Increased urinary indoxyl sulfate (indican): New insights into gut dysbiosis in Parkinson's disease

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Abstract

Introduction: Changes in the composition of gut microflora have been associated with an increase in chronic diseases. Indican urinary concentration is one of the most common and easily assessable markers of intestinal dysbiosis. Little information is available on intestinal dysbiosis in Parkinson’s disease (PD).

Methods: A case—control study including PD patients (N = 68) on treatment with levodopa (PD) or on no pharmacological treatment (De Novo, DPD; N = 34) and an age and gender-matched healthy control group (CTR; N = 50). Main confounders, such as nutritional habits and constipation diagnosed according to Rome III criteria, were also investigated.

Results: Indican urinary concentrations were significantly higher in PD and DPD than in CTR (P < 0.001 and P < 0.01, respectively). In PD patients the concentrations were unrelated to the presence of constipation, whereas this symptom was associated with higher concentrations in controls (P = 0.043). The frequency of dairy product consumption was also positively associated with increased concentrations (P = 0.008). Predictors of indican concentrations were sought by multivariate linear regression analysis. The higher indican urinary concentrations found in both DPD (P = 0.045) and PD (P = 0.023) patients persisted after adjustment for age, gender, BMI, constipation and consumption of dairy products.

Conclusions: Gut dysbiosis seems to be an important issue in PD, independently of the presence of constipation and starting from the early stages of the disease. The role of gut dysbiosis in the pathogenesis of PD deserves further investigation.

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1. Introduction

The human colon hosts about $10^{11}$–$10^{12}$ bacteria/ml of lumen content [1]. These microorganisms include thousands of different bacteria, but 90% belong to only two families: Bacteroidetes and Firmicutes [1]. The composition of the bacterial flora of the gut changes throughout life according to a number of variables related both to the host and the environment [2]. The formation of bacterial flora is influenced by a number of factors, such as diet, antibiotic treatment, type of delivery and breast-feeding [2]. Inter-individual variability of microbiota is considerable among adults and even intra-individual variability is fairly high.

Gut microbiota have a number of beneficial functions in the human body: they prevent colonization by pathogens, synthesize useful substances (e.g. short-chain fatty acids and vitamins), and modulate the local immune response [3]. Moreover, changes in the composition of gut microflora have been associated with an increase in chronic diseases, such as obesity, type 2 diabetes mellitus, metabolic syndrome and atherosclerosis [4–7]. In particular, it appears that the gut microflora may be able to exert pro-inflammatory and pro-atherogenic effects. Indeed, it promotes increased absorption of lipopolysaccharides by enterocytes, resulting in their increase in the bloodstream. This phenomenon
promotes lipogenesis and stimulates pro-inflammatory mechanisms [8].

These observations have suggested that changes in the composition of the microflora of the gut may occur also in other chronic diseases, such as Parkinson’s disease (PD). Starting from the early stages, PD is characterized by dysautonomic gastrointestinal symptoms, such as dysphagia, slow gastric emptying and constipation [9]. In particular, constipation may even precede the onset of PD by many years. Changes in the microflora of the gut have been described in subjects with irritable bowel syndrome [10] and have been associated with systemic inflammation [8]. Very recent investigations have suggested that changes in gut microbiota and increased gut permeability, inflammation and related neuro-inflammation could contribute to the pathogenesis of PD [11–13]. It has been also recently reported that small intestinal bacterial overgrowth (SIBO) occurs in PD patients [14,15].

Growth of gut microbiota is associated with the release of bacterial metabolism products, which are absorbed by the intestine, enter human metabolism and are eliminated in the urine [16]. One of these compounds is indoxyl sulfate (otherwise known as indican). The urinary concentration of indican can be measured by a simple and inexpensive procedure, which has been employed to assess dysbiosis and SIBO [16–19].

In healthy subjects indican urinary concentrations are low (approximately around 10–25 mg/l), since bacterial growth in the upper intestine is very modest. High concentrations (>40 mg/l) indicate changes in bacterial growth in the small intestine, such as SIBO, as well as malabsorption and constipation [17–19]. In the presence of SIBO or malabsorption, the intraluminal concentration of amino acids increases and, in turn, so does bacterial conversion of tryptophan to indican [16,17]. In constipated subjects the increase in indican concentrations is likely the result of changes in gut flora - such as reduction in Lactobacillus strains [16,18].

