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Analysis of human cerebrospinal fluid monoamines and their cofactors by HPLC

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Abstract

The presence of monoamines and their cofactors (the pterins and vitamin B_6 (pyridoxal phosphate)) in human cerebrospinal fluid (CSF) can be used as indicators of the biosynthesis and turnover of dopamine and serotonin in the brain. In addition, abnormalities in the CSF levels of these molecules are associated with various neurological diseases, including genetic diseases leading to dopamime and serotonin deficiency. Here we provide a set of quantitative high-performance liquid-chromatography (HPLC) approaches to determine CSF levels of monoamines and their cofactors. This protocol describes step-by-step procedures for CSF sample preparation for analysis of the different molecules, HPLC calibration and analysis, and data quantification and interpretation. Unlike, plasma/tissue/blood samples, CSF requires minimal sample preparation: in this protocol, only the analysis of pyridoxal phosphate requires mixing with trichloroacetic acid to release the protein-bound vitamin, centrifugation, mixing the supernatant with phosphate buffer and sodium cyanide for derivatization in alkaline conditions. Monoamines are analysed by HPLC with coulometric electrochemical detection, pterins by HPLC with coupled coulometric electrochemical and fluorescence detection, and pyridoxal phosphate by HPLC with fluorescence detection. The quantification of all compounds is achieved by external calibration procedures and internal quality control and standards are analyzed each run. We anticipate that investigation of dopamine and serotonin disturbances will be facilitated by measurements of these compounds in human CSF and other biological samples. The estimated time for the different procedures primarily depends on the electrochemical detector stabilization. Overnight stabilization of this detector is advised, and after that step, preanalytical equilibration rarely exceeds 3 hours.

KEYWORDS monoamine; pterin; pyridoxal-phosphate; coulometric electrochemical detection; fluorescence detection; cerebrospinal fluid; high-performance liquid-chromatography; HPLC-ED; HPLC-FD; UHPLC; cerebrospinal fluid; serotonin; dopamine; homovanillic acid; 5-hydroxyindoleacetic acid; tetrahydrobiopterin; CSF

EDITORIAL SUMMARY The levels of monoamines and their cofactors in cerebrospinal fluid are strong indicators for dopamine and serotonin biosynthesis and turnover. This protocol describes a set of HPLC-based approaches for the quantitative detection of these molecules.

TWEET Detecting #monoamines and their cofactors in human cerebrospinal fluid using #HPLC

COVER TEASER HPLC detection of monoamines and their cofactors.

Please indicate up to four primary research articles where the protocol has been used and/or developed.

1. Ormazabal, A. *et al.* HPLC with electrochemical and fluorescence detection procedures for the diagnosis of inborn errors of biogenic amines and pterins. *J. Neurosci. Methods*. **142**, 153-158 (2005).

2. Ormazabal, A. *et al.* Pyridoxal 5'-phosphate values in cerebrospinal fluid: reference values and diagnosis of PNPO deficiency in paediatric patients. *Mol. Genet. Metab.* **94**, 173-177 (2008).

3. Molero-Luis, M. *et al*. Homovanillic acid in cerebrospinal fluid of 1388 children with neurological disorders. *Dev. Med. Child. Neurol*. **55,** 559-566 (2013).

4. Lam AA, Heales SJ.Nitric oxide accelerates the degradation of tetrahydrobiopterin but not total neopterin in cerebrospinal fluid; potential implications for the assessment of tetrahydrobiopterin metabolism. *Ann Clin Biochem*. **44**:394-396 (2007).

INTRODUCTION

Monoamines, such as dopamine and serotonin, have fundamental roles in motor, perceptual, cognitive and emotional brain functions along with other functions, including vascular tone, temperature regulation, endocrine regulation, and swallowing[1]. The relevant pathways for their synthesis and disease-associated genetic defects are shown in **Supplementary Figure 1** and **Supplementary Table 1**. Several genetic and environmental conditions have been shown to affect the metabolism of dopamine and serotonin or the cofactors that are essential for the proper biosynthesis of these monoamines (pterins and pyridoxal phosphate (PLP))[1]. Aromatic L-amino acid decarboxylase and tyrosine hydroxylase deficiencies are two relevant genetic diseases (see also **Supplementary Figure 1** and **Supplementary Table 1)**. The quantitative detection of monoamines and their cofactors in the cerebrospinal fluid (CSF) can be used as an indicator for dopamine and serotonin biosynthesis and turnover in the brain and is considered of important diagnostic marker for a number the conditions described above. The status of the dopamine and serotonin systems in the central nervous system can for instance be studied by measuring the levels of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA), respectively[2,3]. The CSF concentrations of these primary (and stable) end-metabolites provide insights into the biosynthesis and turnover of dopamine and serotonin. Screening for other dopamine and serotonin metabolites can be done in the same chromatogram and enables a deeper investigation of the monoamine status in a single analysis[2,3]. These metabolites are 3-orthomethyldopa (3-OMD) and methoxyhydroxyphenylglycol (MHPG) for dopamine pathway and 5-hydroxytryptophan (5HTP) for serotonin pathway (see **Figure 1**).In addition, quantitative assessment of the CSF concentrations of cofactors that are essential for monoamine synthesis can provide important diagnostic information. Pterins (neopterin, dihydrobiopterin (BH_2), and tetrahydrobiopterin (BH_4)) are, for example, useful biomarkers for the research of BH_4 metabolism-associated genetic disorders. Neopterin analysis is a powerful tool for identifying inflammatory and immune conditions in the central nervous system[4]. Determining CSF PLP concentrations provides insights into the various genetic and environmental conditions that lead to a brain PLP deficiency[5], which consequently affects dopamine and serotonin biosynthesis. In this protocol, we will describe several HPLC-based biochemical methods for the quantification of CSF concentrations of the aforementioned biomarkers (see **Table 1**). The monoamine quantification procedures specified here can also be adapted for the quantification of other monoamines for diagnostic and research purposes. In addition, this methodology can be used for the investigation of the neurotransmisson status both in animal and celular models, and for the analysis of plasma and urine samples.

