disease severity in the *de novo* PD and PD with only DA groups was mild-to-moderate according to the H&Y stage and UPDRS-III score.

Table 1
Demographic characteristics of participants

	HC	<i>de novo</i> PD		PD with only DA		PD with L/B or L/C	
			<i>p</i> -value		<i>p</i> -value		<i>p</i> -value
Number	40	17		16		218	
Sex (Male: Female)	19:21	7:10	0.660 ^a	6:10	0.495 ^a	111:107	0.711 ^a
Age [years], Mean (SD)	66.9 (11.0)	66.1 (9.18)	0.861 ^b	63.8 (8.26)	0.284 ^b	68.0 (9.62)	0.511 ^b
Disease duration [years], Mean (SD)	-	2.47 (2.09)	-	3.31 (1.54)	-	7.28 (5.35)	<0.0001 ^c
Levodopa administration period [years], Mean (SD)	0 (0)	0 (0)	-	0 (0)	-	4.26 (3.98)	<0.0001 ^c
H&Y stage (each case number)		I (11), II (3), III (3), IV (0)		I (13), II (3)		I (48), II (78), III (64), IV (28)	
H&Y stage, Mean (SD)	-	1.52 (0.799)	-	1.19 (0.403)	-	2.33 (0.961)	<0.0001 ^c
MDS-UPDRS III, Mean (SD)	-	12.7 (9.69)	-	5.94 (3.96)	-	15.7 (10.9)	0.0014 ^c
Abbreviations: HC = healthy controls; PD = Parkinson's disease; DA = dopamine receptor agonist; L/B = levodopa/benserazide; L/C = levodopa/carbidopa; SD = standard deviation; H&Y stage = Hoehn and Yahr staging scale; MDS-UPDRS III = the Movement Disorder Society-sponsored revision of the Unified Parkinson's disease rating scale part III. ^a <i>p</i> -value obtained by chi-square test. ^b <i>p</i> -value obtained by Steel's test compared with healthy controls. ^c <i>p</i> -value obtained by ANOVA among PD groups.							

Analysis of levodopa metabolites

The purpose of administering oral levodopa with an AADC-I and/or COMT-I is to maintain appropriate peripheral blood concentrations of levodopa by reducing conversion to dopamine or 3-methoxytyrosine, respectively. Thus, we measured the concentrations of seven metabolites (levodopa, dopamine, 3-methoxytyrosine, DOPAL, DOPAC, 3-methoxytyramine, and HVA) (Supplementary Fig. 1). Six metabolites could be reliably measured, while the retention time of the peak associated with DOPAL often fluctuated compared with the standard (Supplementary Fig. 2), indicating unsteady chemical characteristics that might include dopamine and/or carbidopa derivatives. Thus, we excluded DOPAL from this study.

Changes to the six metabolites in each PD group

Dopamine and 3-methoxytyramine levels were below the limit of detection in 39/40 and 39/40 HCs, 15/17 and 17/17 of *de novo* PD patients, and 14/16 and 15/16 PD patients with only DA, respectively, while both were detected in all patients in the PD with L/B or L/C group. No significant changes in levodopa, 3-methoxytyrosine, DOPAC, or HVA were identified between HCs and the *de novo* PD or PD with only DA groups, indicating that they were not suitable diagnostic biomarkers for PD at early stages (Table 2). As expected, significantly increased levodopa, 3-methoxytyrosine, DOPAC, and HVA levels were detected in the PD with L/B or L/C group compared with levels in HCs.

Table 2
Alterations in each metabolite in patients in each PD group and HCs

Metabolite	HC	<i>de novo</i> PD		PD with only DA		PD with L/B or L/C	
[pmol/200ml]	level (SD)	level (SD)	<i>p</i> -value	level (SD)	<i>p</i> -value	level (SD)	<i>p</i> -value
Levodopa	3.83 (1.78)	4.00 (1.46)	0.915	4.11 (2.30)	0.995	702 (847)	<0.0001
Dopamine	-	-	-	-	-	0.556 (0.597)	-
3-methoxytyrosine	31.7 (12.8)	39.3 (23.9)	0.787	29.2 (23.2)	0.257	5,270 (5,460)	<0.0001
DOPAC	3.64 (3.75)	2.13 (1.81)	0.179	1.99 (1.68)	0.153	50.9 (82.8)	<0.0001
3-methoxytyramine	-	-	-	-	-	0.118 (0.123)	-
Homovanillic acid	15.7 (9.22)	17.5 (10.8)	0.911	12.4 (6.88)	0.373	213 (219)	<0.0001
Abbreviations: PD = Parkinson's disease; HC = healthy controls; DA = dopamine receptor agonist; L/B = levodopa/benserazide; L/C = levodopa/carbidopa; SD = standard deviation; DOPAC = 3,4-dihydroxyphenylacetic acid. <i>p</i> -value obtained by Steel's test compared with healthy controls.							

