Analysis of circulating metabolites to differentiate Parkinson’s disease and essential tremor

Elena A. Ostrakhovitch a, Eun-Suk Song a, Jessica K.A. Macedo b, Matthew S. Gentry b, Jorge E. Quintero c, Craig van Horne c, Trittia R. Yamasaki a,d,e,*

a Department of Neurology, University of Kentucky, Lexington, KY 40536, USA
b Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536, USA
c Department of Neurosurgery, University of Kentucky, Lexington, KY 40536, USA
d Department of Neuroscience, University of Kentucky, Lexington, KY 40536, USA
* Corresponding author at: 740 South Limestone St., University of Kentucky, Department of Neurology, Ste. J401, Lexington, KY 40536-0284, USA.

1. Introduction

Parkinson’s disease (PD) and essential tremor (ET) are two common adult-onset tremor disorders in which prevalence increases with age. PD is a neurodegenerative condition with progressive disability. In ET, neurodegeneration is not an established etiology. We sought to determine whether an underlying metabolic pattern may differentiate ET from PD. Circulating metabolites in plasma and cerebrospinal fluid (CSF) were analyzed using gas chromatography-mass spectroscopy. There were several disrupted pathways in PD compared to ET plasma including glycolysis, tyrosine, phenylalanine, tyrosine biosynthesis, purine and glutathione metabolism. Elevated α-synuclein levels in plasma and CSF distinguished PD from ET. The perturbed metabolic state in PD was associated with imbalance in the pentose phosphate pathway, deficits in energy production, and change in NADPH, NADH and nicotinamide phosphoribosyltransferase levels. This work demonstrates significant metabolic differences in plasma and CSF of PD and ET patients.

Abbreviations: PD, Parkinson’s disease; ET, essential tremor; CSF, cerebrospinal fluid; MetPA, Metabolomic Pathway analysis; GC–MS, gas chromatography-mass spectrometry; MSTFA, N-methyl-N-trimethylsilyl-trifluoroacetamide; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NAMPT, Nicotinamide phosphoribosyltransferase; PUFAs, polyunsaturated fatty acids; PPF, pentose phosphate pathway; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NAM, nicotinamide; NNM, nicotinamide mononucleotide; NADK, NAD+ kinase.

E-mail address: tyamasaki@uky.edu (T.R. Yamasaki).

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2. Materials and methods

2.1. Patients and consent

Samples were collected during DBS surgery in PD (n=11) and ET (n=7) patients 55–78 years of age. Study group characteristics are detailed in Supplementary S1. Patients were followed by movement disorder specialists at Kentucky Neuroscience Institute and consented under an IRB-approved protocol at the point of candidacy for DBS as determined by clinical care standards.

2.2. Sample collection

After breaching dura and prior to placement of electrodes, cerebral CSF was collected by syringe with a sterile soft-tube catheter. Peripheral blood was collected during surgery (EDTA tubes) and plasma obtained determined by clinical care standards.

2.3. Metabolite extraction/derivatization and GC–MS analysis

Extraction, derivatization and analysis protocols were described previously [34,40]. Briefly, 30 µl of plasma or CSF were mixed with 1 ml of 100% ice cold methanol with 200 µM L-norvaline, placed on a cell disruptor (3000 rpm for 5 min), incubated on ice for 30 min, then centrifuged at 15000 rpm for 10 min. Supernatant containing polar metabolites was dried by SpeedVac. Pellets containing proteins were washed with 50% methanol, hydrolyzed with 3 N HCl at 95 °C for 2 h and dried by SpeedVac. Samples were derivatized with methoxymamine and incubated 90 min at 30 °C [13]. 20 mg/ml N-methyl-trimethylsilylation (MSTFA) in pyridine was added. Samples were incubated for 20 min at 37 °C. Derivatized samples were transferred to a v-shaped glass chromatography vial and analyzed by GC–MS on an Agilent GC with Agilent 5977A quadrupole MS instrument (Agilent). Sample (1 µl) was injected onto a GC column (30 m × 250 µm internal diameter, 0.25 µm film thickness) in a 1:10 split mode. Initial temperature was 130 °C, held for 4 min, rising at 6 °C/min to 243 °C, rising at 60 °C/min to 280 °C, held for 2 min. Full scan mode (m/z: 50–800) was used for target metabolite analysis. Relative abundances were normalized using internal standard L-norvaline, adjusted to protein input from pellet analysis. Results were analyzed with MetaboAnalyst 4.0.

