

# A Set of Reliable Samples for the Study of Biomarkers for the Early Diagnosis of Parkinson's Disease

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## Methodology

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# Abstract

**Background:** The identification of biomarkers for the early diagnosis of Parkinson's Disease (PD) might improve treatment and avoid complications. However, the search of such biomarkers is a real challenge. The main reason for this is that finding samples for that purpose is not an easy task. In this work, we propose a set of reliable samples for the identification of biomarkers for the early diagnosis of PD. In fact, the original source of these samples can be searched for similar purposes when dealing with other diseases.

**Materials and methods:** Case-control study included 12 plasma samples of subjects that subsequently developed PD and 21 plasma samples of matched healthy controls from the Spanish EPIC-Navarra cohort, part of the European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain study. All the case samples were provided by healthy volunteers who were followed up for a mean of 15.9 ( $\pm$  4.1) years and developed PD disease later on. Therefore, these sample are ideal for the search of early biomarkers of PD. We used an analytical multiplatform based on liquid chromatography and tandem mass spectrometry in order to find significant differences in the amounts of previously reported metabolites that are altered when the disease has been established and diagnosed.

**Results:** Out of all 40 analytes that were studied, seven significant metabolites were observed. Benzoic acid, palmitic acid, oleic acid, stearic acid, myo-inositol, sorbitol and quinolinic acid were significantly changed in subjects that developed PD. These metabolites are closely related to mitochondrial dysfunction, the oxidative stress and the mechanisms of energy production, which might indicate that these mechanisms are already affected before disease onset.

**Conclusions:** We propose the samples from the EPIC study as reliable and priceless samples for the search of early biomarkers of PD, although many other diseases can be also studied as long as the information stored about the participants recorded such details. As a proof of concept, we showed that these samples were useful for the study of metabolites that may be altered before the participants developed PD. This might also be a starting point in the establishment of a well-founded panel of metabolites that can be used for the early detection of this disease.

## 1. Background

Metabolomics is an important well established tool able to provide useful insights of unknown biochemical mechanisms and possible biomarkers for various disorders [1]. Understanding altered metabolic pathways and metabolites provides a better knowledge of underlying biological alterations. This information might improve the treatment strategies for these alterations and, more importantly, can be used for disease prediction and diagnosis. The metabolic profiles for neurodegenerative and neuropsychiatric disorders and the related metabolic pathways are still unclear [2, 3], as it is the case for Parkinson's disease (PD). However, not everything is unknown in this challenge and some studies have

shown an association between certain metabolites and several metabolic pathways in PD [2–4], which are summarized here:

- Alterations in the tryptophan and kynurenine metabolism have been associated with the appearance of psychiatric symptoms and the development of PD. Certain metabolites, as part of this metabolic pathway, showed decreased levels in several biological fluids, such as tryptophan [4, 5], kynurenic acid (KA) [5, 6] and quinolinic acid (QA) [5, 6], while kynurenine [2], hydroxytryptophan [2] and xanthurenic acid showed an elevation in early stage PD [2]. Besides, the ratios of kynurenine/QA, KA/kynurenine and 3-hydroxykynurenine/KA seemed to be altered in the development of PD [4–6]. Due to the significance of this metabolic pathway, it is assumed that disruption of tryptophan and kynurenine metabolism might lead to a neurotoxicity associated to PD [7].
- Dopamine and norepinephrine metabolism plays an important role in PD development and progression. It is known that dopaminergic loss in the *substantia nigra* is one of the biggest hallmarks of PD [4, 5]. All the metabolites associated to this metabolic pathway that have been observed to be altered in PD were decreased, including dopamine [6], 3,4-dihydroxyphenylalanine (LDOPA) [5], 3,4-dihydroxyphenylacetic acid (DOPAC) [5], 3,4-dihydroxyphenylglycol [5] and norepinephrine [6].
- The involvement of the tricarboxylic acid cycle (TCA) with dopamine metabolism has been observed. It is assumed that TCA cycle might be associated with dopaminergic loss due to mitochondrial dysfunction and alterations in energy production [5]. In fact, several metabolites involved in the TCA cycle have been observed to be altered in PD, such as pyruvic acid [4, 5], citric acid [5], isocitric acid [8], succinic acid [5] and malic acid [8].
- Sugars and its derivatives, such as fructose [9], mannose [4, 5, 9], galactitol [10], sorbitol [5], threonic acid [9], myoinositol [8] and gluconic acid [8] were also reported as increased in subjects with PD. Monosaccharides has an important role in protein glycosylation and glycation. It is assumed that increased levels of fructose might indicate pathological accumulation of so called “advanced glycation products”, which cause oxidative stress in the early stage of PD due to production of reactive oxygen species. Moreover, alterations in threonic acid together with fructose and mannose might be helpful due to their role in immunity, oxidative stress and processes of glycosylation/glycation in the early stage of PD [9].
- Several amino acids and related compounds have been observed at altered levels in PD too, including valine [4, 5], methionine [4], threonine [4], serine [4], alanine [4, 5], pyroglutamic acid [11], while ratio of uric acid and creatinine was changed in different stages of PD [12]. Alterations in amino acid metabolism indicate involvement of mitochondrial dysfunction in PD [4]. Changes in branched chain amino acids (valine, leucine, isoleucine) might also implicate on changes in protein synthesis, mitochondrial biogenesis, autophagy, as well as with other disease, including mitochondrial respiratory disease [4]. These findings largely indicate involvement of mitochondrial dysfunction in PD pathogenesis.