Regulation of levels of levodopa and its downstream metabolites

Because much higher levels of six metabolites were identified in the PD with L/B or L/C group compared with other groups (Table 2), we first estimated levels of the six metabolites for each H&Y stage in the PD with L/B or L/C group. As expected, most variables correlated with disease severity because of progressive medication intensity (Supplementary Table 3); therefore, because of their peripheral mechanisms of action, we investigated the effects of each anti-PD drug (levodopa, entacapone, selegiline, choice of AADC-I, and equivalent dose of DA) on the levels of each metabolite as explanatory variables in linear regression analyses. The levels of five and six metabolites were significantly regulated by each dose of levodopa and entacapone, respectively (Table 3). Levels of dopamine and 3-methoxytyramine, which are converted to DOPAL/DOPAC or HVA, respectively, by MAOB, were significantly correlated with selegiline in a dose-dependent manner (Table 3). The DA equivalent dose did not contribute to any levodopa, dopamine, or 3-methoxytyrosine level in the PD with L/B or L/C group (Table 3) but contributed to HVA levels in the PD with only DA group with mild significance ($p = 0.0499$) (Supplementary Table 4). Likewise, in the PD with L/B or L/C WITH DA group, DAs mildly contributed only to 3-methoxytyrosine levels with statistical significance ($p = 0.0402$) (Supplementary Table 5), implying little effect of DA on levodopa metabolism. Levodopa and dopamine levels in the PD with L/B or L/C group were not related to disease severity assessed by H&Y stage (Table 3) or UPDRS-III score (Supplementary Table 6), consistent with the unchanged levodopa pharmacokinetics throughout the course of the illness.^{19,20} In Europe and Japan, levodopa (100 mg)/benserazide (25 mg) and levodopa (100 mg)/carbidopa (10 mg) are clinically prescribed. As shown in Table 3, choice of AADC-I significantly affected the levodopa level, implying differences in the effects of the administered levodopa and/or AADC-I dose. Therefore, we compared each metabolite concentration between the PD with L/B and with L/C groups without statistical correction (Supplementary Table 7). In contrast to the significantly higher dose of levodopa in the L/C group than in the L/B group, lower levodopa and 3-methoxytyrosine levels, as well as higher dopamine, DOPAC, 3-methoxytyramine, and HVA levels, were detected in the L/C group compared with the L/B group, indicative of imperfect AADC inhibition in the L/C group. Consistently, the linear regression analysis shown in Table 3 revealed that levels of levodopa and three downstream metabolites (3-methoxytyrosine, DOPAC, and HVA) in the PD with L/B or L/C group were significantly affected by the choice of AADC-I. As shown in Supplementary Table 6, linear regression analysis using the UPDRS-III score (NOT H&Y stage) showed similar contributions of the choice of AADC-I to levodopa concentration ($F = 2.75$, $p = 0.0988$). In addition, the choice of AADC-I contributed to the levodopa level in the PD with L/B or L/C with DA group (Supplementary Table 5). Taken together, we concluded that 25 mg benserazide might have greater inhibitory efficacy on AADC than 10 mg carbidopa.

Table 3
Effects of each variable on six metabolites in PD patients treated with L/B or L/C

Metabolite	Variables											
	dose of levodopa		dose of entacapone		dose of selegiline		choice of AADC-I		equivalent dose of DA		H&Y	
	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value
Levodopa	40.3	< 0.0001	20	< 0.0001	2.04	0.155	4.52	0.0347	1.19	0.276	0.775	0.38
Dopamine	8.22	0.0046	10.5	0.0014	4.44	0.0362	0.00717	0.789	0.858	0.355	0.439	0.508
3-methoxytyrosine	53.6	< 0.0001	21.5	< 0.0001	0.177	0.675	9.78	0.002	0.119	0.731	3.99	0.0471
DOPAC	2.04	0.155	8.38	0.0042	3.22	0.0074	13.9	0.0002	3.29	0.0713	4.1	0.0441
3-methoxytyramine	24.2	< 0.0001	16	< 0.0001	15.8	< 0.0001	1.11	0.293	0.0827	0.774	0.0131	0.909
Homovanillic acid	28.7	< 0.0001	15	0.0001	0.0036	0.953	40.3	< 0.0001	0.174	0.677	0.0699	0.792

