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RESEARCH ARTICLE

Nonmercaptalbumin as an oxidative stress marker in Parkinson's and PARK2 disease

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Abstract

Objective: To investigate the oxidized albumin ratio, which is the redox ratio of human nonmercaptalbumin (HNA) to serum albumin (%HNA), as a biomarker in idiopathic Parkinson's disease (iPD) and related neurodegenerative disorders. **Methods**: This prospective study enrolled 216 iPD patients, 15 patients with autosomal recessive familial PD due to *parkin* mutations (PARK2), 30 multiple system atrophy (MSA) patients, 32 progressive nuclear palsy (PSP) patients, and 143 healthy controls. HNA was analyzed using modified high-performance liquid chromatography and was evaluated alongside other parameters. **Results**: iPD and PARK2 patients had a higher %HNA than controls (iPD vs. controls: odds ratio (OR) 1.325, $P < 0.001$; PARK2 vs. controls: OR 1.712, $P < 0.001$). Even iPD patients at an early Hoehn & Yahr stage (I and II) showed a higher %HNA than controls. iPD patients had a higher % HNA than MSA and PSP patients (iPD vs. MSA: OR 1.249, $P < 0.001$, iPD vs. PSP: OR 1.288, $P < 0.05$). When discriminating iPD patients from controls, % HNA corrected by age achieved an AUC of 0.750; when discriminating iPD patients from MSA and PSP patients, an AUC of 0.747 was achieved. Furthermore, uric acid, an antioxidant compound, was decreased in iPD patients, similar to the change in %HNA. **Interpretation**: %HNA was significantly increased in iPD and PARK2 patients compared with controls, regardless of disease course and severity. Oxidative stress might be increased from the early stages of iPD and PARK2 and play an important role in their pathomechanisms.

Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by bradykinesia, tremor, and rigidity.[1] Although dopaminergic therapies can partially ameliorate motor dysfunction, nonmotor symptoms, including depression, cognitive impairment, sleep disorders, and autonomic dysfunctions can also affect the quality of life in PD patients.[2,3] Therefore, disease-modifying therapies against disease progression, which lead to these motor and nonmotor symptoms, are needed. It is thus essential to elucidate robust biomarkers associated with disease progression and pathomechanisms of PD.[4]

The main pathomechanisms of PD are believed to be environmental factors, aging, and genetics.[5,6] Oxidative stress is a critical factor in the dopaminergic neurodegeneration that occurs in the substantia nigra in PD.[7,8] Functional interactions between parkin/autosomal recessive familial PD due to *parkin* mutations (PARK2) and PTEN-induced putative kinase (PINK1)/autosomal recessive familial PD due to *PINK1* mutations (PARK6) are essential for mitochondria quality control.[9] Additionally, our previous investigations have shown that oxidative

stress markers are relevant in the evaluation of idiopathic PD (iPD) and PARK2, and examining oxidative or redox markers might be useful to understand the pathomechanisms of these diseases.[10,11]

Human nonmercaptalbumin (HNA) is the directly oxidized form of human serum albumin (HSA).[12] The redox ratio of HNA to HSA, defined as %HNA, reflects the systemic oxidative stress.[13–15] Recently, we generated a method to accurately measure HSA using high-performance liquid chromatography (HPLC).[16,17] In this study, we hypothesized that %HNA might be a robust biomarker of a hyperoxidative state in iPD and PARK2 and investigated %HNA in parkinsonism and related disorders.

Methods

Participants

We enrolled 216 patients with iPD (mean age 66.0 ± 0.63 years, female 114, male 102), 15 patients with PARK2 (mean age 52.1 ± 4.56 years, female 9, male 6), 30 multiple system atrophy (MSA) patients (mean age 64.6 ± 1.69 years; female 21, male 9), 32 progressive nuclear palsy (PSP) patients (mean age 73.3 ± 1.58 years; female 19, male 13), and 143 healthy controls (mean age 63.6 ± 0.85 years; female 70, male 73). Participants with high serum creatinine (>1.0 mg/dL), uncontrolled diabetes mellitus (glycoalbumin ≥ 24.0%), or chronic liver failure were excluded. All participants with iPD, PARK2, MSA, or PSP had been treated as outpatients at Juntendo University in Tokyo, Japan. Control participants were randomly selected among the patients' spouses and volunteers who were free of neurological and psychiatric illnesses. Movement disorder specialists (T.H. and SI.U.) diagnosed all iPD, PARK2, MSA, and PSP patients according to the Movement Disorder Society (MDS) clinical diagnostic criteria for PD, Gilman's criteria for MSA, and the MDS criteria for PSP.[18–20] All PARK2 patients were confirmed to carry homozygous or compound heterozygous mutations in *parkin* by the genetic analysis. The genetic analysis methods used to detect *parkin* mutations have been described previously.[21]

Ethics statement

Written informed consent was obtained from all subjects participating in this study, according to the Declaration of Helsinki. The study was approved by the Ethics Committee of the Juntendo University School of Medicine (No. 2018095).

