ORIGINAL ARTICLE

# Increased bilirubin levels in *de novo* Parkinson's disease

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**Keywords:**
bilirubin, biomarker, diagnosis, heme, HO, motor, Parkinson, progression

Received 1 November 2014
Accepted 29 December 2014

*European Journal of Neurology* 2015, **22:** 954–959

doi:10.1111/ene.12688

**Background and purpose:** Oxidative stress is a central pathogenic mechanism of Parkinson's disease (PD), and the heme oxygenase (HO) bilirubin pathway is one of the main mammalian antioxidative defences. Indeed, there is growing evidence of HO–bilirubin upregulation from early phases of PD. Our aim was to investigate bilirubin as a possible biomarker of PD diagnosis and progression.

**Methods:** A cross-sectional case–control study was performed to evaluate differences in bilirubin levels between newly diagnosed, drug-naïve PD subjects and controls. Afterwards, PD subjects were included in a 2-year longitudinal study to evaluate disease progression in relation to baseline bilirubin levels.

**Results:** Seventy-five *de novo* PD subjects were selected and matched with 75 controls by propensity score. Analysis of variance showed higher bilirubin levels in PD patients compared with controls ($P < 0.001$). Linear regression analysis failed to show a relationship between bilirubin and Unified Parkinson's Disease Rating Scale (UPDRS) part III ($P = 0.283$) at baseline evaluation. At 2-year follow-up, indirect relationships between bilirubin levels and UPDRS part III ($P = 0.028$) and between bilirubin levels and levodopa-equivalent daily dosage ($P = 0.012$) were found.

**Conclusions:** Parkinson's disease subjects showed higher levels of bilirubin compared with controls. Bilirubin increase might be due to HO overexpression as a compensatory response to oxidative stress occurring from early stages of PD.

## Introduction

Several studies investigating the pathogenic mechanism of Parkinson's disease (PD) have increasingly focused on oxidative stress pathways [1]. Oxidative balance is frequently impaired in PD with subsequent damage of proteins, lipids, carbohydrates and nucleic acids, up to cell death [1,2]. Therefore, identifying biomarkers of oxidative stress is of primary importance not only to increase our knowledge on PD pathogenesis but also to support PD diagnosis and to track PD motor and non-motor progression [3].

Amongst different oxidative pathways, there is increasing evidence that heme oxygenase (HO) is one of the main antioxidative defences in mammalia and is active since early phases of the adaptive response to stress [2,4]. Considering that HO converts heme molecules into iron, carbon monoxide and biliverdin that is further converted to bilirubin by biliverdin reductase [5], it has been suggested that plasma total bilirubin can serve as a marker for HO activity [6] and can have direct antioxidative properties within the brain [5–7].

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Considering these findings, HO–bilirubin might represent an additional pathway involved in both PD pathogenesis and progression. In support of this hypothesis, there is evidence of a sustained HO upregulation within dopaminergic cells exposed to oxidative stress [8,9]. In particular, overexpression of HO might increase neurotrophic factors, protect neurons from toxic substances (i.e. methyl-phenyl-tetrahydropyridine) [10] and prevent the toxic aggregation of alpha-synuclein [9]. Finally, increased bilirubin levels have been shown in PD patients compared with controls [11]. However, such results have never been replicated, fully discussed or related to PD diagnosis and progression.

The present study aimed to investigate (i) differences in bilirubin levels between newly diagnosed, drug-naïve PD subjects and healthy controls; (ii) relationships between bilirubin levels and motor symptoms in *de novo* PD patients at first evaluation and after 2-year follow-up; (iii) relationships between bilirubin levels and non-motor symptoms (NMS) in *de novo* PD patients at first evaluation and after 2-year follow-up.

## Patients and methods

### Study design

In the first part of the study, a cross-sectional case–control analysis was performed to evaluate differences in bilirubin levels between newly diagnosed, drug-naïve PD subjects and healthy controls. In the second part, PD subjects were included in a 2-year longitudinal study to evaluate motor and non-motor progression in relation to baseline bilirubin levels. The Federico II University Hospital Ethical Committee approved the study and all subjects provided written informed consent. The study was performed in accordance with good clinical practice and the Declaration of Helsinki.