- Furthermore, metabolites involved in fatty acid metabolism, including fatty acids, glycerophospholipids, carnitines and bile acids have also been shown certain impairments, indicating inflammation, increased rate of oxidative stress, impaired brain metabolism or mitochondrial dysfunction [3, 4, 13–16]. Fatty acids mostly show reduction in PD patients, possibly due to their vulnerability to the oxidative stress that cause lipid peroxidation and structural damages of fatty acids [17]. In particular, the fatty acids and related compounds that showed changes in PD subjects were stearic acid [4, 5], oleic [4, 5], linoleic acid [5], palmitic acid [4, 5], palmitoleic acid [5], methylmalonic acid, ethylmalonic acid, and suberic acid [8].
- Implication of oxidative stress in PD development and progression has also been seen in increased levels of markers of oxidative stress, such as 8-hydroxyguanosine (8-OHG) and 8-Hydroxy-2'-deoxyguanosine (8OHdG) [5], but also in reduced levels of endogenous antioxidant, uric acid [4]. However, as it is already mentioned, changes of metabolites, part of different metabolic pathways, such as fatty acid and sugar metabolism have also shown alterations that might be a result of oxidative stress.
- Uric acid, together with xanthine, hypoxanthine, inosine and adenine is part of purine metabolism. It represents the final product of the aforementioned metabolic pathway and has a protective role against cell death and damages caused by oxidative stress [4, 5, 18]. It is observed that people with lower levels of uric acid in the brain, serum or plasma have higher risks to develop PD. Therefore, low levels of uric acid might be a potential biomarker for the early diagnosis of PD [18–21]. Other metabolites belonging to the pathway of the purine metabolism, such as guanosine, inosine, xanthine and hypoxanthine have been reported at reduced levels [4].
- Other compounds, including alcohols, hydroxy acids and amines, including methylhistamine [22], propylene glycol [5], dehydroascorbic acid [9] and trimethylamine [23] have also been altered in PD patients. However, mechanisms that lead to these changes are still unclear.

It is noteworthy that these metabolic pathways have been related to PD from subjects with an already established disorder. This means these metabolites have been found in patients who had already developed and had a positive diagnostic for PD at the time when those studies were carried out. A fundamental reason for this is that the symptoms start with the neurodegeneration and this is a difficulty when looking for reliable samples to study the earliest stages of the disease. Unsurprisingly, for PD as well as for other neurodegenerative disorders, the early diagnosis of the disorder is the greatest challenge. The main point is to detect the disorder before the neurodegeneration starts in order to proceed with an adequate early treatment. In these regards, the scientists have not been able to find a set of metabolites that are altered before the disease is established.

The complication of finding biomarkers for detecting a disease before it emerges is to find reliable samples to be used. The studies published so far are mainly focused on biomarkers for early stages of PD [24–26]. The difficulty of this studies relies on the identification of patients who have recently developed PD, in the earliest possible stage. The diagnosis must be done carefully, in order to avoid confounding symptoms that might relate with other diseases or conditions. For that reason, the diagnosis

must always wait longer than desired, until the symptoms are unequivocal. Sadly, this implies the neuron degeneration has already started. This is not, therefore, an early diagnosis of PD, done before the disease is settled and any symptoms are shown. Anyhow, this approach entails a great advance, since diagnosing PD in a very early stage can also improve the treatment and life quality of the patients.

In this scenario, a set of samples is highlighted for their capacity to be used as true samples for the study of early stages of PD. The plasma samples used in this study were obtained from the European Prospective Investigation into Cancer and Nutrition (EPIC). In this study, focused on investigating the effects of several factor and the incidence of cancer and other chronic diseases, more than half a million participants from 10 European countries were recruited and followed up for almost 15 years. It is possible to research the bank sample in order to find who of those donors were not diagnosed for a particular disease at the time of sample collection and developed the disease afterwards, during the monitoring period of the study, over those mentioned 15 years. We used a subset of these samples from healthy participants (not diagnosed of PD or showing any PD-related symptoms) at baseline, that were researched by expert epidemiologists in order to identify donors who had not a diagnosed PD and did not show any PD-related symptoms at the time of sample collection, but who developed PD later on. These samples are, therefore, of an extraordinary value for studying biomarkers that are altered before PD shows any symptoms and the neurodegeneration begins. Counting on those samples, we considered that investigating those metabolites that are known to be altered when PD is established can be a good starting point for finding biomarkers for the early diagnosis of the disease.