Abbreviations: PD = Parkinson's disease; L/B = levodopa/benserazide; L/C = levodopa/carbidopa; AADC-I = aromatic amino acid decarboxylase inhibitor; DA = dopamine receptor agonist; H&Y = Hoehn and Yahr stage; DOPAC = 3,4-dihydroxyphenylacetic acid. *F*-value and *p*-value obtained by linear regression analysis.

Concomitant effects of entacapone and/or selegiline with L/B or L/C on levodopa metabolism

To confirm the difference in efficacy of AADC-Is, benserazide, and carbidopa, the correlation obtained by linear regression analysis was separately examined for each PD group treated with L/B or L/C. In this cohort, no differences were observed in the *COMT* SNVs, rs4680 and rs4818, between the L/B and L/C groups (Supplementary Table 7). Although the mean doses of levodopa and entacapone in the L/C group were higher than those in the L/B group, the levodopa levels in the L/C group were inferior to those in the L/B group, indicating a lower AADC inhibitory effect of L/C than with L/B as mentioned above (Supplementary Table 7). Consistently, the entacapone dose significantly contributed to levodopa levels only in the PD with L/B group according to both H&Y stage (Table 4) and UPDRS-III score (Supplementary Table 8). Likewise, the leakage tendency of three downstream metabolites (dopamine, DOPAC, and HVA) was more prominent in PD patients treated with L/C with the addition of entacapone. Taken together, the effects of entacapone on levodopa preservation were more pronounced in PD patients treated with 25 mg benserazide than with 10 mg carbidopa. This was confirmed by the observation that levodopa levels were significantly influenced by the choice of AADC-I for PD treated with L/B or L/C with entacapone (Supplementary Table 9, 10) but not WITHOUT entacapone (Supplementary Table 11, 12). Likewise, for PD treated with L/B, a greater correlation was detected between the dopamine and 3-methoxytyramine levels and the selegiline dose, indicating that excessive leakage of dopamine

in the PD with L/C group might not be overcome by the peripheral MAOB inhibitory effects of selegiline (Table 4). In accordance with the lack of association between disease stage and levodopa level previously reported,¹² no association of the H&Y stage (Table 4) with levodopa level was detected in this study. However, a mildly significant correlation between UPDRS-III score and levodopa level was identified only in the PD with L/B group (Supplementary Table 8).

Table 4
Effects of each variable on six metabolites in each PD group treated with L/B or L/C

Metabolite	Variables										
	dose of levodopa			dose of entacapone		dose of selegiline		equivalent dose of DA		H&Y	
		<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value	<i>F</i> -value	<i>p</i> -value
Levodopa	L/B	20.4	< 0.0001	38.2	< 0.0001	1.82	0.182	0.0792	0.7793	0.0016	0.968
	L/C	21.2	< 0.0001	2.01	0.158	0.947	0.332	1.18	0.28	1.64	0.202
Dopamine	L/B	4.13	0.0464	2.01	0.161	12.9	0.0007	6.41	0.0139	0.013	0.91
	L/C	5.68	0.0184	9.54	0.0024	1.55	0.215	5.14	0.0249	0.473	0.493
3-methoxytyrosine	L/B	17.9	< 0.0001	2.3	0.135	0.107	0.745	1.03	0.315	0.0007	0.979
	L/C	33.9	< 0.0001	21.3	< 0.0001	0.336	0.563	0.154	0.695	5.49	0.0204
DOPAC	L/B	4.41	0.0399	5.27	0.0251	1.27	0.265	0.238	0.627	0.0042	0.949
	L/C	1.96	0.164	8.96	0.0032	2.47	0.118	4.21	0.042	3.94	0.0491
3-methoxytyramine	L/B	17.8	< 0.0001	22.2	< 0.0001	13.6	0.0005	1.86	0.178	3.54	0.0648
	L/C	13.5	0.0003	5.05	0.0261	9.66	0.0023	1.64	0.203	0.691	0.407
Homovanillic acid	L/B	9.59	0.003	4.82	0.032	0.0007	0.979	1.74	0.192	0.201	0.656
	L/C	22.3	< 0.0001	11	0.0012	0.0469	0.829	0.358	0.551	0.0672	0.796
Abbreviations: PD = Parkinson's disease; L/B = levodopa/benserazide; L/C = levodopa/carbidopa; DA = dopamine receptor agonist; H&Y = Hoehn and Yahr stage; DOPAC = 3,4-dihydroxyphenylacetic acid. <i>F</i> -value and <i>p</i> -value obtained by linear regression analysis.											