### Parkinson's disease subjects

*De novo* drug-naïve patients with parkinsonism who were consecutively referred to the Department of Neurosciences at the University Federico II of Naples, Italy, between 1 January 2008 and 30 June 2009 were enrolled. Inclusion criteria were the presence of a parkinsonian syndrome according to the UK Parkinson's Disease Society Brain Bank Diagnostic Criteria [12,13]; onset <2 years before; and no previous or current treatment with dopaminergic drugs. Additional criteria for inclusion were lack of significant cerebral lesions on magnetic resonance imaging or computed tomography. Exclusion criteria were a diagnosis of secondary (such as vascular or drug-induced) or familial parkinsonism or a diagnosis of atypical parkinsonism, according to current diagnostic criteria [14–18].

At baseline evaluation, motor features were evaluated by means of the Unified Parkinson's Disease Rating Scale (UPDRS) part III. Disease duration (months since reported motor onset) was recorded. All patients completed the non-motor symptom questionnaire (NMSQ), a validated tool for detection of NMS [19]. The NMSQ consists of 30 questions with dichotomous (yes/no) answers and a total score that can be determined, with higher scores reflecting more NMS (from 0 to 30). At baseline visit, none of the patients was treated with anti-parkinsonian drugs, anticholinergic agents, choline esterase inhibitors, antidepressants, anxiolytic drugs or other centrally acting substances that might have affected both motor and non-motor evaluation.

Due to the observational nature of the study, after baseline visit dopaminergic and non-dopaminergic treatments (i.e. serotoninergic antidepressants) were then started according to the discretion of each supervising physician. After 2 years from enrolment, a clinical evaluation was performed to confirm the diagnosis of PD. UPDRS part III was evaluated during off phase (12 h off drugs) and during on state, and NMS were checked by means of the NMSQ, as at baseline. Dopaminergic treatment was recorded and levodopa-equivalent daily dose (LEDD) was calculated for each drug class [20]. Moreover, for statistical purposes patients were also classified as requiring or not requiring levodopa. Patients taking drugs other than dopamine agonists, monoamine oxidase inhibitors type B, catechol-*O*-methyltransferase inhibitors and levodopa were excluded from the analyses.

### Controls

Controls were recruited amongst subjects visiting the same hospital within the same period for their scheduled visit at the Occupational Medicine Unit. Visits were not related to current health problems but were performed in accordance with occupational Italian regulations. Concomitant diseases and treatments were recorded. All controls underwent physical examination according to clinical practice.

### Laboratory assessment

Serum was collected at baseline visit for both cases and controls and appropriately stored, in order to be subsequently analysed with standardized procedures. In particular, bilirubin was determined in serum obtained

from fasting blood by the BILTS method with the COBAS® c501 analyser (Roche Diagnostic, Mannheim, Germany). All subjects were evaluated for possible confounding factors in determining bilirubin levels. Exclusion criteria were concomitant liver or gallbladder disease in clinical records or in standard laboratory procedures (i.e. aspartate aminotransferase > 35 U/l, alanine aminotransferase > 35 U/l, gamma-glutamyl transpeptidase > 36 U/l, presence of HBsAg, presence of anti-HCV antibody, previous gallbladder removal surgery, previous gallbladder attack), abnormal cholesterol or triglyceride metabolism in standard laboratory procedures (i.e. total cholesterol > 220 mg/dl, triglycerides > 180 mg/dl), use of cholesterol or triglyceride lowering agents (i.e. statins), recent anaemia or haemolysis in clinical records or in standard laboratory procedures (i.e. haemoglobin < 12 g/dl, haematocrit < 35% in females or < 37% in males, iron < 45 μg/dl, presence of reticulocytes in peripheral blood, mean corpuscular volume <80 fl or >97 fl), abnormal body mass index (BMI) (overweight, BMI > 25 kg/m$^2$, or underweight, BMI < 19 kg/m$^2$).

### Statistical analysis

First, PD subjects and controls were respectively extracted from databases of the Department of Neurosciences and of the Occupational Medicine Unit, according to the exclusion and inclusion criteria, and matched considering the outcome measure (PD diagnosis) and covariates such as age and gender by using propensity score matching (PSM), with a case–control matching ratio of 1:1. Demographic differences between groups were evaluated by $\chi^2$ or $t$ test, as appropriate. Differences in bilirubin levels between cases and controls were evaluated by $t$ test and subsequently analysis of variance (ANOVA) corrected for age and gender.