This work is a proof of concept based, therefore, in two premises. The first one is the ability of the EPIC samples to reveal early biomarkers for PD, considering the unique nature of those samples. The second premise is that some of these early biomarkers for PD can also observed altered in later stages of the developed disease, meaning that some of the markers for stablished PD could also be used as biomarkers for the early diagnosis of PD. Based on these two premises, the aim of this article was to study the EPIC samples using liquid chromatography and mass spectrometry methodologies for finding metabolites that can be used as early biomarkers of PD.

## 2. Results

The analytical performance of the five chromatographic methods is shown in the supplementary material in the section "Method validation". Out of all 40 analytes that were analyzed, seven significant metabolites were observed (t-test,  $p < 0.05$ ). Benzoic acid, palmitic acid, oleic acid, stearic acid, myo-inositol, sorbitol and quinolinic acid were significantly changed in subjects that later developed PD. While fatty acids, myo-inositol and sorbitol were significantly decreased, benzoic and quinolinic acid were significantly increased in PD subjects. Despite the assumption that decreased levels of uric acid represent risk for PD development [21], in this study the difference in uric acid levels between subjects that later developed PD and healthy control subjects was not observed (Table 1).

**Table 1.** List of analyzed metabolites together with their p-values, log<sub>2</sub>FC values, VIP, p(corr) scores and AUC scores. In green, the significant metabolites found in this work. In blue, other compound that did not fulfill criteria for signification but are also included in the discussion because of their relatively significant values.

Metabolic pathway/ class	Metabolites	p-value	log <sub>2</sub> FC	VIP	p(corr)	AUC
<i>Amino acids and derivatives</i>	D-methionine	0.3653	0.10	0.28	0.09	0.606
	Serine	0.6553	0.03	0.89	0.26	0.577
	Threonine	0.3898	-0.08	0.63	0.22	0.614
	Valine	0.3725	-0.06	0.40	0.13	0.571
	Alanine	0.3450	-0.19	0.90	0.35	0.613
	Creatinine	0.0619	0.21	1.34	0.31	0.688
	Pyroglutamic acid	0.2814	-0.13	0.71	0.28	0.651
<i>Tryptophan and kynurenine metabolism</i>	Tryptophan	0.9767	0.01	0.11	0.05	0.503
	Kynurenic acid	0.9877	-0.01	0.68	0.12	0.505
	3-hydroxykynurenine	0.0789	0.67	1.28	0.38	0.701
	Quinolinic acid	0.0440	0.38	1.62	0.34	0.728
	Kynurenine	0.1335	0.15	1.43	0.33	0.690
<i>Benzoic acids and derivatives</i>	Benzoic acid	0.0385	0.31	1.30	0.34	0.693
<i>Bile acids</i>	Deoxycholic acid	0.3085	0.24	0.32	0.03	0.619
<i>Purine metabolism</i>	Uric acid	0.5773	-0.10	0.81	0.25	0.536
	Hypoxanthine	0.6508	-0.71	0.72	0.28	0.553
	Xanthine	0.9366	-0.01	0.36	0.01	0.548
	Inosine	0.4830	-1.53	0.76	0.26	0.582
	Guanosine	0.7943	-0.08	0.31	0.00	0.503
	<i>Fatty acid and dicarboxylic acid metabolism</i>	Palmitic acid	0.0013	-1.31	1.91	0.68
Oleic acid		0.0036	-1.25	1.86	0.68	0.854
Stearic acid		0.0076	-1.02	1.62	0.62	0.792
Suberic acid		0.0593	0.32	1.03	0.28	0.720
Methylmalonic acid		0.2769	0.04	1.30	0.28	0.627
Ethylmalonic acid		0.5404	-0.10	0.95	0.32	0.571
<i>Sugars and others</i>		Galactitol	0.0570	-0.90	1.21	0.29
	Sorbitol	0.0040	-2.20	1.13	0.42	0.836
	D-Gluconic acid	0.5855	-0.10	0.67	0.06	0.545
	Threonic acid	0.7249	0.07	0.60	0.09	0.582
	Myo-inositol	0.0211	-0.27	1.45	0.52	0.765
	<i>TCA cycle</i>	Pyruvic acid	0.4958	-0.37	0.40	0.02
α-ketoisocaproic acid		0.6346	0.03	0.76	0.02	0.556
Succinic acid		0.6186	0.03	0.95	0.11	0.558
Malic acid		0.0936	0.11	1.08	0.14	0.656
<i>Amines</i>		Methylhistamine	0.9611	-0.03	0.15	0.06
	Trimethylamine	0.3191	-0.18	1.17	0.45	0.610
	Propylene glycol	0.0607	-0.49	1.50	0.40	0.683
<i>Alcohols and polyols</i>	Propylene glycol	0.0607	-0.49	1.50	0.40	0.683
<i>Dopamine and norepinephrine metabolism</i>	Dopamine	0.9609	-0.06	0.66	0.21	0.505
	3,4-dihydroxyphenylacetic acid	0.2210	-0.23	0.78	0.10	0.643
<i>Gamma butyrolactones</i>	Dehydroascorbic acid	0.9639	0.06	0.33	0.10	0.505