The objective of the study was to measure indican urinary concentrations, as surrogate of changes in the gut microflora, in three groups of subjects: PD patients at the onset of disease (de novo, DPD); PD patients on levodopa treatment; age and gender-matched healthy controls (CTR). 2. Material and methods

2.1. Ethics

The study was approved by the local Ethics Committee and all subjects gave their informed consent in writing.

2.2. Patients

The following groups of subjects were recruited for the study: 1) de novo (no ongoing treatment) PD patients (DPD group; N = 34); levodopa-treated PD patients (PD group; N = 68); age and gender-matched healthy controls (CTR; N = 50). Diagnosis of PD was made according to UK Brain Bank criteria [20]. In addition, as the diagnosis in DPD may be limited by the lack of supportive prospective criteria, only patients followed-up for at least one year were included. PD cases were recruited consecutively (response rate, 99%) among the patients seen at the ICP Parkinson Institute in Milano (DPD, between January and December 2013; PD group, between February and March 2013). Healthy controls were recruited over the same period among the people living with patients seen at the Parkinson Institute (spouses, caregivers). The assumption was that their dietary habits were at least fairly similar to the dietary habits of the patients. Exclusion criteria were: any-stage acute or chronic heart failure (according to NYHA classification system), liver failure (according to Child-Pugh criteria) and/or kidney failure (creatinine >1.2 mg/dl); history of cancer; history of chronic intestinal disease or gastrointestinal surgery; previous neurosurgical procedure for PD; antibiotic treatment or diarrhea in the last month; probiotic treatment in the last month.

2.3. Clinical assessments

The following information was collected in PD patients and healthy controls:

- Medical history: attention was focused on pharmacological therapy, disease duration, type of delivery (cesarean or natural) and breast-feeding.
- Anthropometry: weight (Kg) and height (m) were measured according to standard procedures and body mass index (BMI) was then calculated as the ratio between weight and height squared (kg/m²) [21].
- Dietary habits: they were assessed by means of a food frequency questionnaire: an additional 3-day dietary recall just before urine sample collection was also considered.

Diagnosis of constipation was made according to Rome III Criteria [22]. In particular, it was confirmed one week after discontinuation of any rescue medication.

2.4. Indoxyl sulfate urinary determination

Fresh urine samples (20 mL) were collected in the morning after fasting overnight and analyzed at the laboratory Centro Analisi Monza (CAM). In patients using rescue medication for constipation, samples were collected one week after discontinuation of any treatment.

Indoxyl sulfate was assessed in triplicate by means of an internally validated spectrophotometric assay (limit of quantitation, 3 mg/l; intra- and inter-assay coefficients of variation were 2.8% and 5.2%, respectively). Three mL of urine, were mixed with 3 mL of the Obermeyer reactant (1333 mM ferric chloride hexahydrate in concentrated HCl) and the mixture was allowed to stand for 5 min at room temperature. The product of this reaction is indigo, which is intensely blue. Four mL of chloroform were then added and the sample was centrifuged at 4000 rpm for 10 min in order to separate the lower colored layer from the oily phase. Thereafter, optical density of the colored layer was determined at 600 nm against the reagent blank (distilled water) using a Agilent 8453 UV–Vis spectrophotometer (Agilent Technologies; Santa Clara, CA, US). In the blank, distilled water was treated in the same way as the sample. The calibration curve included 6 values between 3.125 mg/l and 100 mg/l.

2.5. Statistics

Non sensitive data of all the subjects were entered into a password-protected database. Continuous variables were reported as mean and standard deviation (SD) or errors (SE), as appropriate. Categorical variables were described as counts and percentages. Comparisons between groups of continuous variables were performed using parametric and non-parametric tests, as appropriate. Fisher’s exact test was used for the analysis of categorical variables. Independent variables associated with indican concentrations were investigated by multivariate linear regression analysis. The statistical analysis of the data was carried out using JMP® software version 3.2.6, (SAS Institute Inc.; USA). The value of p < 0.05 was set as the limit of statistical significance.

3. Results

All the subjects who entered into the study reported that they had been delivered naturally and breastfed. The general characteristics of the three study groups are shown in Table 1.