Mathematical expression of two metabolite levels (dopamine and 3-methoxytyramine)

As shown in Table 3, each dopamine or 3-methoxytyramine level was significantly influenced by the levodopa, entacapone, and selegiline doses. Thus, we selected appropriate variables with the AIC to clarify which variables are important for estimating the serum concentration of dopamine or 3-methoxytyramine. The resultant models are given in (1) and (2).

$$\text{dopamine [nM]} = 0.109 + 0.000720 \times \text{levodopa dose [mg]} + 0.000581 \times \text{entacapone dose [mg]} + 0.0320 \times \text{selegiline dose [mg]} \quad (1)$$

$$\text{3-methoxytyramine [nM]} = 0.0105 + 0.000239 \times \text{levodopa dose [mg]} - 0.000144 \times \text{entacapone [mg]} + 0.0115 \times \text{selegiline dose [mg]} \quad (2)$$

In both cases, levodopa, entacapone, and selegiline were selected by the AIC. For dopamine estimation, all metabolites had positive impacts. However, for 3-methoxytyramine estimation, entacapone had a negative impact, consistent with the levodopa metabolic pathway characteristics, in that 3-methoxytyramine production from dopamine is dependent on COMT (Supplementary Fig. 1).

Motor complications and levodopa concentration

The degree of nigrostriatal degeneration and the mode of drug administration are important factors of motor complications [wearing off (WO) and levodopa induced dyskinesia (LID)].^{21, 22} The well-established risks of motor complications are age at onset, disease severity, levodopa treatment duration, and levodopa, entacapone, selegiline, or DA dose.²³ Fluctuating serum levodopa levels may be translated into peaks and troughs in striatal dopamine concentration; therefore, we examined the relationship between levodopa concentration and motor complications. As shown in Supplementary Table 13, levodopa levels did not correspond to the presence or absence of WO or LID.

AADC rs6263 A/G affects the response to carbidopa

The clinical response to L/B or L/C is variable, and approximately 25% of PD patients show a poor response to levodopa because of unknown causes.²⁴ Thus, we examined six SNVs (rs6950777, rs3735273, rs6263, rs4947580, rs1157457, and rs4490786) in the AADC gene, which are observed in more than 1% of the Japanese population (jMorp, <https://jmorp.megabank.tohoku.ac.jp/202001/>). As shown in Supplementary Table 14, there was no significant change in AADC activity (dopamine/levodopa ratio) for any SNV in all PD patients. Considering the difference in inhibitory (binding) activity against AADC between benserazide and carbidopa, we then determined the difference in AADC activity among groups treated with each AADC-I. AADC activity was not changed by AADC SNVs except that AADC activity was preserved by rs6263 (A→G) in patients treated with L/C, indicative of their poor response to carbidopa (Table 5).