### 3. Discussion

Parkinson's disease is a complex, heterogeneous neurodegenerative disorder with an expected risen prevalence up to 9 million in 2030 [27]. Thus, an increasing number of PD patients might cause a high financial and social burden [28]. Clinical diagnosis of PD is usually established when first parkinsonian symptoms appear and when there is already a significant dopaminergic loss [9]. Therefore, due to the lack of biomarkers for early diagnosis of PD, targeting metabolites that could be involved in PD

development and progression might improve therapeutic efficiency and provide a better understanding of the underlying molecular mechanisms that lead to PD development [27], as well as improving the quality life of patients and relieve pressure on medical services.

### **3.1. A multi-platform for the analysis of metabolites associated to PD**

To find metabolites that are related to PD in samples from healthy volunteers, who developed PD over the time after the sample collection, is a real challenge that requires analytical sensitivity and selectivity. In this study we developed and partially validated five liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for the analysis of metabolites that are known to be altered in PD. Ion pairing chromatography, reverse phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) have been used in order to provide a wide coverage of the analytes that were selected to be analyzed, which have many different chemical natures. Tandem mass spectrometry has been chosen in order to provide the highest selectivity and sensitivity in the analysis. The analytical performance of the methods has been adequate for most of the analyzed metabolites, which has been evaluated by studying the linearity, the repeatability, the intermediate precision and the sensitivity in terms of limit of detection (LOD) and limit of quantitation (LOQ). All this ensures the quality of the obtained data for the studied metabolites.

### **3.2. The altered metabolites**

Out of the 40 studied compounds related to PD, seven statistically significant ( $p < 0.05$ ) metabolites have been observed in PD subjects compared with healthy control subjects. Benzoic acid, palmitic acid, oleic acid, stearic acid, myo-inositol, sorbitol and quinolinic acid were significantly changed in PD subjects.

Altered levels of sugar alcohols, galactitol and sorbitol, might indicate alterations in the sugar metabolism, especially in galactose metabolism. It is assumed that increased glucose levels could overcome glycolysis capacity, which cause conversion of glucose to sorbitol. Another altered metabolite that was significantly altered is myo-inositol, which also plays an important role in sugar metabolism. It is known that altered levels of myo-inositol, together with altered levels of sorbitol might be associated with changes in polyols metabolic pathways, including glucose metabolism and glycolysis [8]. Alterations of glycolysis and sugar metabolism imply on potential involvement of metabolic pathways that participate in the energy production in pathogenesis of PD [10]. Besides, malabsorption of sorbitol might be associated with gastrointestinal dysfunction. Bacterial overgrowth causes changes in gut mucosa, what leads to sugar malabsorption [29]. Bacterial overgrowth, as well as other gastrointestinal dysfunctions are typical among subjects with PD. Up to 80% of PD patients show some signs of gastrointestinal impairments, which usually appear in the early stage of PD [30]. Altered levels of galactitol and sorbitol in subjects with PD have been observed in other studies too [10]. While we obtained decreased levels of galactitol and sorbitol, Ahmad and colleagues (2009) showed decreased levels of galactitol but increased levels of sorbitol [8]. However, even inconsistent results indicate possible association of PD pathogenesis

with dysfunction of sugar metabolism and energy production and therefore represent potential biomarkers for early diagnosis of PD. While we found decreased levels of myo-inositol, Ahmad and colleagues (2009) found its levels increased in subjects with Parkinson [8]. Such changes indicate possible involvement of mitochondrial dysfunction in altered energy metabolism in the early stage of Parkinson's disease or even before the illness has appeared.

Decreased levels of fatty acids in subjects with PD have been observed in this study. Palmitic, oleic and stearic acids were significantly decreased. Such a result is in correspondence with other studies that found reduction in fatty acids levels among PD patients [4]. Havelund et al. (2017) found, among other, decreased levels of palmitic, oleic and stearic acid in subjects with PD [4]. The metabolism of fatty acids has repeatedly been associated to the development and pathogenesis of PD. Changes in fatty acids might be associated with mitochondrial dysfunction [4, 5, 31], neuroinflammation [13], alterations in apoptotic signaling [32], as well as with oxidative stress [13]. Oxidative stress cause production of reactive oxygen species that affect fatty acids, making them more vulnerable for lipid peroxidation and causing membrane impairments, as well as loss of integrity [17]. Therefore, these changes in the metabolism of fatty acid could help to understand the progression of PD and the involved species could be potential biomarkers for early diagnosis of the disease.