Patients suffering from PD, taken as a whole, had indican urinary concentrations that were about twice as high as in controls (46.4 ± 4.1 mg/l vs. 24.9 ± 4.6, P < 0.001). The difference vs. controls was significant both in DPD patient (42.5 ± 7.6 mg/l; P = 0.040) and PD patients (48.3 ± 4.9 mg/l; P = 0.001) (Fig. 1).

The prevalence of constipation was significantly higher in PD patients than in controls (57% vs 20%; P < 0.001). Indican urinary concentrations in PD patient groups (PD and DPD) were unrelated.

Table 1

<table>
<thead>
<tr>
<th>Clinical and demographic features of the study population.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Subject number</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Age (year)</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
</tr>
<tr>
<td>Disease duration (year)</td>
</tr>
<tr>
<td>Levo-Dopa (mg/die)</td>
</tr>
</tbody>
</table>

Data are reported as mean ± standard error or counts.

Abbreviations: Controls (CTR); De Novo Parkinson’s Disease (DPD); Parkinson’s Disease patients (PD).

Significant differences between groups (p < 0.05) by Student’s t test are reported as follows.

- DPD vs. CTR.
- DPD vs. PD.
to the presence of constipation. On the contrary, this symptom was associated with higher indican urinary concentrations in controls (42.8 ± 16.6 mg/l vs 20.1 ± 3.5; P = 0.043; Fig. 2). Moreover, most differences between PD groups and CTR were largely due to controls without constipation.

In respect to dietary patterns, PD patients followed a protein redistribution regimen with a low-protein breakfast and lunch and high-protein dinner, using products of animal origin as primary source \cite{23,24}. DPD and healthy subjects followed a balanced Mediterranean diet. All subjects consumed vegetables regularly (at least one portion at every meal), fruits (about three pieces a day) and drank about a liter of water daily. Dietary habits and hydration did not appear to affect indican urinary concentrations, except for frequency of dairy product consumption, which was positively associated with increased concentrations (P = 0.008; Fig. 3).

Predictors of indican urinary concentrations were sought by multivariate linear regression analysis (Table 2). The higher indican urinary concentrations found in both DPD and PD patients persisted after adjusting for age, gender, BMI, presence of constipation and consumption of dairy products.

4. Discussion

In patients suffering from PD indican urinary concentrations were significantly higher than in healthy controls (approximately twice as high). The difference did not appear to be influenced by differences in BMI, dietary habits or intestinal function, since concentrations were not affected by the presence of constipation. Indican urinary concentrations were significantly higher also in de novo PD patients. This suggests that disease duration and pharmacological therapy of PD do not play a role.

The validity of our findings is supported by the consistency of our data with the results of the few studies published in the literature, which report that the mean indican urinary concentration in healthy men is around 10–25 mg/L \cite{18,19}. In addition, although the recruitment of patients at a specialized tertiary care setting does not enable us to exclude inclusion bias completely, our study

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**Table 2**

Multivariate linear regression analysis of predictors of urinary indican concentrations.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Beta coefficient</th>
<th>Standard error</th>
<th>Student’s t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)(^a)</td>
<td>0.2</td>
<td>0.3</td>
<td>-0.62</td>
<td>0.538</td>
</tr>
<tr>
<td>Male gender</td>
<td>-0.3</td>
<td>6.8</td>
<td>-0.04</td>
<td>0.968</td>
</tr>
<tr>
<td>Body mass index (Kg/m(^2))(^b)</td>
<td>-0.9</td>
<td>0.7</td>
<td>-1.15</td>
<td>0.253</td>
</tr>
<tr>
<td>Constipation (yes)</td>
<td>4.7</td>
<td>7.5</td>
<td>0.63</td>
<td>0.532</td>
</tr>
<tr>
<td>Consumption of dairy products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(times/week)(^a)</td>
<td>11.5</td>
<td>4.5</td>
<td>2.56</td>
<td>0.012</td>
</tr>
<tr>
<td>PD group(^a)</td>
<td>18.2</td>
<td>7.9</td>
<td>2.30</td>
<td>0.023</td>
</tr>
<tr>
<td>DPD group(^a)</td>
<td>9.8</td>
<td>4.8</td>
<td>2.03</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Abbreviations: Parkinson’s Disease patients (PD); De Novo Parkinson’s Disease (DPD).