Sample collection

All fasting blood samples were collected in the outpatient department of Juntendo University Hospital between January 2013 and February 2019. Following an overnight fast (12–14 h), a serum sample was obtained in 8-mL blood collection tubes (78447 SIM-L1008SQ3, Kyokuto Pharmaceutical Ind. Co. Ltd.) followed by two or three inversions. The samples were then allowed to stand for 30–60 min at 4 °C followed by centrifugation for 10 min at 2,660*g*. The serum was then separated, placed in collection tubes, and stored in a deep freezer until analysis.

HNA analysis

HNA was measured using a recently developed anion-exchange HPLC system, as described previously.[16,17] Briefly, the anion exchange column (50 × 7.6 mm ID) was packed with a polyvinyl alcohol gel introduced with diethyl amine. The conditions were as follows: eluent A was a solution of 25-mM phosphoric acid buffer containing 60-mM sulfuric acid sodium (pH 6.0), and eluent B was a high-concentration magnesium chloride solution. The flow rate was 1 mL/min after equilibrating the column for 4.5 min with eluent A. The linear gradient time from eluent A (100%) to eluent B (100%) was programmed for 7.5 min, and the total measurement time was 12 min per sample. The sample size was 3 μL, and the temperature was 40 °C. The excitation and emission wavelengths were 280 nm and 340 nm, respectively, and the repeatability (within-day variability) and reproducibility (day-to-day variability) were 0.3% and 0.27% (coefficient of variation), respectively. The measured values were expressed as %HNA = HNA area/total HSA area × 100 using the area value (μV × seconds) of each peak of the chromatogram obtained as a result of HPLC.

Statistical analysis

Statistical analysis was performed using JMP 13.2 software for Mac (IBM Corp., Armonk, NY) and Prism 8 for MacOS (GraphPad Software, Inc., San Diego, CA). Demographic and clinical data were examined for normality with visual histograms and the Shapiro–Wilk test. Data were compared using unpaired t-tests or one-way analysis of variance, followed by the Wilcoxon rank sum test or Kruskal–Wallis test, or Steel's test for non-normally distributed data. Categorical variables were analyzed with a Chi-square test.

Associations between age and %HNA or uric acid (UA) were determined using Spearman's rank correlation analysis. The relationships between %HNA and disease severity, disease duration, serum albumin, serum γ-globulin, and antiparkinsonian drugs were determined using multiple regression analysis and logistic regression analysis for continuous and ordinal variables. Receiver operating characteristic (ROC) curve analysis using Youden index

maximums (sensitivity + specificity − 1) was conducted to assess the discrimination capacity of the measurements of %HNA for patients with iPD versus control subjects and for iPD patients versus MSA or PSP patients. A P value < 0.05 was considered statistically significant.

Data Availability Statement

Anonymized data can be obtained by request from any qualified investigator for purposes of replicating procedures and results.

Results

Patient profiles

Table S1 shows the demographic characteristics of the 216 iPD patients, 15 PARK2 patients, 30 MSA patients, 32 PSP patients, and 143 healthy controls. Sex did not differ between the controls and iPD, PARK2, MSA, and PSP patients. However, age at sampling was significantly older in PSP patients than in controls. In addition, the disease duration from the onset of initial motor symptoms was longer in the PARK2 group than in the iPD, MSA, and PSP groups. The Hoehn & Yahr (H&Y) stage, unified Parkinson's disease rating scale (UPDRS) part III score, levodopa daily dose (LDD), %HNA, and mutations in *parkin* are displayed in Table S1. UA, IgG, aspartate aminotransferase, and glycoalbumin levels in all participants were within normal limits.

%HNA was positively correlated with age and was higher in iPD and PARK2 patients than controls

The %HNA was positively correlated with age at sampling in each group except for PSP patients, whose average age was older than that of the others. The iPD group had a higher %HNA than controls, whereas the PARK2, MSA, or PSP groups showed no significant difference in %HNA compared with controls. Additionally, iPD patients had a higher %HNA than MSA and PSP patients (Fig. 1A–C).

To exclude the influences of age and sex, we analyzed a logistic regression model that was corrected for both factors. As expected, the %HNA of iPD patients was higher than that of the controls. Furthermore, in PARK2 patients, the %HNA was also significantly higher than in the controls. For the parkinsonian disorders, including both MSA and PSP, the %HNA of PD patients was significantly higher than that of MSA and PSP patients.