In the second part of our study, PD subjects only were evaluated. For statistical purposes, variables from differences in scores of UPDRS part III (Δ-UPDRS-off and Δ-UPDRS-on), and NMSQ total score (Δ-NMSQ) between 2-year follow-up and baseline visit were generated. After testing for normal distribution, linear regression analysis was performed to assess the relationship between bilirubin levels and continuous variables (age, disease duration, UPDRS part III, Δ-UPDRS-off, Δ-UPDRS-on, NMSQ, Δ-NMSQ, LEDD). $t$ test and ANOVA analysis were performed to evaluate categorical variables (gender, requiring or not requiring levodopa). All models were subsequently adjusted for age and gender. When evaluating motor symptoms during on state, analyses were adjusted for LEDD.

STATA 12.0 and Microsoft Excel 2011 software were used for data processing and statistical analysis. Results were considered statistically significant for $P < 0.05$.

## Results

In the first part of the study, 75 PD subjects were selected and matched with 75 healthy controls by PSM. No significant differences were found in age and gender between cases and controls (Table 1). Bilirubin levels were found to be significantly higher in PD subjects at $t$ test ($P < 0.001$) and ANOVA after correction for age and gender ($P < 0.001$; adjusted $R^2 = 0.269$) (Table 1; Fig. 1). In the latter model, gender but not age appeared to affect bilirubin levels ($P = 0.001$ and $P = 0.092$, respectively), with males presenting higher levels than females (Table 1).

In the second part of the study, PD subjects only were evaluated. Considering the baseline visit, linear regression analysis found no different bilirubin levels in relation to age ($P = 0.703$) and disease duration ($P = 0.901$). The $t$ test confirmed lower bilirubin levels in females ($P = 0.021$).

With regard to motor symptoms, linear regression did not show a relationship between bilirubin levels and UPDRS part III ($P = 0.283$) at baseline evaluation; a relationship between these variables only emerged after correction for age and gender ($P = 0.043$; coefficient 0.078; 95% confidence interval 0.001–0.015). Regression analysis failed to show any difference in bilirubin levels in relation to Δ-UPDRS part III (defined as the difference between baseline score and 2-year follow-up evaluation) performed during off phase, before ($P = 0.114$) and after correction for age and gender ($P = 0.714$). Regression analysis showed different bilirubin levels in relation to

**Table 1** Demographics and serum bilirubin levels of PD cases and healthy controls, matched by propensity score

| | PD subjects ($n$ = 75) | Controls ($n$ = 75) | $P$ values |
|---|---|---|---|
| Age, mean ± SD (range) | 59.7 ± 7.9 (45–72) | 58.7 ± 5.9 (48–69) | 0.594 |
| Gender, males / females | 45/30 | 45/30 | 0.799 |
| Bilirubin levels, mean mg/dl ± SD | | | |
| Males | 0.79 ± 0.25 | 0.58 ± 0.32 | 0.001 |
| Females | 0.64 ± 0.22 | 0.42 ± 0.20 | 0.001 |
| Total (range) | 0.74 ± 0.25 (0.24–1.32) | 0.51 ± 0.29 (0.14–1.62) | <0.001 |

PD, Parkinson's disease.
$P$ values are shown from $\chi^2$, $t$ test or analysis of variance, as appropriate.

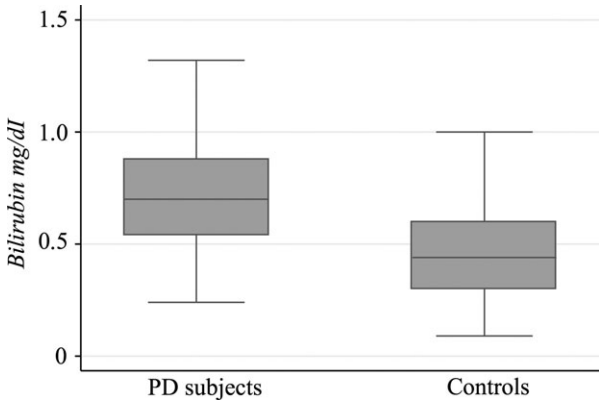


**Figure 1** Box and whisker plot showing the difference in bilirubin levels between cases and controls. Parkinson's disease (PD) subjects presented higher bilirubin levels than healthy controls at *t* test ($P < 0.001$) and analysis of variance corrected for age and gender ($P < 0.001$).

