Original Article

Evidence-Based Discriminant Analysis: A New Insight into Iron Profile for the Diagnosis of Parkinson's Disease

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Abstract

Introduction: Parkinson's disease is the second most common neurodegenerative disorder. Neurochemical studies have implicated metals in pathogenesis of PD. **Objectives:** To examine the association of serum iron, transferrin, ferritin, transferrin saturation and UIBC in PD patients and to derive the Discrimination Function with scores of these variables to correctly classify PD cases and healthy controls. **Methods:** In the present study, identification of biomarker pool in case-control study involving 79 PD cases and 80 healthy controls were performed. **Results:** The results of independent t-test analysis showed that PD cases presented significantly higher ($P < 0.01$) level of transferrin, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and urea than controls. As only one-third of transferrin is saturated with iron, so the transferrin present in serum has the extra binding capacity (67%), this is called UIBC. Discriminant analysis was performed to determine the factors that best discriminate between the categories of an outcome variables (Disease status = PD and Control) and total of five biochemical independent variables (UIBC, transferrin, iron, transferrin saturation, and copper) were taken into consideration. UIBC has emerged out to be highest discriminating, powerful and independent variable among considered independent variables, which indicates iron deficiency. After development of Discriminant Function (Z) and calculation of discriminant function cut points, a cross-validation analysis of PD cases and controls were conducted. The sensitivity of the developed model was 98.73% and specificity 83.75%. Receiver operating characteristics (ROC) was plotted, and the findings of ROC curve corroborated with the results obtained from discriminant function analysis. **Conclusion:** Prospective validation of Discriminant model in large cohort is warranted in future studies.

Keywords: Discriminant analysis, ferritin, iron profile, Parkinson's disease, ROC curve, transferrin

Introduction

With increasing life expectancy/span, there has been increased prevalence of age-related neurodegenerative disorders in the society. Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting 2% of the population over the age of 65 years.[1] There have been multiple factors like age, dietary habits, genetics, occupational and environmental factors, playing major role in the pathology of PD.[2] Neurochemical studies have implicated metals in pathogenesis of PD and include Manganese, Aluminium, Zinc, Copper and Iron.[3] Iron is the most abundant transition metal in the body and has a unique distribution in the brain.[3] It plays a pivotal role in many physiological functions in brain, including neurotransmission and myelination.[3] Iron is found in abundance in the basal ganglion. It is a cofactor for the enzyme tyrosine hydroxylase which is involved in the dopamine synthesis pathway.[4] In addition to this, iron also serves as an electron donor as well as acceptor. Hence, it is also associated with increased oxidative stress,[5] oligomerisation of alpha-synuclein protein and formation of Lewy bodies. Iron has been investigated extensively and is implicated in the pathogenesis of PD.[2] This is further stressed by reports showing association of low dietary iron and iron deficiency anaemia with PD.[6]

Transferrin transports iron in ferric form, from liver to different tissues via circulation. It also prevents iron from reacting with other molecules by attenuating their redox activity. Raised total iron binding capacity (TIBC) and transferrin levels during iron deficiency may increase the risk of PD.[7] Ferritin stores iron in ferric form, which is a nontoxic form of iron. Ferritin also acts as an antioxidant. Decreased level of ferritin has been demonstrated in the substantia nigra (SN) of PD patients as compared to controls.[8]

Various epidemiological studies have explored the relationship between serum/plasma iron levels and PD risk,[2] as it is easier to measure iron levels in blood as compared to brain/CSF. Presently, limited data is available regarding the association of metals in serum with PD, with inconsistent results.[2] In the present study, we determined the iron profile in serum of PD cases and healthy control to assess the association

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of these independent variables in these two groups. Linear Discriminant Analysis (LDA) was applied on these parameters to differentiate PD cases and healthy controls. This can be used as a diagnostic tool to classify PD cases and healthy subjects based on iron and it's profile.

Materials and Methods

Clinical phenotype assessment

In the present case control cohort study, 79 PD cases and 80 healthy controls were enrolled in the Department of Neurochemistry, Institute of Human Behavior and Allied Sciences (IHBAS), New Delhi, India. PD cases were diagnosed as per UK Brain Bank Criteria. The control samples collected from the community did not have recent history of stroke, cerebrovascular surgery, head injury, depression or any other mental disorder. The study was approved by the ethical committee of IHBAS. Written consent was obtained from all the PD cases and healthy controls. In all participants copper, iron, iron profile and ceruloplasmin levels were measured in serum along with routine laboratory tests.