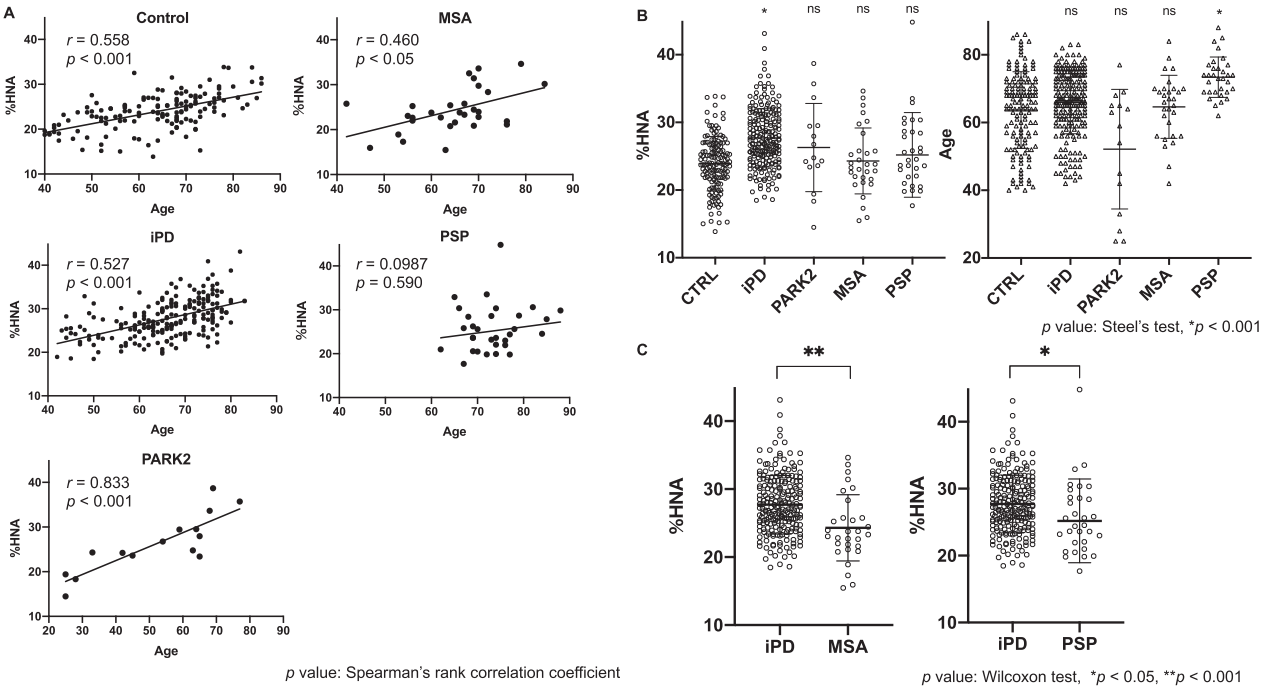

Figure 1. %HNA was positively correlated with age and was higher in iPD patients than in controls. (A) %HNA was positively correlated with age in each group, except for the PSP group. (B) %HNA was higher in the iPD group than the controls. Mean values are displayed. (C) %HNA was higher in the iPD group than in the MSA and PSP groups. %HNA: the ratio of human nonmercaptalbumin to human serum albumin, iPD: idiopathic Parkinson's disease, CTRL: control subject, MSA: multiple system atrophy, PSP: progressive supranuclear palsy, ns: not significant.

Additionally, the %HNA of MSA and PSP patients did not significantly differ from controls even when accounting for age and sex (Table 1).

Association of %HNA with disease progression and levodopa dose

To compare controls with each iPD type categorized by disease severity, we used H&Y stages to classify iPD patients into two subgroups: a mild group (H&Y I, II) and a severe group (H&Y III, IV, V). The %HNA was significantly higher in each iPD subgroup than in controls (Fig. 2A–D). In the severe group, age was significantly higher than in controls, but there was no difference in age between the mild group and controls.

PARK2 patients with H&Y stage III, but not stages I and II, had a significantly higher %HNA than controls. The mean age of PARK2 patients with H&Y stages I and II was significantly younger than that of the controls (Fig. 2A–D). To investigate whether dopaminergic treatment, disease severity, or duration might affect %HNA in iPD and PARK2, we performed a multiple regression analysis. UPDRS-III scores, H&Y stage, disease duration, and LDD did not correlate with %HNA (Table 2).

Table 1. %HNA was higher in iPD and PARK2 compared with controls, regardless of age and sex.

Independent variable	OR (95% CI)	*P* value
iPD vs. controls		
% HNA	1.325 (1.231–1.436)	<0.001
Age	0.961 (0.935–0.988)	<0.01
Sex (female:male)	1.300 (0.810–2.097)	0.276
PARK2 vs. controls		
% HNA	1.712 (1.358–2.314)	<0.001
Age	0.815 (0.731–0.885)	<0.001
Sex (female:male)	1.470 (0.388–5.884)	0.568
MSA vs. controls		
% HNA	1.108 (0.911–1.141)	0.744
Age	1.004 (0.961–1.049)	0.852
Sex (female:male)	2.442 (1.074–5.598)	<0.05
PSP vs. controls		
% HNA	0.968 (0.906–1.097)	0.522
Age	1.123 (1.065–1.195)	<0.001
Sex (female:male)	1.582 (0.682–3.775)	0.289
PD vs. MSA		
% HNA	1.249 (1.115–1.415)	<0.001
Age	0.974 (0.923–1.025)	0.329
Sex (female:male)	1.846 (0.776–4.673)	0.167
PD vs. PSP		
% HNA	1.288 (1.153–1.465)	<0.001
Age	0.831 (0.769–0.889)	<0.001
Sex (female:male)	0.635 (0.257–1.548)	0.316

iPD, idiopathic Parkinson's disease; %HNA, the ratio of human nonmercaptalbumin to human serum albumin; MSA, multiple system atrophy; PSP, progressive supranuclear palsy; OR, odds ratio; CI, confidence interval. *P* values were obtained by logistic regression analysis.