Statistically significant estimates (P<0.05) have been highlighted in bold characters.
\(^a\) For 1-unit increase.
\(^b\) For increase over categories (0–2 times/week; 3–5 times/week; 6–7 times/week).
\(^c\) Compared to healthy controls.

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sample was likely representative of the general PD population, since the prevalence of constipation (60%) was consistent with the data in the literature [9,25,26]. Main confounders were excluded by study design. Although indican production clearly relies on amino acid availability, dietary factors did not explain the difference between patients and controls. It could be argued that increased indican concentrations are associated with gut dysbiosis, which—in turn—is responsible for impaired triptophan metabolism within the intestine. We are not able to establish which is the main determinant of this phenomenon, but it could reasonably be hypothesized that both SIBO and alterations in the microbial intestinal profile contribute to this [12,14–18].

Indeed, our findings also suggest that dysbiosis has already occurred at the onset of PD, independently of the presence of constipation, and is not affected by disease duration and/or pharmacological treatment of PD. Thus, it could even have a role in the pathogenesis of the disease. This hypothesis is supported by recent literature. It has been observed that tryptophan metabolism is impaired in PD patients, with a significant reduction in its metabolites in both plasma and cerebrospinal fluid, and that normalization of tryptophan metabolism may result in neuroprotection [27]. The common mechanism of action may reflect the presence of protein and nitrogen compound putrefaction mainly induced by some bacterial species, such as Proteus and Klebsiella. Pathogenic gut microbiota likely induce barrier dysfunction, promoting inflammation and neuro-inflammation due to increased lipopolysaccharide and pro-inflammatory cytokines [8]. A preliminary case-control study on intestinal microbiota has also shown that the intestinal bacterial pattern characterized by significant reduction in Prevotella and increased in Enterobacteriaceae, is associated not only with PD, but also with motor phenotype [12]. The maintenance of healthy gut microflora was found to have also a genetic basis [11]. In any case, also the interaction between gut flora and dietary habits could play a role. It is noteworthy that the incidence of PD is higher amongst subjects who consume high quantities of dairy products [28], which are an important source of triptophan. Indeed, we found that such products are associated with higher urinary indican concentrations.

The limitations of the present study should be acknowledged. Although adjustment for multiple variables enabled us to control for important confounders, there are now high-quality methods to directly characterize fecal microbe profiles (e.g. pyrosequencing). The method used in our study provided only indirect information on gut microbial flora. Moreover, although our findings are consistent with the literature, it is difficult to make any direct comparisons on account of methodological differences (e.g. triptophan load or 24 h urine collections). None of the previous studies have addressed the impact of constipation on indican urinary concentrations and alterations in gut microbial flora associated with intestinal dysfunction, such as irritable bowel syndrome, have been usually evaluated in the last few years through the use of other direct assessment procedures (e.g. quantitative PCR assays or in situ hybridization or microarray technology, etc.) [29]. The same applies to the evaluation of SIBO. Although no longer in vogue, the culture of jejunum aspirate for bacterial counts is considered the gold standard for its diagnosis. On the other hand, most of the literature is based on the less precise breath testing using 14C-o-xylene, glucose, lactulose or 14C-glycoallic acid [30]. Consequently, the present investigation could reasonably be considered a proof-of-concept study. Risk of inclusion bias cannot be completely excluded. Although most conditions associated with increased urine indican concentrations were taken into account (e.g. liver and kidney failure, use of antibiotics or probiotics) during study design, specific diagnostic procedures for these conditions (e.g. celiac disease or malabsorption) were not performed. In particular, indican may indicate partial failure to digest dietary protein even in the presence of normal intestinal bacterial populations [16]. On the other hand, we would expect lower indican values in patients adhering to protein redistribution dietary regimens, but this was not the case. Finally, we acknowledge that the reliability of PD diagnosis in DPD is limited by the absence of levodopa response data.

In conclusion, we have described a significant increase in indican urinary concentrations in PD patients independently of BMI, dietary factors, constipation, levodopa treatment and disease duration. These findings are indicative of gut dysbiosis in PD patients, which seems to occur starting from the early stages of the disease. Further studies on fecal microbiota are warranted to investigate the potential role of gut dysbiosis in the pathogenesis of PD.

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References


Rome Criteria [E3ca href=“http://www.romecriteria.org”].