Biochemical assessment

Non-fasting blood samples (10–12 ml) were collected by venipuncture in plain evacuation tubes from PD cases and healthy controls, following all universal precautions. After 30 minutes of collection, all samples were centrifuged for 15 minutes at 1500 rpm at room temperature. After centrifugation, serum was collected and stored at -20°C for analysis of copper, iron, iron profile (transferrin, ferritin, Total iron binding capacity and unsaturated iron binding capacity) and ceruloplasmin along with routine laboratory tests on fully automated autoanalyser AU 480 by Beckmann Coultre Pvt. Ltd. Serum copper, iron and transferin were measured by spectrophotometery using kits from Fortress diagnostic Ltd, UK and ceruloplasmin by immunoturbidimetry using kits from Randox Pvt. Ltd. Serum ferritin was estimated by electrochemiluminiscence immunoassay on Cobas e601 from Roche Diagnostics India Pvt. Ltd. TIBC was calculated mathematically from the estimated serum transferrin (TRF) using formula: TIBC (μmol/L)=25.0×TRF g/L. UIBC is calculated by using formula: UIBC (μmol/L) = TIBC (μmol/L) - Serum Iron (μmol/L).

Statistical analysis

All the analysis were carried out by SPSS software package, version 17, (SPSS Inc., Chicago, IL, USA). Descriptive statistics (Mean, SD and Percentage) was applied to describe the data of PD cases and healthy control groups and independent student-t test/Chi-square test was used to find out the statistically significant difference. The Discriminant Function analysis was applied to develop a linear model (Discriminant Function) of independent biochemical marker variables by which PD cases and controls could be correctly classified. The Kolmogrorow-Smirnov's (K-S) test was applied to verify, if the variables had normal distribution, $P < 0.05$. Receiver Operating Characteristics (ROC) curve was also plotted to verify the classification rule developed by Discriminant Function model.

Results

Demographic and clinical characteristics

Table 1 shows that all the three socio-demographic variables (age, gender and place of residence) statistically matched ($P > 0.05$) in PD cases and healthy control groups. Use of alcohol and dietary pattern had significant association with disease status ($P < 0.05$).

Association of biochemical biomarkers

The comparison of biochemical parameters including iron profile among PD cases and healthy controls is shown in Table 2. Significant differences were observed among the two groups in the distribution of bilirubin, copper, iron, transferrin, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), transferrin saturation and copper. In particular, higher levels of transferrin, TIBC, UIBC and urea were observed in PD cases as compared to controls ($P < 0.01$), whereas iron, copper, transferrin saturation, bilirubin, alanine transferase, aspartate transferase and HDL-C were low in PD group ($P < 0.01$).

Discriminant analysis was performed to determine the factors that best discriminates between the categories of an outcome variables (Disease status = PD and Control). Major assumption of discriminant analysis is data, generated by each predictor variable and should follow the normal distribution. The normality of each biochemical variable (UIBC, transferrin, serum iron, transferrin saturation, and copper) was examined by K-S test in PD cases and healthy controls. It was found

Table 1: Socio-demographic Profile of PD patients and Healthy Controls

Variables	PD (n = 79)	Control (n = 80)	P-value
Age (yrs.) Mean (SD)	54.57 (10.02)	52.11 (8.75)	0.10*
Gender			0.92
Male	48 (60.80)	48 (60.00)	
Female	31 (39.20)	32 (40.00)	
Habitat			0.48
Urban	63 (79.70)	60 (75.00)	
Rural	16 (20.30)	20 (25.00)	
Alcohol			0.00
Yes	5 (6.30)	19 (23.80)	
No	74 (93.70)	61 (76.30)	
Smoking			0.11
Yes	11 (13.90)	19 (23.80)	
No	68 (86.10)	61 (76.30)	
Diet			0.00
Vegetarian	34 (43.00)	53 (66.30)	
Non-vegetarian	45 (57.00)	27 (33.80)	
Drinking Water			0.48
Tap	63 (79.70)	60 (75.00)	
Underground	16 (20.30)	20 (25.00)	