Diagnostic performance

In discriminating iPD patients from controls, serum % HNA corrected by age achieved an AUC of 0.750 ($P < 0.001$, 95% CI 1.227–1.431), with a sensitivity of 68.9% and specificity of 70.7%. In addition, in discrimination of iPD patients from both MSA and PSP patients, serum %HNA corrected by age achieved an AUC of 0.747 ($P < 0.001$, 95% CI 1.153–1.366), with a sensitivity of 81.9% and specificity of 62.9%. Furthermore, in discrimination of iPD patients from MSA and PSP patients separately, serum %HNA corrected by age achieved AUCs of 0.714 ($P < 0.001$, 95% CI 1.131–1.436) and 0.820 ($P < 0.001$, 95% CI 1.143–1.432), with sensitivities of 78.7% and 73.6% and specificities of 63.3% and 81.2%, respectively (Fig. 3).

Association of %HNA with UA

To confirm that %HNA is a potential oxidative stress marker, we analyzed the correlation between %HNA and UA, which is an antioxidant compound. The iPD, MSA, and PSP groups had lower UA serum levels than controls. Then, to discriminate the influence of sex, we analyzed the correlation between %HNA and UA in each sex. In both females and males, the iPD and PSP groups had lower UA levels than controls (Fig 4A).

Next, we analyzed the correlation between UA and % HNA in each sex. Females in the iPD, control, and MSA groups had positive correlations between UA and %HNA, whereas males in the iPD and PARK2 groups tended to have negative correlations between UA and %HNA (Fig 4B).

Discussion

In this study, we investigated the usefulness of %HNA as an oxidative stress marker in iPD and PARK2. We identified a strong correlation between this compound and age in a relatively large cohort. The correlation was present not only in control subjects, but also in iPD and PARK2 patients. In addition, patients with iPD or PARK2 had a higher %HNA than control subjects, even after age was taken into consideration. In both iPD and PARK2 patients, %HNA was not correlated with disease duration or antiparkinsonian drug treatment.

Oxidative damage is closely associated with cellular damage and results in several disorders including neurodegenerative diseases, cancer, diabetes mellitus, liver dysfunction, and renal diseases. Oxidative stress may also

play an important role in the pathomechanisms of cellular senescence.[22] Oxidative stress generates reactive oxygen species (ROS) such as superoxide (O_2^-), hydroxyl radicals (HO^-), hydroperoxyl radicals (HO_2), hydrogen peroxide (H_2O_2), nitric oxide (NO), and nitrogen dioxide (NO_2). ROS can generate oxidative damage in macromolecules such as lipids, DNA, and proteins. In addition, a hyperoxidative stress state might lead to the disruption of redox-regulated signaling mechanisms.[22]

Previous investigations have revealed an association between aging and increasing oxidative stress markers in the serum, plasma, and urine.[23] Compounds that reflect systemic oxidative damage can therefore be considered candidate biomarkers of aging. In the present study, we investigated HNA, which is the oxidized form of HSA. The cysteine-34 (Cys-34) thiol of serum albumin is a stabilizer of NO in plasma and has been defined as a marker of redox status.[24] In our cohort, %HNA demonstrated a marked correlation with aging, consistent with a previous report;[16] therefore, this compound is an excellent biomarker of oxidative stress. Patients with PD might have an earlier, faster aging speed after disease onset than healthy individuals, suggesting that aging is the pivotal risk factor for pathogenesis of iPD and PARK2.

Table 2. %HNA was not correlated with disease severity, duration, or levodopa dose.

	Dependent variable: %HNA		
iPD	*P* value	PARK2	*P* value
Age	<0.001	Age	0.0781
H&Y	0.828	H&Y	0.948
UPDRS	0.251	UPDRS	0.985
Disease duration	0.831	Disease duration	0.714
LDD	0.536	LDD	0.920

iPD, idiopathic Parkinson's disease; %HNA, the ratio of human nonmercaptalbumin to human serum albumin; SE, standard error; H&Y, Hoehn & Yahr; UPDRS, Unified Parkinson's Disease Rating Scale; LDD, Levodopa dose. *P* values were obtained by multiple regression analysis.

In contrast, disease severity, disease duration, and LDD were not correlated with %HNA in a multiple regression analysis. However, aging was associated with disease severity. iPD patients with H&Y I and II and PARK2 patients with H&Y III had higher %HNA than controls, even though their average age was similar to that of controls. Aging is one of the putative risk factors for developing iPD. Considering the increased %HNA in iPD compared with controls regardless of disease duration,