2.4. NAD+ and NADP+/NADPH levels

Total plasma nicotinamide adenine dinucleotide (NADH) levels were assessed from 25 µl of plasma, using a NAD+ /NADPH kit (Cayman). Nicotinamide adenine dinucleotide phosphate (NADP+ /NADPH) levels were assessed from 25 µl plasma using a NADP+/NADPH kit (Cell Biolabs). A 10KDa spin filter deproteinated plasma. Samples were analyzed in triplicate.

2.5. Dot blot assay

Bio-Dot Apparatus (Bio-Red, Hercules) was assembled with a pre-wet 0.4 µm nitrocellulose membrane. Plasma (25 µl) was diluted 2-fold and CSF (2 µl) was diluted 25-fold with TBS, and applied to the membrane (50 and 25 µl). Membranes were blocked for one hour at 25 °C and probed with anti-α-synuclein Clone42 (BD 610787), rabbit monoclonal MJFR1 (Abcam, ab138501), rabbit monoclonal αSer129 (Abcam, ab138501), rabbit monoclonal αSer129 (Abcam, EP1536Y), and NAMPT (Invitrogen PA5-23198) antibody overnight at 4 °C. Membranes were washed with 50% methanol, hydrolyzed with 3 N HCl at 95 °C for 2 h and dried by SpeedVac. Samples were derivatized with methoxymamine and incubated 90 min at 30 °C [13]. 20 mg/ml N-methyl-trimethylsilylation (MSTFA) in pyridine was added. Samples were incubated for 20 min at 37 °C. Derivatized samples were transferred to a v-shaped glass chromatography vial and analyzed by GC–MS on an Agilent GC with Agilent 5977A quadrupole MS instrument (Agilent). Sample (1 µl) was injected onto a GC column (30 m × 250 µm internal diameter, 0.25 µm film thickness) in a 1:10 split mode. Initial temperature was 130 °C, held for 4 min, rising at 6 °C/min to 243 °C, rising at 60 °C/min to 280 °C, held for 2 min. Full scan mode (m/z: 50–800) was used for target metabolite analysis. Relative abundances were normalized using internal standard L-norvaline, adjusted to protein input from pellet analysis. Results were analyzed with MetaboAnalyst 4.0.

2.6. Data analysis/statistics

Statistical analyses utilized GraphPad Prism. Data are presented as mean ± SD. Column analysis was performed using one-way ANOVA or t-test; p-values < 0.05 were considered statistically significant.
3. Results

3.1. α-Synuclein in plasma and CSF

α-Synuclein in plasma and CSF samples from patients with PD and ET was measured by dot blot assay. PD plasma and CSF α-synuclein levels were significantly higher than in ET (Fig. 1A-D). Plasma levels of Ser129-phosphorylated α-synuclein (pSer129 α-syn) and the ratio of pSer129 α-syn/total α-synuclein were significantly higher (p < 0.01 and p < 0.05) in PD than ET (Fig. 1E, F). There was no significant difference for pSer129 α-syn in CSF (Fig. 1G, H).

3.2. Plasma metabolites

Polar metabolites in the plasma of PD and ET patients were assessed by GC–MS. Supervised clustering analysis was utilized to assess the overall metabolic profile in each cohort. Distinct clustering was observed in ET (red) and PD (green) samples (Fig. 1A-D). Plasma levels of Ser129-phosphorylated α-synuclein (pSer129 α-syn) and the ratio of pSer129 α-syn/total α-synuclein were significantly higher (p < 0.01 and p < 0.05) in PD than ET (Fig. 1E, F). There was no significant difference for pSer129 α-syn in CSF (Fig. 1G, H).

3.3. Abnormal metabolic pathways in PD as compared to ET

We performed pathway enrichment analysis of metabolic pathways in plasma. Six metabolic pathways were identified as altered in PD: tyrosine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; quinone biosynthesis; glycolysis; and phenylalanine, purine and amino acid metabolism.