In this study reduced levels of propylene glycol has been observed, while Ahmad et al. (2009) found propylene glycol levels in PD statistically elevated [8]. Propylene glycol is part of several metabolic pathways including glycine, serine, tyrosine and pyruvate metabolism, which dysregulation are associated to Parkinson's disease [2, 3]. These metabolic pathways are related to dopamine and energy metabolism. It is known that the dysregulation of dopamine metabolism is characteristic for PD, including dopaminergic loss [4, 5]. Through mitochondrial dysfunction and dysregulation of energy metabolism, TCA cycle is associated with dopamine metabolism, while pyruvate is one of the metabolites that plays an important role in the TCA cycle. Therefore, altered levels of propylene glycol and its implication in several metabolic pathways, such as pyruvate, glycine metabolism, which are mutually interconnected, might indicate that the energy and dopamine metabolism are disrupted in the early stage of PD. Another altered metabolite indicates involvement of TCA cycle in PD pathogenesis. In this study, increased levels of succinic acid have been observed in PD subjects. Succinic acid is a part of TCA cycle, which is assumed to be downregulated in the early phases of PD [5]. These alterations of the TCA cycle might be result of mitochondrial dysfunction, as well as impairments in energy production. However, due to some inconsistent results, further research of potential implication of this metabolic pathway is necessary.

Quinolinic acid is an intermediate compound in the tryptophan-kynurenine metabolic pathway [7]. Kynurenine is the main intermediate compound and it can be metabolized in two ways by two kynurenine aminotransferase isoenzymes to kynurenic acid, which acts as neuroprotective agent, or to 3-hydroxykynurenine, and quinolinic acid, which are neurotoxic. Increased levels of quinolinic acid cause neuron excitation by activation of NMDA receptors, which consequently leads to excitotoxicity, increased inflammation and eventually to the neuronal death [33]. It is known that degeneration of dopaminergic

neurons in the substantia nigra in Parkinson's disease is a result of excitotoxicity. Recent studies [6, 34] showed that altered kynurenine pathways and its metabolites are present in plasma and cerebrospinal fluid respectively in subjects with PD. This is in correspondence with our finding of altered levels of quinolinic acid. Dysfunction of tryptophan and kynurenine pathway might result in increased oxidative stress, as well as neuroinflammation that would lead to neurodegenerative processes characteristic for PD. Therefore, increased levels of quinolinic acid in the subjects that later developed PD might indicate alterations in the kynurenine pathway in the early stage, before first PD symptoms appear and might represent potential biomarker for early diagnosis or future improved treatment, that could target kynurenine to 3-hydroxykynurenine conversion [7].

Globally, the metabolites that have been found to be significantly altered in this study are related to mitochondrial dysfunction, the oxidative stress and the mechanisms of energy production. Considering that all the volunteers enrolled in this study were healthy at the time of sample collection, these finding might imply that these processes begin to be affected before PD shows any symptoms. This information is of great relevance for a disease such as PD, in which the definition of a metabolite panel that can be used for the early diagnosis of the disease has been sought for decades. Counting with the necessary tools for defining and studying such panels enables effective clinical managements, ensures early treatments and reduces the chances of complications.

### **3.3. EPIC samples for finding biomarker for the early diagnosis of PD**

We are aware of the limitations of the study, in particular, in terms of the sample size. Therefore, these metabolites should be validated in larger studies. However, what we consider the most important aspect of the present work, is the use of true reliable samples that allow for well-founded biomarkers for the early diagnosis of PD. The samples used in this work are of invaluable importance. They were obtained from the large prospective EPIC study, in which volunteers were followed up for years, not just for cancer events, which was the main objective of the study, but for the development of many other chronic diseases such as cardiovascular disease, type 2 diabetes, PD and also mortality or even healthy ageing. We want to highlight that the samples included in this study, which include plasma, serum, leukocytes, and erythrocytes, are searchable and researchers can apply for their collection and use in their research studies. This work used samples from the EPIC cohort in Navarra (Spain). Among the 8084 participants enrolled in this cohort (Fig. 1), 36 were found to be suitable for our study. Two other EPIC-Spain cohorts have undergone the ascertainment of PD cases, for a total of 25,016 participants and 69 PD cases occurred during over 15 years of follow-up. However, Spain has a total of 5 cohorts, counting up to 41438 participants, who donated the different sample types mentioned before. And ours is just one of the 10 participating countries. All this, considered together, provides multiple options worth of investigation. In fact, the EPIC study can be branched in endless ways, which keep the project well alive.

One of the premises of this work was that some of the metabolites that are altered in conditions of established PD might also be altered before and, therefore, could be used as biomarkers for the early

diagnosis of PD. In order to study such a premise, reliable samples must be used. In these regards, the samples from the EPIC study were a great opportunity for the evaluation of panels of metabolites that might be altered in subjects before they developed a disease. Being aware of the small cohort studied in this work, we have also limited our study to certain compounds that were found altered in previous studies. This does not exclude the possibility that other metabolic pathways could be altered before the disease is established and other studies of different natures should be performed, such as untargeted studies, which aims for the blind discovery of differential metabolites.