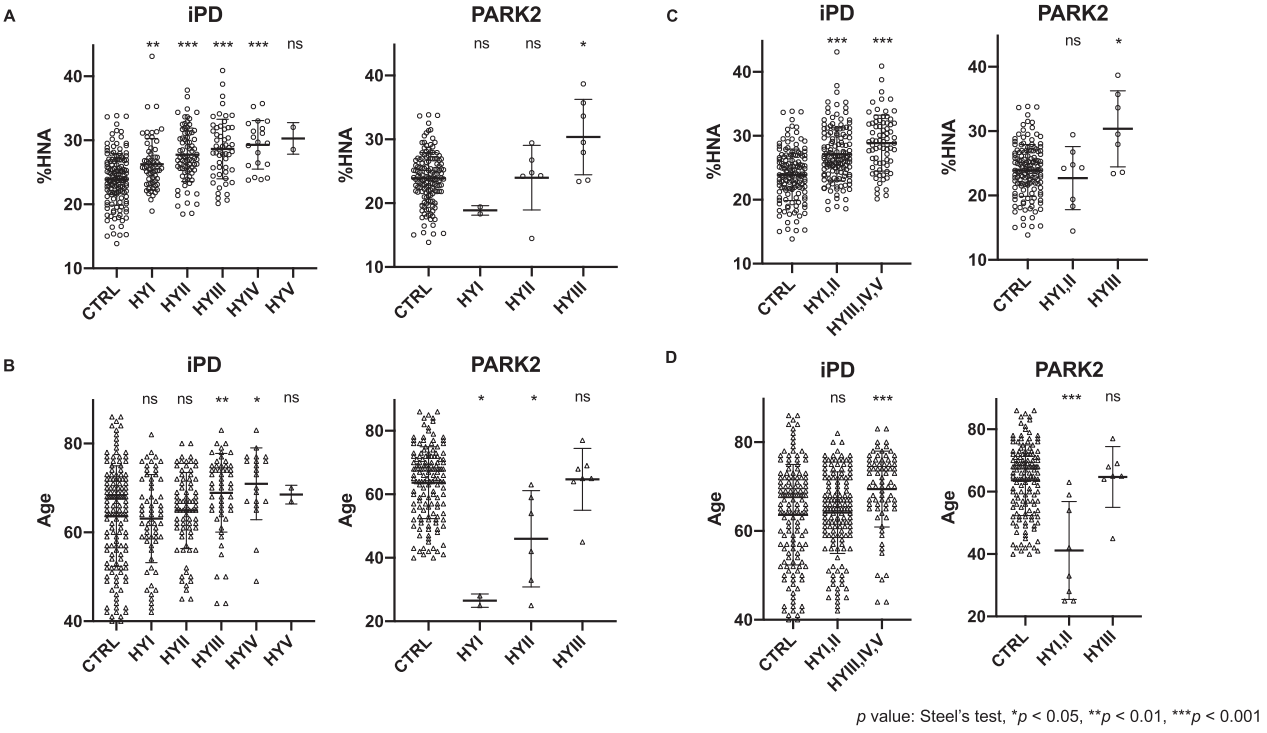

Figure 2. Correlation between %HNA and disease severity. (A–D) To compare controls and patients with iPD and PARK2 for disease severity, we classified patients by H&Y stage into two groups: a mild group (H&Y I, II) and a severe group (H&Y III, IV, V). %HNA: the ratio of human nonmercaptalbumin to human serum albumin, iPD: idiopathic Parkinson's disease, CTRL: control subject, H&Y: Hoehn & Yahr, ns: not significant.