Δ-UPDRS part III performed during on phase before ($P = 0.028$; coefficient −0.057; 95% confidence interval −0.001 to −0.016) and after correction for age, gender and LEDD ($P = 0.013$; coefficient −0.066; 95% confidence interval −0.001 to −0.013) (Fig. 2). Linear regression analysis showed a negative relationship between bilirubin levels and LEDD at 2-year follow-up, before ($P = 0.012$; coefficient −0.053; 95% confidence interval −0.009 to −0.012) and after correction for age and gender ($P = 0.017$; coefficient −0.045; 95% confidence interval −0.001 to −0.008) (Fig. 3). No differences were found between subjects requiring or not requiring levodopa in relation to bilirubin levels at *t* test ($P = 0.093$) and ANOVA corrected for age and gender ($P = 0.071$).

When analysing NMS, linear regression models failed to show any difference in bilirubin levels in relation to NMSQ total score at baseline before ($P = 0.631$) and after correction for age and gender ($P = 0.368$), and to Δ-NMSQ before ($P = 0.765$) and after correction for age and gender ($P = 0.462$).

## Discussion

This is the first study specifically designed to investigate differences in bilirubin levels between newly diagnosed, drug-naïve PD subjects and healthy controls, and to evaluate bilirubin levels in relation to clinical progression of PD.

Considering the main objective of the present study, increased bilirubin levels in PD patients compared with controls were found. Our results are in line with previous findings by Scigliano and colleagues [11]. Accordingly, it is speculated that HO upregulation within the substantia nigra might be an adaptive response to increased oxidative stress occurring in PD [9] and is likely to be responsible for increased bilirubin levels [9]. Interestingly, circulating bilirubin is in

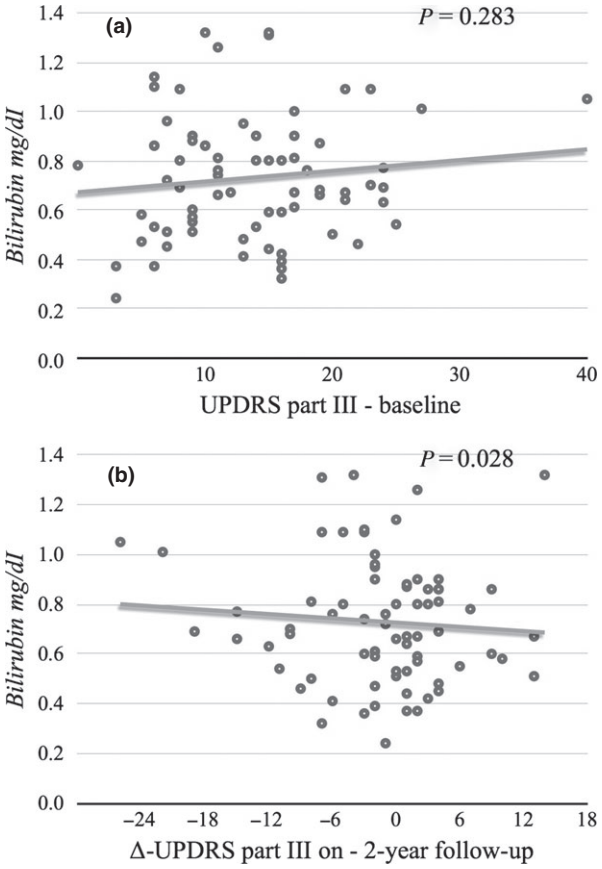


**Figure 2** Scatter plot showing the relationships between bilirubin and Unified Parkinson's Disease Rating Scale (UPDRS) part III performed at baseline (a) and at 2-year follow-up during on state (Δ-UPDRS) (b). *P* values are shown from linear regression analysis.

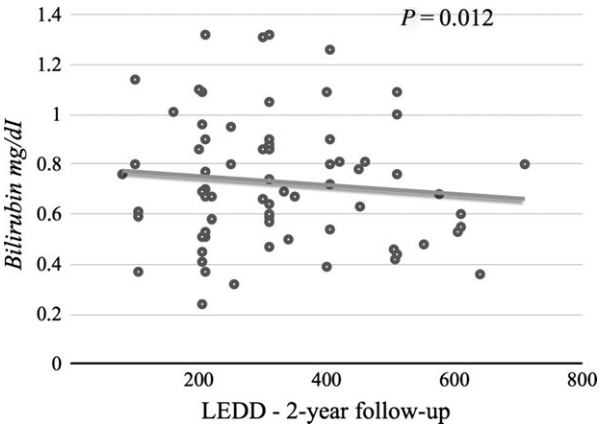


**Figure 3** Scatter plot showing the relationships between bilirubin and levodopa-equivalent daily dosage (LEDD). *P* value is shown from linear regression analysis.