## **4. Conclusion**

This work was conceived as a proof of concept that shows the possibilities of the EPIC samples for finding biomarkers for the early diagnosis of PD. The fact that all the donors of these samples were healthy at the time of the sample collection make these sample of trusted value for such a purpose. We consider the samples stored in the different cohorts of the EPIC study can also be used for many other purposes, since massive data, such as life conditions, development of diseases, time of death or survival are stored for all the participants, who were followed up for 15 years. These samples can be searched in order to find healthy donors for a particular disease, who developed that particular disease over the time. In our opinion, the possibilities of these samples are almost endless, and these samples are of extreme value for those researchers looking for small changes that occur before a disease is established.

We provided adequate analytical techniques for the metabolites to be studied, which has ensured to have a wide panel of metabolites to be evaluated. We have confirmed that some of the metabolites that are altered in PD seem to be also altered before the disease shows any symptoms. These significant metabolites indicate possible association of mitochondrial dysfunction, alteration in energy metabolism and tryptophan metabolism, as well as oxidative stress as the molecular mechanisms that could lead to the development and progression of a complex neurodegenerative disorder, such as PD.

We are aware of the limitations of this study: these findings should be confirmed in bigger studies and other biological pathways should also be investigated for finding reliable biomarkers for the diagnosis of PD, a disease of which the early diagnosis has always been a great challenge.

## **5. Methods**

### **5.1. Subjects recruitment**

The study was conducted at the Centre for Metabolomics and Bioanalysis (CEMBIO) in Madrid, Spain. This case-control study included plasma samples from one Spanish cohort (EPIC-Navarra), part of the EPIC study. EPIC is a prospective multi-center cohort study, including around half a million healthy volunteers collected from 23 different centers in 10 European countries. Subjects were recruited from 1992 to 2000 and monitored for 15 years with the aim of researching chronic disorders. Biological samples, including plasma, serum, erythrocytes and leukocytes were collected and stored in liquid

nitrogen. Our study included 12 case plasma samples, from subjects who had developed Parkinson's disease from the sampling time to June 2011, and 21 corresponding control samples. Incident PD cases were ascertained by record linkage with health databases to identify potential cases, followed by individual revision of the medical history by expert neurologists in order to establish the diagnosis based on the available clinical records [35]. Cases and controls have been matched by follow-up time, age, sex, body mass index and center. Diagnosis of Parkinson's disease was established through record linkage with health databases, for participants fulfilling at least two of the following criteria: 1) Primary Care records using either codes 332 of ICD-9, or codes N87 of the International Classification of Primary Care; 2) registration of prescriptions, including subjects with at least one prescription of any of the N04-antiparkinsonian drugs of the ATC/DDD index (N04-antiparkinsonian drugs; N04A-anticholinergic agents; N04B-dopaminergic agents); 3) mortality record using codes 332 of the ICD-9 for PD; 4) the Minimum Basic Data Set (CMBD) using codes 332 of the ICD-9 for the EP; 5) death certificates with the G20 code of the ICD-10.

## 5.2 Sample preparation

The straws containing the plasma of each sample were removed from the freezer and slowly thawed on ice. Subsequently, they were opened and transferred to 500  $\mu$ L Eppendorf tubes, which were kept constantly on ice. They were vortexed for 2 minutes. 100  $\mu$ L of plasma were transferred to an Eppendorf tube and 300  $\mu$ L of a cold mixture (-20°C) of methanol:ethanol (1:1, v/v) were added for deproteinization. After stirring the samples for 1 minute, they were incubated on ice for 5 minutes and vortexed for another 1 minute. Samples were centrifuged for 20 minutes, at 13200 rpm and at 4°C. Finally, 100  $\mu$ L of supernatant were transferred to an HPLC vial for analysis.

## 5.3. Preparation of blanks and calibration samples

Blank samples were prepared in the same way as the plasma samples. 300  $\mu$ L of methanol:ethanol (1:1, v/v) were added to 500  $\mu$ L Eppendorf tubes with 100  $\mu$ L of Mili-Q water. The protocol continued with a centrifugation at 13200 rpm for 20 min. The supernatant was transferred to the HPLC vial for analysis. Calibration samples were prepared from 1000 ppm stock solutions in methanol:ethanol (1:1, v/v).

## 5.4. Analytical setup

The study was conducted using 5 different LC-MS/MS methods according to the detectability of the analytes (see Table 2 and section "Chromatographic method" in the supplementary material). Briefly, one ion-pairing method, two HILIC methods and two RPLC methods were used combined with tandem mass spectrometry in a triple quadrupole. These methods were partially validated according to the information provided in the section "Methods validation" in the supplementary material.

Table 2

Analytical methods used and their corresponding systems, columns and mobile phases.