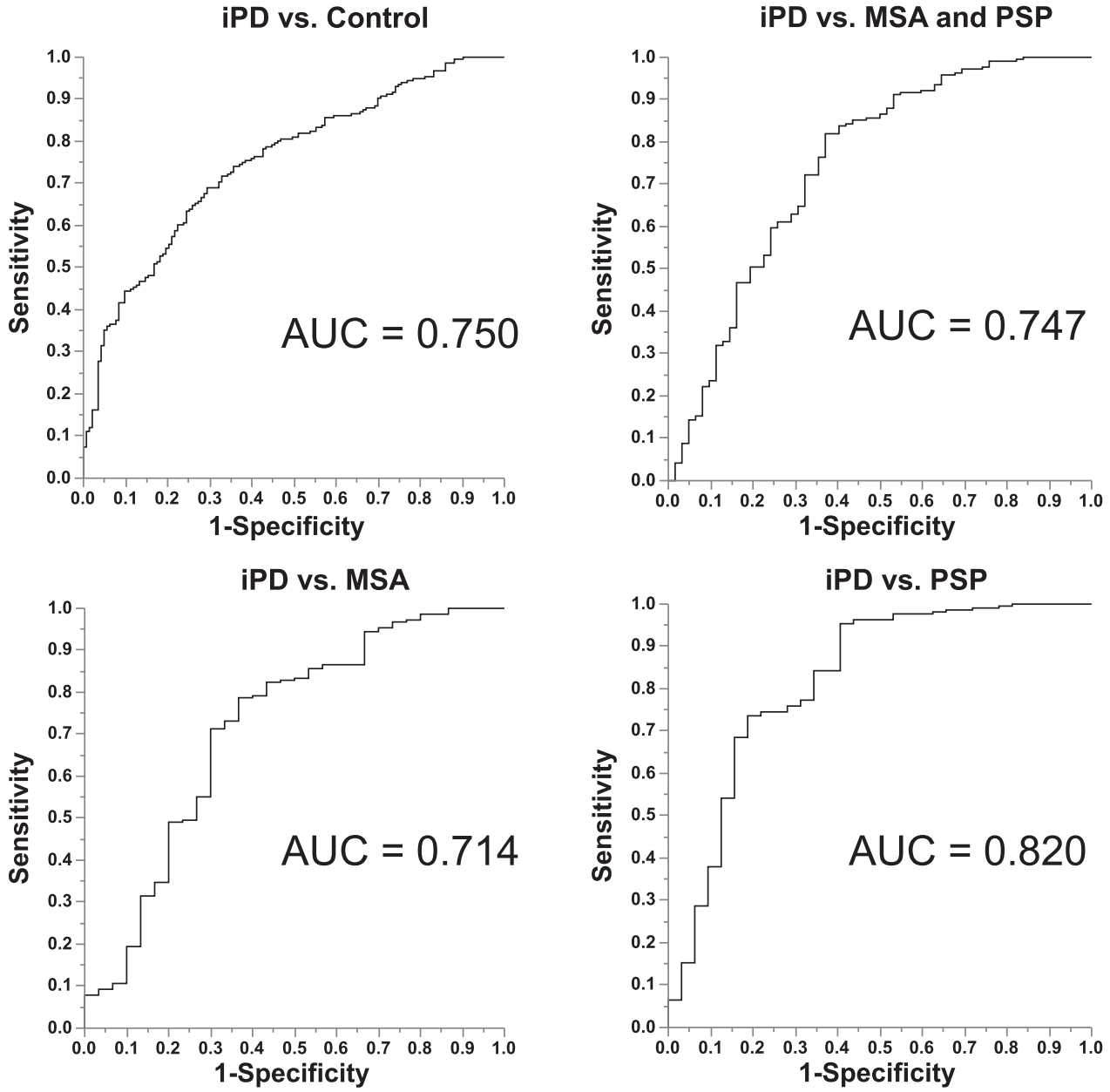

Figure 3. %HNA may be a reliable biomarker for distinguishing iPD patients from controls and MSA and PSP patients. The ROC curve and corresponding AUC were analyzed using the Youden index. The optimal cutoff point was measured by the maximum sensitivity and specificity. %HNA: the ratio of human nonmercaptalbumin to human serum albumin, iPD: idiopathic Parkinson's disease, CTRL: control subject, MSA: multiple system atrophy, PSP: progressive supranuclear palsy, ROC: receiver operating characteristic, AUC: area under the curve.

age-induced oxidative stress may trigger disease onset.[25] Previously, several investigations have revealed oxidative stress-related compounds as diagnostic biomarkers of iPD.[26,27] Our study confirmed the significance of lower serum levels of UA, which is a powerful antioxidant in iPD[28] (Fig. 4A). Therefore, the %HNA may reflect systemic oxidative stress, similar to other oxidative stress-related compounds. Interestingly, the correlation between %HNA and UA tended to be opposite in men and women in all groups (Fig. 4B). A sex difference of the UA levels in PD has been also reported in several epidemiological studies.[29–31] This discrepancy might be caused by sex hormones, age, and environmental and/or genetic factors. Additionally, UA plays a direct role in antioxidation, whereas oxidative albumin results from oxidative stress. Thus, UA and oxidative albumin are distinct oxidative