There were perturbations in the pentose phosphate pathway (PPP). The level of ribose was significantly downregulated (0.45-fold) and sedoheptulose was significantly upregulated (39.7-fold) in PD compared to ET (Fig. 2B). The increase in sedoheptulose and sedoheptulose-7-phosphate levels suggest dysfunction in the PPP (Fig. 2C). The PPP is divided into an oxidative branch and non-oxidative branch (Fig. 2D). The irreversible oxidative branch utilizes glucose 6-phosphate to produce ribose-5-phosphate and NADPH. Therefore, we measured NADPH and NADP+, which were significantly higher in PD plasma compared to ET (Fig. 2E), implying a more active oxidative arm of the PPP in PD. Consistent with activation of the non-oxidative segment of the PPP, the level of ribose was lower in PD than in ET plasma (Fig. 2F).

Plasma uridine was also significantly diminished (0.45-fold) (Fig. 2B, F) which could reflect ATP depletion and glucose hypometabolism since uridine homeostasis is tightly related to glucose homeostasis and lipid and amino acid metabolism [39].
glutathione metabolism (Fig. 3A). We detected a higher plasma level of lactate and a 25% decrease of glutathione derivative cysteinylglycine, suggesting glutathione depletion in PD (Fig. 3B).

Oxidative stress results from the failure to maintain redox homeostasis and is observed in PD [16]. The pyridine nucleotide redox system impacts the oxidative state and includes nicotinamide adenine dinucleotide (NADH/NAD$^+$) [8,28,38]. We measured levels of NADH in the plasma samples of PD and ET patients. We detected a significant increase in NADH in PD plasma (Fig. 3C). Nicotinamide phosphoribosyltransferase (NAMPT) increases the NAD$^+$ pool and plays an essential role in lipid and glucose metabolism (Fig. 3D). We found a significant increase in NAMPT in PD plasma samples (Fig. 3E-F) by dot blot analysis.

### 3.4. CSF metabolic profile

In CSF samples, supervised clustering analysis demonstrated clear separation between PD (green) and ET (red) (Fig. 4A) with 66 metabolite peaks identified in PD and ET patients (Supplementary S3). Eight metabolites were statistically altered in PD compared to ET. There were reduced levels of lactic acid (0.376-fold) and malic acid (0.43-fold), and increased levels of beta-hydroxyisovalerate (2.4-fold), monosaccharide (2.64-fold), urea (2-fold) and sucrose (2.64-fold) (Fig. 4B). Pathway enrichment analysis demonstrated alterations in glycine, serine and threonine metabolism, glyoxylate metabolism, and t-RNA biosynthesis in PD (Fig. 4C). Similar to plasma, purine and glutathione metabolism were altered in PD CSF. NAMPT levels did not show a statistically significant change in PD versus ET (Fig. 4D).

The PD:ET ratio of specific metabolite concentrations in CSF and plasma showed significant variability (Fig. 4E). Pyruvate, urea, and dehydroascorbic acid levels showed elevated ratios, whereas oleic acid demonstrated reduction in PD versus ET in both CSF and plasma, implying concordance of the central and peripheral metabolomic states. Most striking was a significant elevation in eicosapentanoic acid and malic acid in plasma (but not CSF) in PD compared to ET. Discordance was also observed between CSF and plasma levels for lactate, serine, glycine, stearic and palmitic acid for PD versus ET which may imply a more specific CNS-based metabolomic change for those metabolites.

### 4. Discussion

PD is the most common age-related neurodegenerative movement disorder, whereas ET can occur at any age but is most common in older people [12]. Although ET is a common neurological disorder, there are surprisingly few studies in this area. We detected higher levels of total $\alpha$-syn and pSer129 $\alpha$-syn in PD plasma compared to ET. This is consistent with many studies investigating PD compared to controls [7,9,21]. We also detected higher levels of total $\alpha$-syn in PD compared to ET in cerebral CSF samples. There was no significant difference in pSer129 $\alpha$-syn levels for PD and ET. Other groups have been unable to detect pSer129 $\alpha$-syn in PD CSF samples obtained through standard lumbar puncture [9]. In our study, we utilized cerebral CSF taken in proximity to the brain, which may have different characteristics than lumbar CSF. One limitation of our study, given the foundational nature, was the small sample size of PD and ET samples obtained. Another limitation, given cerebral CSF collection in the setting of brain surgery, was the lack of a...
similarly-treated control group, although we abstracted values found in literature for comparison (Supplementary S4).