	<b>Ion-pairing</b>	<b>HILIC A</b>	<b>HILIC B</b>	<b>RPLC A</b>	<b>RPLC B</b>
<b>System</b>	1200 Infinity 6460 QqQ	1260 Infinity II 6470 QqQ	1260 Infinity II 6470 QqQ	1260 Infinity II 6470 QqQ	1260 Infinity II 6470 QqQ
<b>Column</b>	Zorbax Extended C18 (2.1x150 mm, 1.8 µm)	XBridgeBEH Amide 2.5 micron (2.1 x 100 mm, 2.5 µm)	XBridgeBEH Amide 2.5 micron (2.1 x 100 mm, 2.5 µm)	Zorbax Eclipse, XDB, C18 (4.6 x 150 mm, 5 µm)	Zorbax Eclipse, XDB, C18 (4.6 x 150 mm, 5 µm)
<b>Mobile phase A</b>	97% water and 3% methanol, 10mM TBA, 15mM acetic acid	0.1% Formic acid prepared in water, pH 9 (NH <sub>3</sub> )	5mM Ammonium formate prepared in water	0.1% Formic acid prepared in water	0.5% Formic acid prepared in water
<b>Mobile phase B</b>	10mM TBA, 15mM acetic acid in methanol	0.1% Formic acid prepared in ACN	Acetonitrile	0.1% Formic acid prepared in methanol	0.5% Formic acid prepared in methanol

## 5.5. Metabolites and methods

According to the information provided in the introduction, the following analytes were included in this study. Due to the chemical diversity of these metabolites, the analytes were distributed in the 5 analytical methods above-described based on their detectability and selectivity, as it is shown in Table 3.

Table 3

List of studied metabolites and their retention times in the assigned chromatographic method.

<b>Metabolic pathway/ class</b>	<b>Metabolites [RT*]</b>	<b>Methods</b>
<i>Amino acids and derivatives</i>	Creatinine [1.193]	<b>Ion-pairing</b>
	Pyroglutamic acid [6.937]	
<i>Benzoic acids and derivatives</i>	Benzoic acid [15.113]	
<i>Bile acids</i>	Deoxycholic acid [20.894]	
<i>Purine metabolism</i>	Hypoxanthine [1.950]	
	Xanthine [2.523]	
	Inosine [4.623]	
	Guanosine [4.896]	
<i>Fatty acid and dicarboxylic acid metabolism</i>	Palmitic acid [22.446]	
	Oleic acid [22.559]	
	Stearic acid [23.252]	
	Suberic acid [14.951]	
	Methylmalonic acid [12.097]	
	Ethylmalonic acid [13.059]	
<i>Sugars and others</i>	Myoinositol [1.294]	
<i>TCA cycle</i>	Succinic acid [12.097]	
	Malic acid [12.806]	
<i>Tryptophan and kynurenine metabolism</i>	Tryptophan [7.513]	
	Kynurenic acid [14.589]	
<i>Amino acids and derivatives</i>	Valine [12.068]	<b>HILIC A</b>
	Alanine [10.207]	
<i>Amines</i>	Methylhistamine [11.941]	
	Trimethylamine [4.409]	
<i>Purine metabolism</i>	Uric acid [8.763]	
<i>Amino acids and derivatives</i>	D-methionine [3.298]	<b>HILIC B</b>
*RT = retention time expressed in minutes		

Metabolic pathway/ class	Metabolites [RT*]	Methods
	Serine [4.155]	
	Threonine [3.893]	
<i>Tryptophan and kynurenine metabolism</i>	3-hydroxykynurenine [3.439]	
<i>Alcohols and polyols</i>	Propylene glycol [3.375]	<b>RPLC A</b>
<i>Dopamine and norepinephrine metabolism</i>	Dopamine [3.482]	
	3,4-dihydroxyphenylacetic acid [5.345]	
<i>TCA cycle</i>	Pyruvic acid [2.625]	
	α-ketoisocaproic acid [5.339]	
<i>Sugars and others</i>	Threonic acid [2.314]	
<i>Gamma butyrolactones</i>	Dehydroascorbic acid [6.229]	<b>RPLC B</b>
<i>Sugars and others</i>	Galactitol [2.227]	
	Sorbitol [2.227]	
	D-Gluconic acid [2.249]	
<i>Tryptophan and kynurenine metabolism</i>	Quinolinic acid [3.807]	
	Kynurenine [5.312]	
*RT = retention time expressed in minutes		

## 5.6. Data treatment and statistical analysis

After the chromatogram inspection, the obtained data were treated with the MassHunter Quantitative Analysis software (Agilent MassHunter Quantitative Analysis 10.0) for the determination of the area of each peak. Microsoft Office Excel was used for quantitation, doing also a blank subtraction. The p-values (t-test or Wilcoxon/Mann-Whitney test, Microsoft Office Excel and SPSS, respectively) were calculated for each metabolite. Log<sub>2</sub>FC was calculated according to the following formula:

$$\log_2\text{FC} = \log_2 (\text{averageCASES} / \text{averageCONTROLS})$$

Multivariate statistics were also performed in this study. Supervised Orthogonal Partial Least Square – Discriminant analysis (OPLS-DA) was performed. Volcano plots plotting variable importance in the projection (VIP) in OPLS-DA model against corrected p-values [p(corr), loading values scaled as correlation coefficients values] were generated. Variables with absolute p(corr) lower than 0.3 show a low correlation, while value between 0.3 and 0.5 show an intermediate correlation.