*t-independent test

Table 2: Biochemical Profile and Biological Variables of Metals in PD cases and Healthy controls

Variables (Units) (In Serum)	Mean (SD)		*P*
	PD (*n*=79)	Control (*n*=80)	
Transferrin (g/L)	3.64 (0.62)	3.30 (0.48)	0.00*
TIBC (µmol/L)	91.48 (15.67)	82.75 (12.02)	0.00*
Iron (µmol/L)	13.55 (2.49)	23.25 (4.44)	0.00*
UIBC (µmol/L)	77.96 (15.19)	59.50 (12.60)	0.00*
Transferrin saturation	15.09 (3.34)	28.72 (7.06)	0.00*
Copper (µg/dL)	114.05 (10.21)	144.13 (30.74)	0.00*
Ceruloplasmin (mg/dL)	36.78 (7.90)	38.78 (7.27)	0.10
Ferritin (µg/L)	65.62 (45.09)	95.71 (107.22)	0.23
Urea (mg/dL)	31.68 (11.48)	24.78 (7.85)	0.00*
Creatinine (mg/dL)	0.99 (0.19)	0.92 (0.30)	0.10
Uric Acid (mg/dL)	5.33 (1.46)	5.08 (1.16)	0.23
Bilirubin (mg/dL)	0.57 (0.27)	0.79 (0.31)	0.00*
SGOT/AST (IU/L)	20.43 (6.83)	31.28 (17.81)	0.00*
SGPT/ALT (IU/L)	14.52 (7.37)	31.44 (16.55)	0.00*
ALP (IU/L)	95.97 (29.43)	98.46 (34.23)	0.62
Total Protein (g/dL)	7.33 (0.87)	7.10 (0.68)	0.06
Albumin (g/dL)	4.25 (0.40)	4.20 (0.50)	0.54
GGT (IU/L)	32.19 (28.55)	30.58 (15.70)	0.66
Total Cholesterol (mg/dL)	175.95 (48.90)	186.69 (42.64)	0.14
Triglyceride (mg/dL)	138.96 (77.62)	136.79 (53.04)	0.84
HDL-C (mg/dL)	42.43 (7.94)	47.41 (10.97)	0.00*
LDL-C (mg/dL)	101.49 (32.39)	111.69 (40.24)	0.08

*Significant (P<0.01)

that the null hypothesis i.e., sample data are not significantly different than a normal population, was accepted ($P > 0.05$) for each predictor.

In the present study, only iron profile variable (UIBC, transferrin, serum iron, transferrin saturation, and copper) were considered for discriminant analysis due to their strong association with outcome variable. The canonical relation, which gives a correlation between the discriminant score and the level of outcome variable was found to be 0.83. The Eigen value, related to the canonical correlation, was found to be 2.15. It explains how best discriminating ability the function possesses. The Wilks' Lambda test was performed to assess whether the discriminating power of the developed function is statistically significant or not. It was observed that Wilks' Lambda for said function was 0.32 ($p < 0.01$). This indicated that the group means were different from each other.

During stepwise LDA, serum iron was excluded from the model as it failed minimum tolerance level (0.001). Hence, predictive equation was followed from the unstandardized canonical discriminant function coefficients, which is as follows:

Discriminant Function (Z) = (2.00) + (0.327) × UIBC (µmol/L) + (-7.215) × Transferrin (g/L) + (0.055) Transferrin Saturation + (-0.005) × Copper (µg/dL)

UIBC (µmol/L) emerged out to be highest discriminating and powerful independent variable among considered independent variables.