Table 5
AADC activity of each SNV in PD patients treated with L/B or L/C

AADC activity of each SNV in PD patients treated with L/B or L/C							
L/B (N = 67)				L/C (N = 151)			
SNV	nucleotide	number	AADC activity (dopamine/l-dopa) Mean (SD)	p-value	number	AADC activity (dopamine/l-dopa) Mean (SD)	p-value
rs6950777	C/C	28	0.00245 (0.00516)	0.579 ^a	65	0.00173 (0.00406)	0.617 ^a
	C/T	33	0.00147 (0.00211)		63	0.00191 (0.00432)	
	T/T	6	0.00165 (0.00127)		23	0.000983 (0.000811)	
rs3735273	G/G	28	0.00245 (0.00516)	0.579 ^a	65	0.00173 (0.00406)	0.617 ^a
	G/A	33	0.00147 (0.00211)		63	0.00191 (0.00432)	
	A/A	6	0.00165 (0.00127)		23	0.000983 (0.000811)	
rs6263	A/A	58	0.00188 (0.00367)	0.908 ^b	135	0.00158 (0.00370)	0.0212 ^b
	G/A	9	0.00203 (0.00380)		14	0.00285 (0.00539)	
	G/G	0	-		2	0.000682 (-)	
rs4947580	G/G	53	0.00204 (0.00405)	0.799 ^b	134	0.00155 (0.00321)	0.293 ^a
	G/A	13	0.00129 (0.00148)		14	0.00321 (0.00800)	
	A/A	1	0.00254 (-)		3	0.000914 (0.000259)	
rs11575457	T/G	32	0.00135 (0.00213)	0.405 ^a	72	0.00237 (0.00542)	0.115 ^a
	T/T	20	0.00276 (0.00593)		47	0.000993 (0.00113)	
	G/G	15	0.00192 (0.00189)		32	0.00118 (0.00101)	
rs4490786	G/G	43	0.00169 (0.00215)	0.845 ^b	99	0.00161 (0.00345)	0.79 ^a
	G/A	23	0.00118 (0.000742)		47	0.00195 (0.00482)	
	A/A	1	0.0277 (-)		5	0.000873 (0.000246)	
Abbreviations: PD = Parkinson's disease; AADC = aromatic amino acid decarboxylase; L/B = levodopa/benserazide; L/C = levodopa/carbidopa; SNV = single nucleotide variant; SD = standard deviation. ^a p-value obtained by ANOVA, ^b p-value obtained by Wilcoxon's test.							

rs4680 and changes in COMT activity

Of 251 PD patients, 155 were not treated with entacapone. The Val158Met polymorphism in the *COMT* gene (rs4680) affects COMT activity (G/G: high, G/A: intermediate, A/A: low).²⁵ Additionally, rs4818 is highly variant in the Japanese population; therefore, we examined associations of COMT activity (3-methoxytyrosine/levodopa) with the two SNVs. Consistent with previous reports, a trimodal distribution of high (G/G), intermediate (G/A), and low (A/A) COMT activity was identified (Supplementary Table 15). No significant changes of COMT activity were detected.

Discussion

In this study, we analyzed levodopa metabolic pathway during the systemic circulation, corresponding to about 90% metabolism of it. The absolute serum concentrations of levodopa and five of its downstream metabolites were significantly higher in PD patients receiving levodopa than in HCs. As expected, levels of five and six metabolites significantly correlated with the levodopa and entacapone doses, respectively. The dose of the MAOB-I selegiline did not contribute to the serum levels of levodopa but did contribute to the serum dopamine and 3-methoxytyramine levels, consistent with its blocking point, MAOB, in the pathway. Importantly, significant differences in levodopa, 3-methoxytyrosine, DOPAC, and HVA levels between the L/B and L/C groups were detected that were compatible with the higher area under the curve (AUC) of levodopa in response to 25 mg benserazide compared with 10 mg carbidopa.²⁶ Likewise, concomitant use of L/B with entacapone or selegiline preserved levodopa or dopamine levels, respectively, compared with L/C. Additionally, mathematical models could partially express the correlation of dopamine or 3-methoxytyramine levels with three medications (levodopa, entacapone, and selegiline). Finally, the rs6263 A/G variant in the *AADC* gene conferred higher AADC activity (dopamine/levodopa ratio) than the wild-type (G/G), indicating a decreased response to carbidopa.

In addition to the positive correlation between total daily levodopa dose and plasma levodopa concentration in PD,¹¹ pharmacokinetic studies have shown that the T_{max} is between 30 and 60 min, and the half-life is approximately 3 hours.^{12, 19} In this study, blood was obtained within 4 hours after the most recent levodopa administration. The sampling time was randomly allocated every 30 min; therefore, the levodopa levels in the PD with L/B or

L/C groups were averaged and might reflect the mean area under the serum concentration curve. According to a recent report,¹⁹ a larger AUC was observed for L/B treatment than for L/C treatment, consistent with our results.