Metabolites with p-values less than 0.05, VIP score > 1 and  $p(\text{corr}) \geq 0.3$  were considered significant.

ROC curves and the area under the curve (AUC) for the studied metabolites were obtained in Metaboanalyst 5.0 [36].

## List Of Abbreviations

AUC, area under the curve.

DOPAC, 3,4-dihydroxyphenylacetic acid.

EPIC, European Prospective Investigation into Cancer and Nutrition.

HILIC, hydrophilic interaction liquid chromatography.

HPLC, high performance liquid chromatography.

KA, kynurenic acid.

LC-MS/MS, liquid chromatography-tandem mass spectrometry.

LDOPA, 3,4-dihydroxyphenylalanine.

LOD, limit of detection.

LOQ, limit of quantitation.

OPLS-DA, Supervised Orthogonal Partial Least Square – Discriminant analysis.

PD, Parkinson's Disease.

QA, quinolinic acid.

RPLC, reverse phase liquid chromatography.

RT, retention time.

TCA, tricarboxylic acids cycle.

VIP, variable importance in the projection.

## Declarations

- Ethics approval and consent to participate

The EPIC study protocol was approved by the International Agency for Research on Cancer (IARC) Ethics Committee. The current research has been conducted according to the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

All participants voluntarily agreed to take part and gave written informed consent.

- Consent for publication

Not applicable.

- Availability of data and materials

All data generated or analyzed in this study are included in this article. Other data that are relevant to this article are available from the corresponding author upon reasonable request. Competing interests

The authors declare that they have no competing interests.

- Funding

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- Authors' contributions

MK prepared the samples, developed the analytical methods, analyzed the samples, did the corresponding data analysis and wrote the first draft of the manuscript. JS assisted with the method development and corrected the manuscript draft. CB supervised the whole analytical process and helped with the data evaluation.

AB, PA, EA, JMH and JMI were involved in study concept and design.

EA, JMH, PA, AB, MDC, MEE and AVA were involved in data acquisition.

AB, PA, EA, JMH, JMI and EA were involved in data interpretation.

AB, PA and JMI were involved in the work supervision.

AB, PA, EA, JMH, MEE, AVA, MDC, JMI and EA were involved in critical revision of the manuscript for important intellectual content.

All authors read and approved the final manuscript.

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## Figures

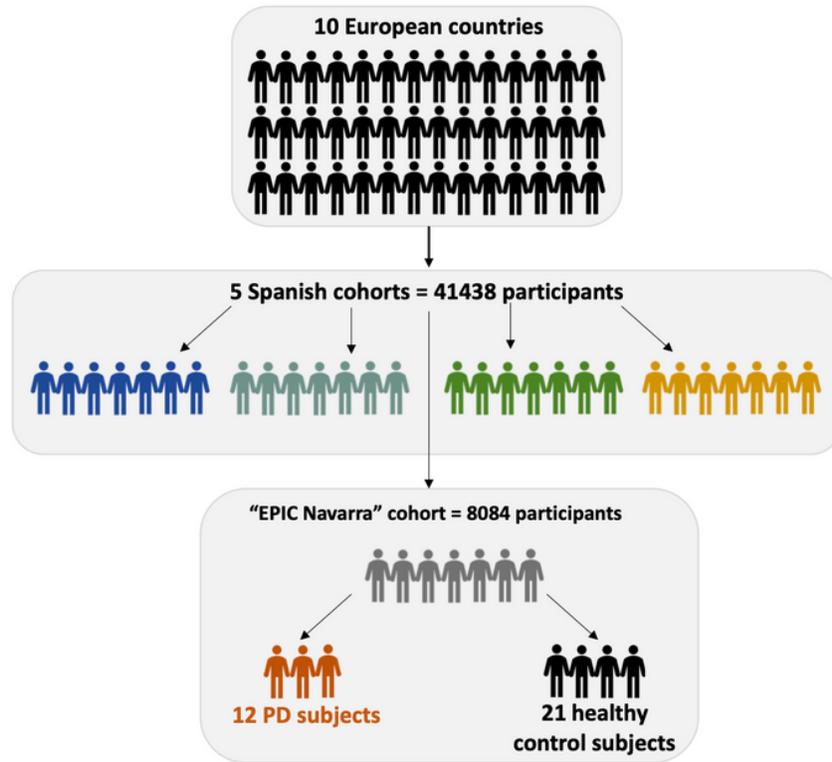


Figure 1

Schematic structure of the EPIC study and the samples used in the present study.

## Supplementary Files

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