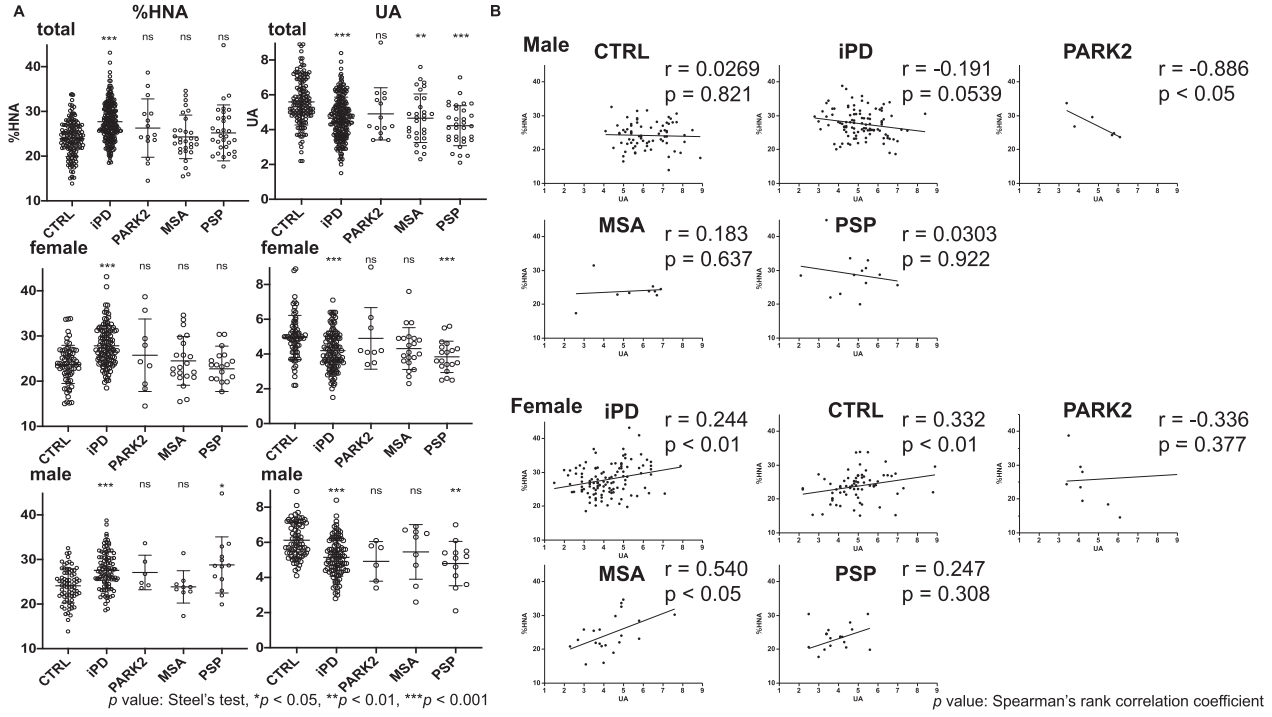

Figure 4. Association between %HNA and UA, which is an antioxidant compound. (A) Analysis of UA in all groups and in each sex. (B) Analysis of the correlation between UA and %HNA in each sex. %HNA: the ratio of human nonmercaptalbumin to human serum albumin, UA: uric acid, iPD: idiopathic Parkinson's disease, CTRL: control subject, MSA: multiple system atrophy, PSP: progressive supranuclear palsy, ns: not significant.

stress markers. Previously, we have shown that the levels of urine 8-hydroxydeoxyguanosine, which is regarded as an indicator of mutagenesis consequent to oxidative stress, and the bilirubin/biliverdin ratio, which reflects ROS levels, are linked with disease progression in iPD.[10,27] In both iPD and PARK2 patients in our current cohort, a longer disease duration and the progression of disease severity were also linked to aging. Disease severity of iPD and PARK2 patients tends to be associated with higher %HNA; however, to elucidate the relationship between oxidative stress and disease progression and severity, a longitudinal investigation should be conducted.

Furthermore, we revealed that PARK2 patients had a higher %HNA than controls. Parkin functions as an E3 ubiquitin ligase, which monoubiquitinates and polyubiquitinates target proteins.[9] Many groups, including our own, have previously shown that parkin also participates in quality control of damaged mitochondria, together with PINK1.[32–34] Parkin is widely distributed in systemic tissue; therefore, impairment of the mitochondrial quality control system because of dysfunction of parkin might be associated with general oxidative stress, resulting in the elevation of oxidized albumin. In this context, the elevation of ROS levels in iPD and PARK2 might reflect mitochondrial dysfunction. We speculated that the decreased parkin levels in iPD patients might be associated with the elevation of oxidized albumin. Therefore, we investigated the protein levels of parkin in serum using Western blots. However, we could not determine the protein levels of parkin in human serum. Because the molecular weight of parkin protein is approximately 50 kDa, which is similar to those of IgG heavy chain and albumin, these proteins might interfere in the detection of parkin (Supplementary File). Additionally, the serum levels of parkin might be quite low (Supplementary File). Therefore, further investigations focusing on the role of parkin in the pathomechanisms of iPD are needed.

Oxidative stress might be also associated with the pathogenesis of atypical parkinsonism, including MSA and PSP.[35,36] However, it remains unknown whether systemic oxidative stress might induce the brain damage observed in these disorders. In this study, we did not find elevation of oxidative albumin in MSA and PSP patients. However, these patients showed decreased serum UA levels compared with the controls, similar to previous reports.[37,38] These results might indicate that systemic oxidative stress may be involved in the pathomechanisms of atypical parkinsonism but to a lesser extent than in iPD and PARK2. Furthermore, these findings suggest that combining systemic oxidative stress markers, including %