dynamic equilibrium with extravascular tissues, including the central nervous system [21], and PD subjects do not present any difference in peripheral HO-1 mRNA levels evaluated in blood mononuclear cells [22]. Therefore increased bilirubin production within the brain may be responsible for systemic bilirubin variations. Moreover, as HO upregulation occurs since first PD diagnosis, it can be part of early pathological mechanisms underlying PD.

With regard to the relationships amongst bilirubin levels and clinical variables at baseline evaluation, slightly worse motor symptoms were found in PD patients with higher bilirubin levels. Sustained HO immunoreactivity is observed in dopaminergic neurons of PD patients where cytoplasmic Lewy bodies displayed intense peripheral HO staining [9,23,24]. It has been found that chronic upregulation of HO in the PD brain may exacerbate the degenerative process by promoting iron deposition and oxidative mitochondrial damage [9], explaining the baseline relationship between motor symptom severity and higher bilirubin levels. However, upregulation of HO in the PD substantia nigra may also represent an adaptive response, and may at least in part counteract the impact of motor symptoms by preventing the toxic aggregation of alpha-synuclein. In this view, HO inducibility and subsequent bilirubin levels may correlate with improved health outcomes [6].

At 2-year follow-up evaluation, PD subjects with higher bilirubin levels required fewer dopaminergic drugs and, at least as a trend, less levodopa, and presented lower motor scores during on state despite similar motor scores during off. The latter result is intriguing and could at least in part be attributed to potential interactions between drug pharmacokinetics and bilirubin levels at the peripheral level.

Afterwards, no relationship was found between bilirubin levels and NMS presence or progression. However, further investigations on larger populations should be strongly encouraged by means of the NMS scale, which was unfortunately still not available when the present study was designed [19].

Finally, some limitations need to be addressed. First, the sample size is small and data generalizability can be difficult. Further studies on larger populations are needed to specifically address the unmet issues (i.e. NMS and bilirubin levels). In addition, bilirubin variations during PD course were not evaluated. Two previous studies investigated this issue and found lower bilirubin levels in Alzheimer's disease [7,25]. However, both studies included subjects with different disease durations. As variability in bilirubin levels during the time course of the disease cannot be excluded, subjects with possible confounding factors were excluded from the analysis. Furthermore, although highly selective inclusion criteria for both cases and controls were used, the presence of other factors possibly interfering with bilirubin levels cannot be completely excluded. Finally, it has to be reported that bilirubin presents a selective neurotoxicity for basal ganglia, as it occurs in kernicterus; however, our population did not present bilirubin levels as high as required to be directly neurotoxic [21].

In conclusion, our findings support the fact that bilirubin levels may differentiate PD subjects from healthy controls, possibly because HO overexpression is a compensatory response to oxidative stress, occurring from early stages of PD. The antioxidant efficacy of increased bilirubin levels is evident at 2-year follow-up when PD subjects with higher bilirubin levels presented better motor outcomes, suggestive of more preserved dopaminergic pathways. In this view, the inducibility of the HO pathway could represent an important compensatory mechanism in PD with significant effects on disease progression. In addition, it may be hypothesized that the inducibility of HO can be an important mechanism predisposing the senescent nervous system to PD and to other neurodegenerative disorders [9]. The latter is possibly due to different mechanisms such as iron deposition or mitochondrial damage [9], and it has recently been confirmed by the finding of increased PD risk in subjects presenting HO gene variations [26]. As a consequence, measures increasing intracellular bilirubin (i.e. administration of biliverdin or of HO inducers) or mimicking its activity (i.e. administration of phycocyanobilin) should be investigated.

The present study broadens our knowledge of oxidative stress in PD and suggests that bilirubin may provide useful diagnostic and prognostic information in this disorder. Further studies should be specifically addressed to evaluating variations in bilirubin levels during disease course and possible pathophysiological and clinical consequences.

## Acknowledgement

This work was supported by the University of Salerno, FARB 2012 (ORSA127397).

## Disclosure of conflicts of interest

The authors declare no financial or other conflicts of interest.

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