The discriminant function was constructed with the unstandardized canonical discriminant function coefficient. Since the group size (PD cases = 79, Controls = 80) were unequal, the optimal cut off point was calculated, as the weighted average of the two group centroids, with equation:

Discriminant function cut off point = $[(n_1 \times \text{Lower Centroid}) + (n_2 \times \text{Higher Centroid})]/(n_1 + n_2) = [(80 \times -1.45) + (79 \times 1.47)]/(80 + 79) = 0.00082$

Based on discriminant cut point, the classification of subjects as PD cases and healthy controls is:

(i) Predicted and classified as control, if $-1.45 < Z < 0.00082$ and
(ii) Predicted and classified as PD cases, if $0.00082 < Z < 1.47$

Cross-validation analysis of PD and controls

In present study, the cross validation was done on the same data set from which the model (discriminant function) was developed, considering the diagnosis of experienced Neurologist as Gold Standard and comparison was done between the results of Neurologist and predicted by the model. A high percentage (91.2% after cross validation) of total subjects was correctly classified. The control presented the best classification with only one PD case classified as control and 13 controls were erroneously classified as PD cases. This discriminant function model had 98.73% sensitivity and 83.75% specificity.

Receiver operating characteristics (ROC) curve

Receiver Operating Characteristics (ROC) curve was plotted with true positive rate (sensitivity) on Y-axis against the false positive rate (1-Specificty) on X-axis for developed discriminant function model as well as for each considered biochemical variables (UIBC, Transferrin, Tranferrin Saturation, Copper) to verify the classification rule developed by Discriminant Function model. The value of area under the curve (AUC) ranges from 0 to 1 where a value of 0 indicates almost inappropriate model/variable to discriminate between two groups and a value of 1 may be explained as perfectly appropriate variable to classify across groups. An AUC value of 0.5, indicates that the ROC curve will fall on the diagonal (45°) line and hence suggests that no discriminating ability of plotted model/variable. The AUC along with it's 95% confidence interval for model and each variable were also calculated. As shown in ROC Figure 1, developed discriminant function model in the present study is very good in classification of PD cases as well as Control subjects. The AUC for the same was found to be 0.98 (95% CI: 0.96–0.99). As Figure 1 shows UIBC (µmol/L) was found to be the most powerful variable in classification of PD cases and controls. The AUC (95% CI) for UIBC, Transferrin, Transferrin Saturation and Copper was 0.84 (0.77-0.90), 0.66 (0.57–0.74), 0.03 (0.01–0.06) and 0.16 (0.09–0.22), respectively. Hence, the findings of ROC

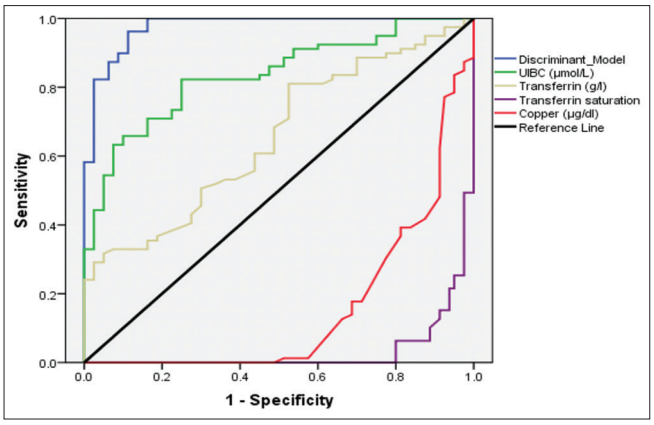

Figure 1: Receiver Operating Characteristics Curve

curve corroborated with the results obtained from discriminant function analysis.

Discussion

In recent years, there has been keen interest to study the role of metals especially aluminium, iron and copper in pathogenesis of Parkinson's disease. It is still not confirmed whether the altered levels of metals in brain and plasma are a cause or consequence in the pathology of disease. In the present study iron and copper levels were studied in serum of PD subjects to assess their role in the pathogenesis of PD. Iron and copper have high concentration in brain with excessive presence in the basal ganglia, hippocampus, cerebellum and in the cell bodies of cortical pyramidal and cerebella granular neurons.[9] These metals are redox active transition metals, which are implicated in the pathogenesis of PD due to imbalance in their homeostasis, leading to excessive free radical production during oxidative stress.[10]