Use of benserazide or carbidopa only increases the amount of levodopa reaching the brain to an estimated 10% of an administered dose because blocking AADC shunts levodopa into the COMT metabolic pathway, thereby increasing peripheral formation of 3-methoxytyrosine.¹⁰ Among all cohorts of this study, 3-methoxytyrosine levels in PD patients were elevated more than 100 times those in HCs. Although no differences in efficacy between L/B and L/C were detected in PD patients treated with levodopa without COMT-Is,^{27–29} our data suggested that concomitant use of benserazide with entacapone preserved levodopa concentrations more than carbidopa. These results are supported by correlated levels of dopamine or its downstream metabolites with entacapone doses in the L/C but not the L/B group, showing a high level of AADC leakage associated with COMT inhibition. According to previous therapeutic studies,³⁰ switching from L/B to L/C/entacapone produces similar efficacy in PD as switching from L/C to L/C/entacapone; however, no clinical trials comparing L/B/entacapone and L/C/entacapone in PD have been reported.

Selegiline is primarily metabolized by the liver P450 system (CYP1A2) with some extrahepatic metabolism occurring in platelets.³¹ Although platelet MAOB activity, which is inhibited by more than 85% within 4 hours of selegiline administration (5 mg), can be monitored *in vitro*,³² measurement of MAOB-I efficacy using serum/plasma has not been reported. In our study, dopamine and 3-methoxytyramine levels in PD patients receiving levodopa were significantly enhanced by selegiline, indicating potential responders to MAOB-Is. Further large-scale studies should address possible associations of *MAOB* SNVs, such as rs1799836, with MAOB activity.³³

In our validation cohort, linear regression analyses revealed no correlation between H&Y stage and levodopa concentration in PD treated with L/B and/or L/C (Table 3, 4), consistent with other reports.¹² Importantly, existence of WO or LID was not influenced by levodopa concentration in the PD with L/B or L/C group (Supplementary Table 13). These results were compatible with motor complications that occur when the levodopa therapeutic range is narrowed because of the increase in median effective concentrations at the more advanced stage of PD.^{34,35} Among three PD groups (untreated, stable, and fluctuating), no differences in pharmacokinetics, including mean plasma levodopa peak, were detected.^{11,36} However, some patients in the fluctuating group showed higher levodopa concentrations, consistent with our results of levodopa levels being related to UPDRS-III scores in the L/B group.

According to a systematic review, 26.9% of pathologically proven PD patients were nonresponsive to oral levodopa treatment.²⁴ Misdiagnosis, malabsorption because of insufficient acidification resulting from *Helicobacter pylori* infection, and undesired conversion of intestinal levodopa because of excessive bacterial enzymes, including decarboxylase, have been reported;^{37,38} however, effects of *AADC* SNPs on the enzyme activity have not been identified. One of five haplotypes defined by 23 SNVs (> 1% minor allele frequencies) without *AADC* rs6263 is predictive of AADC activity, as measured by [¹⁸F]-FDOPA positron emission tomography.³⁹ In addition, the common *AADC* polymorphisms (rs921451 and rs3837091) may alter therapeutic responses to levodopa.⁴⁰ According to our data, rs6263 (A→G) preserved higher AADC activity than the wild-type allele in the PD with L/C but not the PD with L/B group, indicating insufficient AADC blockage by carbidopa.

A limitation of this study is that it was conducted at a single university hospital, and severe PD cases (H&Y V) were not fully represented because of the history of aspiration pneumonia or cancer exclusion criteria. Although we performed linear regression analysis to evaluate the effect of each medication on each metabolite level, we could not exclude unknown interactions among the drugs. Other antiparkinsonian medications may influence the metabolism of levodopa; however, at least in our validation cohort, a DA equivalent dose did not correlate with levodopa concentrations. Association studies of levodopa metabolism with genetic background screening for levodopa-associated genes in a large PD cohort should be performed.⁴¹

Methods

Ethics statement

The study protocol complied with the Declaration of Helsinki and was approved by the ethics committee of Juntendo University (#2012157). Written informed consent was obtained from all participants.