Changes in metabolism are a key feature of neurodegenerative diseases like PD [27,37]. ET patients show significant glucose hypermetabolism at the medulla, thalami, and cerebellar cortex, whereas PD patients exhibit cortical glucose hypometabolism [2,15,27]. Pathway enrichment analysis of plasma metabolites found that glycolysis was significantly altered in PD as compared to ET. We observed a trend toward lower glucose levels in PD plasma. Surprisingly, this change was not statistically significant. We found a decreased plasma ribose level in PD which also suggests glucose hypometabolism. It is possible that the advanced age of our participants results in an overall reduction in glycolysis [6,24].

We found prominent dysregulation of the PPP in PD. Under oxidative stress conditions, which are characteristic of PD, glucose metabolism is rerouted from glycolysis through the oxidative segment of the PPP [20] and generates NADPH. In support of this, we found that NADPH and NADP⁺ levels were significantly higher in PD than ET. There was an increase in the level of sedoheptulose and sedoheptulose 7-phosphate, intermediates in the PPP, in PD samples. Both NADP⁺/NADPH and NAD⁺/NADH are essential to the regulation of cellular metabolism, bioenergetic homeostasis, and redox balance. The level of NADH is relatively unaltered in normal aging [11]. We found a higher NADH level in PD plasma compared to ET. However, PD and ET NADH concentrations were significantly lower than those in healthy control subjects (0.44–2.88 µM) [31].

The increased level of NADH was associated with increased NAMPT in PD plasma compared to ET. NAMPT activates pro-inflammatory pathways and NAMPT inhibitors protect neurons against ischemic injury in vivo [4,10,41]. Our findings are consistent with studies that show upregulation of NAMPT mRNA in PD plasma [30]. There was no corresponding increase in CSF NAMPT in PD compared to ET which suggests that CSF NAMPT levels are generally low among this age group.

Tryptophan metabolism along the kynurenine pathway has been implicated in PD [18,25]. The kynurenine pathway has been implicated in neuroinflammation in PD and leads to the production of NAD+ [33]. Increased plasma NADH suggests alteration in tryptophan metabolism and activation of the kynurenine pathway. We were unable to identify kynurenine metabolites with GC–MS. Liquid chromatography-mass spectrometry might allow analysis of tryptophan metabolites of the kynurenine pathway.

We compared the PD: ET ratio of metabolites in plasma and CSF. Specific metabolites did correlate, trending toward an increase or decrease in both plasma and CSF that might indicate a systemic effect. Other metabolites demonstrated discordance between plasma and CSF. In these cases, the CSF value is more likely to be reflective of a CNS-based dysfunction in PD.

5. Conclusions

This study was undertaken to determine whether the metabolic profile differs in PD and ET patients. We utilized plasma and CSF from patients with clinically-established movement disorders. We found that total and phosphorylated α-synuclein levels in plasma and cerebral CSF differentiate these cohorts. PD and ET have different metabolic profiles that are readily detected in plasma and CSF. Changes in metabolic profile were consistent with altered bioenergetics, dysregulation of the pentose phosphate pathway, increase in NADPH/NADH energy substrates, and elevation of NAMPT in PD vs ET plasma. This study provides insight into potential therapeutic targets for PD and suggests that...
differentialization of PD and ET may be possible through utilization of a carefully selected set of plasma-based markers.

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**CRediT authorship contribution statement**

Elena A. Ostrakhovitch: Investigation, Writing – original draft, Conceptualization, Formal analysis. Eun-Suk Song: Investigation, Formal analysis. Jessica K.A. Macedo: Investigation, Methodology, Formal analysis. Matthew S. Gentry: Conceptualization, Methodology, Resources, Funding acquisition, Writing – review & editing, Funding acquisition. Jorge E. Quintero: Resources, Writing – review & editing. Craig van Horne: Resources. Tritia R. Yamasaki: Conceptualization, Resources, Supervision, Writing – review & editing, Formal analysis, Funding acquisition.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2021.136428.

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