In this study, we assessed the plasma levels of iron, transferrin, ferritin, TIBC, UIBC, copper and ceruloplasmin in PD cases and compared them with healthy controls. Our study observed decreased serum iron and copper levels in PD cases as compared to healthy controls. However, Jimenez-Jimenez *et al*. 1998[11] showed no change in serum levels of iron in PD cases and no significant difference in copper levels between PD cases and healthy controls, whereas, Kumidini *et al*. 2014[12] observed elevated copper and iron levels in serum of PD cases. A recent meta-analysis[13] comprising of 11 studies involving 829 PD cases and 1219 healthy controls, revealed significantly higher serum iron levels in PD cases as compared to healthy controls (SMD = 0.27, 98% CI = 0.18, 0.37, $P < 0.001$). Subgroup analysis by ethnicity also showed significantly higher serum iron levels in PD cases both in Asian and European population. Similar results were reported by Wang *et al*. 2016[14] (SMD = 0.60, 95% CI = 0.16, 0.39; $P < 0.001$). Another meta-analysis done by Mariani *et al*. 2013[15] found no variation in serum iron and copper between PD cases and healthy controls. The meta-analysis of 9 studies on copper levels in serum (a pooled total of 425 PD cases and 333 controls) demonstrated no variation between PD cases and healthy controls ($p = 0.691$). Similar results were reported with serum iron as well. Additionally, no variation was reported for serum iron levels in PD cases as compared to healthy controls when replication studies were done by same group on 22 PD cases and 49 healthy controls.

The present study also found significantly higher levels of transferrin, TIBC and UIBC with low transferrin saturation levels in PD cases. Mariani *et al*. 2013[15] found high serum transferrin and transferrin saturation levels in PD cases, whereas Logroscino *et al*. 1999[16] reported low levels of transferrin along with iron and ferritin in serum of PD cases. Transferrin along with it's receptor is considered as a major mechanism for cellular uptake of iron, but Xu *et al*. 2008[17] showed that transferrin or it's receptor did not increase in the PD brain. Hence transferrin and it's receptor might not be responsible for iron accumulation in PD.

We also observed a significant difference in serum ferritin levels in PD cases as compared to controls. Similar results have also been reported by Farhoudi *et al*. 2012[18] and Annanmaki *et al*. 2007.[19] However Logroscino *et al*. 1999[16] found lower concentration of TIBC, transferrin and ferritin in serum of PD cases as compared to healthy controls. They also could not find any relation of them with dietary intake of iron or duration of treatment of PD.

Above mentioned findings and studies, show that measurement of iron proteins (transferrin, transferrin saturation and ferritin) are essential along with serum iron levels, to understand the role of iron and copper in PD cases,[15] as they are indirect measures of iron storage.[20] Such results signify that in PD there is redistribution of iron to the nigral intracellular compartment leading to availability of excess iron for lipid peroxidation.

Excessive iron deposition in CNS has been reported in PD as well as in AD and ALS. Although extensive evidence links the dysregulation of iron homeostasis in PD, it has mostly been found associated with increased age and may involve iron uptake and release, storage and intracellular metabolism.[21] Evidences suggest that dyshomeostasis of brain iron metabolism is one of the initial events that trigger neuronal death in neurodegenerative disorders.[22]

Age induced iron accumulation due to redistribution of iron-containing molecules in different brain areas has been found to be associated with AD and PD.[21] This has been observed especially in cerebral cortex, cerebellum, SN and hippocampus, where they are involved in neuroinflammation observed in neurodegenerative disorders. Various hypotheses have been put forward to explain intracellular accumulation of iron in brain. According to one such hypothesis, blood brain barrier dysfunction may lead to exudation of serum components including iron. Other hypothesis proposes that intracellular iron accumulation may be due to dysregulation of proteins like iron regulatory proteins (IREG), that control

iron levels in cells. This hypothesis is supported by strong experimental findings observed in IREG2 knockout mice, which developed intracellular iron in white matter tracts and nuclei in different brain areas and display signs of neurodegeneration in Purkinje cells.[23]

Conclusion

In conclusion, we demonstrated the ability of four biomarkers to discriminate PD cases from healthy controls. A predictive equation was derived from the unstandardised canonical discriminant function coefficients to discriminate between PD cases and healthy controls after discriminant analysis of iron profile variables (UIBC, transferrin, iron, transferrin saturation and copper). This discriminant function model had 98.73% sensitivity and 83.75% specificity.

Limitation of study

Small sample size is the limitation of this study as it is difficult to get PD patients. However, prospective validation of our findings in large cohort and in other ethnic populations is warranted in future studies.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient (s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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