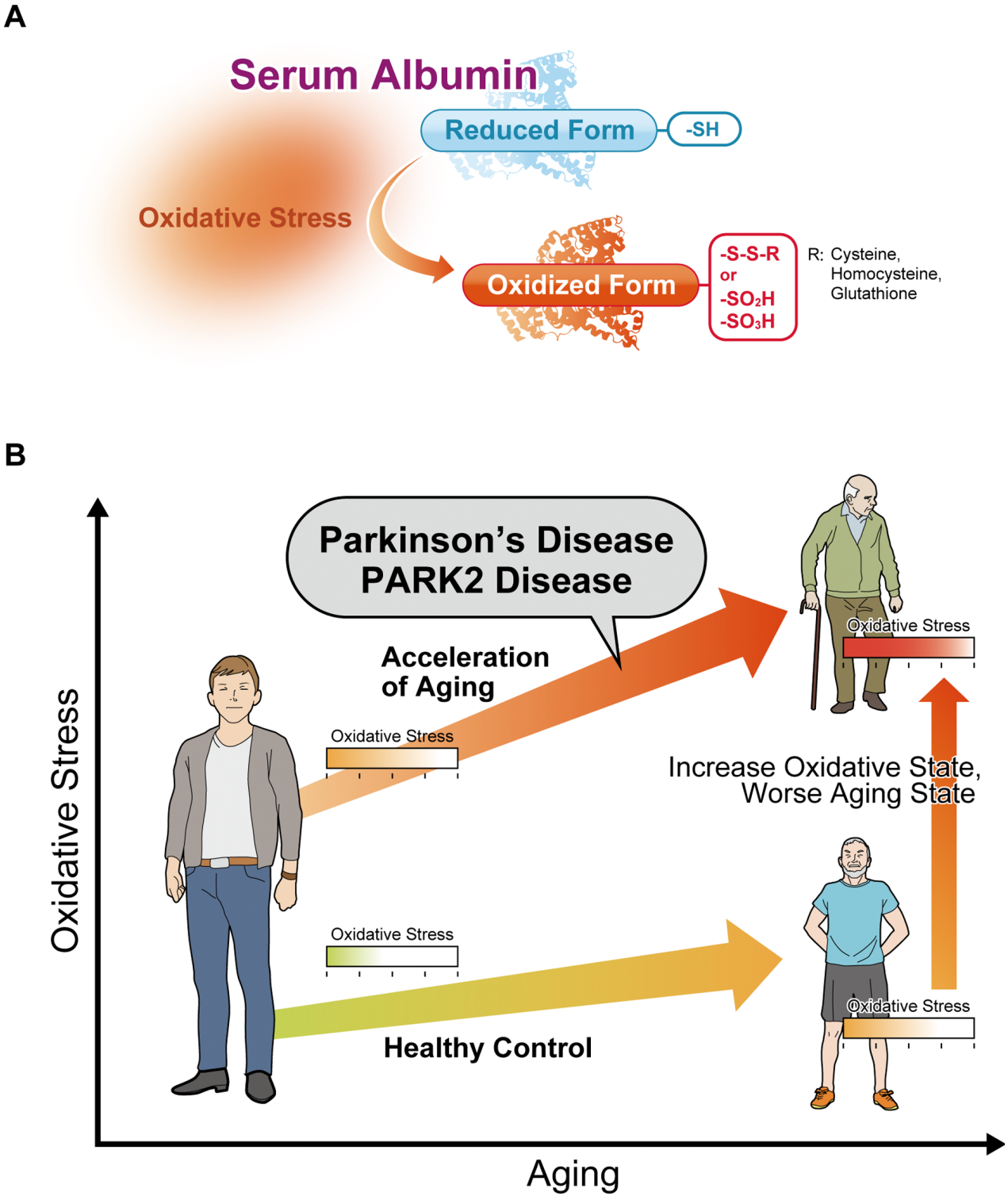

Figure 5. Elevated levels of the oxidized form of albumin accelerate aging and affect the pathomechanisms of Parkinson's and PARK2 disease. (A) Human serum albumin is converted from the reduced form to the oxidized form by oxidative stress in the whole body. (B) Elevation of oxidative stress may accelerate aging and affect the pathomechanisms of Parkinson's and PARK2 disease.

HNA and UA, might be useful for diagnosis and understanding the pathomechanisms of iPD, PARK2, and atypical parkinsonism.

We should consider the limitations of our study. First, the numbers and average age of patients in each group were not matched among healthy controls, iPD patients, PARK2 patients, MSA patients, and PSP patients. However, we adjusted for this using multiple regression tests, which revealed a statistically significant difference in %HNA between the iPD and PARK2 patients and other groups. Second, our patients were diagnosed clinically according to MDS clinical criteria for PD and PSP and Gilman's criteria for MSA,[18–20] but we did not confirm these diagnoses via autopsy. Third, the influence of antiparkinsonian drugs should be considered. However, the LDD was not correlated with %HNA using multiple regression analysis in iPD and PARK2 patients. Although patients with atypical parkinsonism were also taking antiparkinsonian drugs, including levodopa, dopamine agonists, and MAOB inhibitors, the %HNA of these patients was similar to healthy subjects. Additionally, in recent reports, a metabolomics analysis in iPD revealed that antiparkinsonian drugs are less effective on their metabolites;[10,39,40] therefore, we consider that antiparkinsonian drugs had little influence on the results. Fourth, a longitudinal study should be conducted to establish a direct association between hyperoxidative albuminemia and disease progression. However, our results are in line with previous findings from clinical investigations that have shown an association between oxidative stress and PD. Therefore, despite the limitations of our study, we believe that %HNA may be a useful tool for measuring oxidative stress levels in iPD and PARK2.

Conclusion

In this study, %HNA was increased in iPD and PARK2 patients, regardless of disease course and severity. Oxidative stress might be increased from the early disease stage and could be an important factor in the pathomechanisms of iPD and PARK2 (Fig. 5A–B).[41] In particular, parkin is involved in mitochondrial maintenance; therefore, oxidative stress may be caused by mitochondrial dysfunction. In previous reports, it has been controversial whether antioxidants, such as coenzyme Q10 and α-tocopherol, can inhibit disease progression.[42] If oxidative stress could be targeted in the prodromal or very early phase of iPD and PARK2, it may be possible to modify the disease course. In this context, oxidative stress markers, including oxidative albumin, may be useful biomarkers for diagnosing prodromal PD and for monitoring PD treatment.

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Author Contributions

S-I.U., T.H., H.I., and N.H. were involved in the study concept and design. T.H., S-I.U., A.O., S.S., Y.O., A.M., T.K., M.F., H.T-A., A.K., N.Y., T.N., M.K., K.Y., Y.Y., H.I., and N.H. were involved in the acquisition of data. S-I.U. and T.H. were involved in the analysis and interpretation and drafting of the manuscript. T.H., H.I., and N.H. were involved in the critical revision of the manuscript for important intellectual content. N.Y., T.N., M.K., K.Y., Y.Y., and H.I. were involved in technical and material support. T.H., H.I., and N.H. were involved in study supervision.

Conflict of Interest

Nothing to report.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Participants' characteristics.
Figure S1. Biochemical experiments examining serum parkin in the control, Parkinson's disease, and PARK2 disease groups: Western blotting.