Participants

All participants were recruited at the Juntendo University Hospital and examined by board-certified neurologists. Cohort 1 and 2 were previously reported.¹⁴ PD was diagnosed according to the Movement Disorder Society diagnostic criteria.¹ Exclusion criteria for complications (e.g., dementia) were previously described.¹⁶ Hoehn and Yahr (H&Y) stages and Unified Parkinson's disease Rating Scale motor section (UPDRS-III) scores were defined during the “on” phase for practical and ethical reasons. Entacapone or selegiline were used as COMT-Is or MAOB-Is, respectively. Pramipexole, ropinirole, and rotigotine were used as dopamine receptor agonists (DAs) against parkinsonism, and equivalent DA doses were calculated according to a method previously reported.⁴²

Sample collection

All blood samples were collected at the outpatient department of Juntendo University Hospital between October 2014 and March 2018. Venous blood samples for laboratory analysis were collected between 9:00 am and 12:00 pm. All participants were only allowed to have water and medicines from 12:00 am until sampling. Plasma or serum samples were collected using 7 ml EDTA-2Na blood spits (SRL, Tokyo, Japan) or 8 ml INSEPACK tubes (Sekisui Medical, Tokyo, Japan) with two or three inversions, respectively. Samples were then allowed to incubate for 30–60 min at 4°C followed by centrifugation for 10 min at 2,660 g. The plasma and serum were then separated and placed in collection tubes, which were then stored in liquid nitrogen until analysis.

Sample preparation

Sample preparation of plasma for metabolome analysis was previously described.¹⁴ Levodopa and its six downstream metabolites [dopamine, 3-O-methyldopa, 3-methoxytyramine, 3,4-dihydroxyphenylacetaldehyde (DOPAL), 3,4-dihydroxybenzeneacetic acid (DOPAC), and homovanillic acid (HVA)] were extracted from sera.

Serum samples (200 µl) were added to 600 µl 0.1% HCOOH/CH₃CN containing internal standards: 5 pmol 3-methoxytyramine-1,1,2,2-d₄, 200 pmol homovanillic acid-2,2,2',5',6'-d₅ (Isosciences, Ambler, PA, USA), 20 pmol dopamine-1,1,2,2-d₄, and levodopa-2',5',6'-d₃ (Cambridge Isotope Laboratories, Tewksbury, MA, USA). After centrifugation at 14,000 g for 10 min at 4°C, the supernatants were transferred to new tubes, dried under nitrogen, and then kept at –80°C until use. The samples were reconstituted in 40 µl mobile phase A prior to analysis and assayed by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS).

High-performance liquid chromatography-mass spectrometry

LC-MS/MS analysis was performed using a Nexera liquid chromatography system connected to an LCMS-8060 tandem mass spectrometer (Shimadzu, Kyoto, Japan). Levodopa and its six downstream metabolites were separated using a Discovery HS F5-3 column (15 cm×2.1 mm, 3 µm particle; Sigma-Aldrich, St. Louis, MO, USA). The mobile phase consisted of 0.1% HCOOH/2 mM AcONH₄/H₂O as A and 0.1% HCOOH/2 mM AcONH₄/90% CH₃CN as B at a flow rate of 150 µl/min. The gradient conditions were as follows: The mobile phase was 0% B for 3 min, increased by 3%/min to 90% B, and then maintained for 15 min. The column temperature was 40°C. Target compounds were analyzed in multiple reaction monitoring positive and negative ionization mode.

Genomic DNA analysis

During plasma collection, DNA was extracted from peripheral blood according to a standard protocol using a Qiagen kit (Venlo, Netherlands). Primer sequences were designed to amplify the coding exons of *AADC* and rs4818 and rs4680 of *COMT*. PCR products were purified, and DNA sequences were determined by Sanger Sequencing (Genewiz, South Plainfield, NJ, USA). The frequencies of each variant were evaluated using the Japanese Multi Omics Reference Panel (jMorp, <https://jmorp.megabank.tohoku.ac.jp/202001/>).

Statistical analysis

All statistical analyses were performed using JMP13 (SAS Institute, Tokyo, Japan). The chi-square test was used to analyze categorical variables. ANOVA was used to assess the relationships of each clinical parameter among PD groups. Wilcoxon's test or ANOVA was performed to test for statistical significance of enzyme activities between two single nucleotide variants (SNVs) or among three SNVs. Steel's test is a nonparametric, multiple-comparison test and was used to examine participant characteristics and levels of levodopa and its metabolites in PD patients and healthy controls (HCs). Linear regression analysis was performed to reveal the influence of the levodopa, entacapone, or selegiline dose, choice of AADC-I, H&Y stage, and/or UPDRS-III score, followed by variable selection based on the Akaike Information Criteria (AIC). A *p*-value <0.05 was considered statistically significant.

Declarations

Acknowledgements

We thank Jeremy Allen, PhD and Lisa Kreiner, PhD, from Edanz Group (<https://en-author-services.edanz.com/ac>) for editing a draft of this manuscript.

Authors contribution statement:

(1) Research project: A. Conception and Design, B. Acquisition of Data, C. Organization; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript: A. Writing of the First Draft, B Review and Critique; (4) Others: A. Technical and Material Support, B. Management of Data, C. Study Supervision, D. Supervision of Data collection.

AC: 1A, 1B, 1C, 2A, 3A, 3B

MF: 1A, 1B, 1C, 2A, 2B

NK: 1A, 1B, 3A

HTa (Hikari Taka): 1A, 1B, 3A

YL: 1B

S-IU: 1B

HT-A: 1B

MF: 1B

TI: 1B

KM: 1B

HTe (Hirofumi Teranishi): 1B

TH: 1B

K-II: 1B

YO: 1B

AM: 1B

TT: 1B

YIs (Yuta Ishiguro): 1B

KD: 1B

YIm (Yoko Imamichi): 1B

YS: 1B

TK: 1B

MK: 1B

NN: 1B

MI: 1B

TU: 1B

OK: 2A, 2B, 2C, 3A, 3B

YM: 1A, 1B

WA: 1A, 3A, 3B

NH: 1A, 3A, 3B, 4C

SS: 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B, 4B, 4C, 4D

Financial disclosure/Conflict of interest disclosure

Akiko Chukyo reports no disclosures.

Motoki Fujimaki reports no disclosures.

Naoko Kaga received a grant from JSPS (18K07829).

Hikari Taka received a grant from JSPS (19K11752).

Yuanzhe Li received a grant from JSPS (16K09700).

Shin-Ichi Ueno reports no disclosures.

Haruka Takeshige-Amano reports no disclosures.

Manabu Funayama received a grant from JSPS (16K09676).

Tomohiro Ishimaru is an employee of Ohara Pharma Co., Ltd.

Kengo Miyamoto is an employee of Sumitomo Dainippon Pharma Co., Ltd.

Hirofumi Teranishi reports no disclosures.

Taku Hatano received a grant from JSPS (16K09675) and the Japan Agency for Medical Research and Development (19dm0107156h0001).

Kei-Ichi Ishikawa received a grant from JSPS (20K07873).

Yutaka Oji received a grant from JSPS (16H07184).

Akio Mori reports no disclosures.

Taiji Tsunemi received a grant from JSPS (18K07510).

Yuta Ishiguro reports no disclosures.

Kensuke Daida reports no disclosures.

Yoko Imamichi received a research grant from JSPS (16H00625).

Yukiko Sasazawa received a research grant from JSPS (20H05340).

Tetsushi Kataura reports no disclosures.

Mitsuhiro Kitagawa reports no disclosures.

Noriko Nishikawa reports no disclosures.

Masaya Imoto received grants from JSPS (18H02099, 17H06401).

Takashi Ueno reports no disclosures.

Osamu Komori received a grant from JSPS (18K11190).

Yoshiki Miura reports no disclosures.

Wado Akamatsu received a research grant from the Japan Agency for Medical Research and Development (20ak0101112h0002, GAPFREE).

Nobutaka Hattori received personal fees from Hisamitsu Pharmaceutical, Dai-Nippon Sumitomo Pharma, Otsuka Pharmaceutical, Novartis Pharma, GlaxoSmithKline, Nippon Boehringer Ingelheim, FP Pharmaceutical, Eisai, Kissei Pharmaceutical, Janssen Pharmaceutical, Nihon Medi-Physics, and Kyowa Kirin, outside of this work and received research grants from the Japan Agency for Medical Research and Development [program for Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS Beyond)] and JSPS (18H04043).

Shinji Saiki received research grants from the Ministry of Education, Culture, Sports, Science and Technology (25111007, 25111001) and JSPS (18H02744, 18KT0027, 18KK0242).

Study funding: Supported by the Japan Agency for Medical Research and Development-CREST (N.H.), the Advanced Genome Research and Bioinformatics Study to Facilitate Medical Innovation (GRIFIN) (N.H. 20km0405206h0005), the Ministry of Education, Culture, Sports, Science and Technology-Supported Program for the Strategic Research Foundation at Private Universities 2014-2018 (N.H.), Grant-in-Aid for Scientific Research on Priority Area (S.S. 25111007, 25111001), and Grant-in-Aid for Scientific Research (B) (S.S. 18H02744, 18KT0027, 18KK0242).

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