Overview of the procedures

The first critical step towards studying the aforementioned compounds is the CSF sample collection procedure. For most of the diseases shown in **Supplementary Table 1**, analyses of blood or urine sample are unreliable for brain dopamine and serotonin

status estimations. CSF collection should be performed by an expert clinician, and strict follow-up of the pre-analytical protocol is mandatory[6,7].

For the chromatographic separation of the biomarkers presented here, different HPLC methods are used: ion-pair HPLC is based on the modification of a reverse phase column to separate ionized compounds. Reverse phase HPLC allows the separation of compounds by hydrophobic interactions with the stationary phase of the column. With regards to detection, electrochemical detection (ED) is used to quantify redox active compounds by applying a potential and measuring the resulting current produced, which is proportional to the concentration. Fluorescence detection (FD) is used for the quantification of compounds with either native (pterins analysis) or derivatized fluorescent groups (pyridoxal phosphate (PLP) analysis). In fluorescence detection the compound is excited at one wavelength (excitation) and measured at another wavelength (emission) (see **Table 2** for details).

For the CSF monoamine quantification (option A, procedure section) ion-pair HPLC with coulometric electrochemical detection (HPLC-ED) is the gold standard because it enables a highly sensitive separation and quantification of these compounds[2,7].

For the CSF pterin analysis HPLC with fluorescence detection (HPLC-FD) is single approach for the rapid quantification of the oxidized pterins (neopterin, biopterin, primapterin and sepiapterin) as an estimation of the biosynthesis and metabolism of the active pterin (BH_4). This procedure has previously been reported in detail[3] and will not be included in this protocol. In-line HPLC-ED-FD (option B, procedure section), is also available for the quantification of total neopterin, BH_4 and its precursor, BH_2, and it will be detailed in this report[3]. This in-line method allows the simultaneous quantification of both electroactive (BH_4) and fluorescence compounds (BH_2 and neopterin). For the PLP analysis (option C, procedure section), HPLC-FD offers a single-high-sensitivity approach for the PLP quantification[8,9]. The results for each technique are reported as absolute values in the nmol/L range.

Applications of the method

The most common applications for the methods described here include the diagnosis of primary genetic conditions that lead to neurotransmitter deficiencies and other severe early onset neurological diseases that can be associated with disturbances in brain dopamine and serotonin availability[10,11]. The procedures may also be suitable for the quantification of monoamines, such as dopamine, serotonin and related compounds, in experimental animal and cellular models after adequate sample extraction and purification steps, (acid precipitation on ice, centrifugation, and supernatant filtration) as previously reported[12,13,14]. Brain microdialysis procedures also allow the determination of monoamines and other molecules in interstitial tissue fluid[15]. Fukushima et al reported several works studying BH_4 and its metabolites by HPLC with fluorescence detection in different rat tissues (pineal, liver, adrenal, brain and blood)

showing that the same methodology is also useful for human samples (blood and urine)[11].

Elution and quantification of other monoamines are expected to occur in the same chromatogram as the key monoamines metabolites of interest (Figure 1). For the pterins, applications beyond identification of primary genetic causes of BH_4 deficiency may include studies of brain inflammatory/immune events by CSF neopterin quantification. An analysis of PLP can detect a suboptimal vitamin B_6 status due to nutritional deficiencies, increased turnover, increased sequestration by chemical adducts, and other environmental and genetic conditions that promote its deficiency[5,16,17].

Comparison with other commonly employed methods

The methods described here have routinely been used to investigate biogenic amines and pterins in the CSF. Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS-MS) methods are increasingly used in bioanalytical investigations due to their sensitivity, specificity and efficacy in different biological materials[18]. A targeted metabolomics approach is a noteworthy alternative to HPLC-based strategies, and new developments towards future human diagnostic applications are expected. Kovac et al. developed a LC/MS method to assess 5-hydroxytryptamine, 5HIAA, HVA, noradrenaline, adrenaline, dopamine, glutamate, γ-aminobutyric acid, 3,4-dihydroxyphenylacetic acid, and histamine in the CSF of a rat model[18]. Santos-Fandila et al. described an analysis of neurotransmitters, metabolites and derivatives in rat microdialysates by UHPLC-tandem mass spectrometry[19]. LC-MS/MS has also been used for pterin analyses and involves simultaneous detection of BH_4, BH_2 and biopterin in urine samples, cell extracts and the rat brain[20,21,22]. However, according to aforementioned authors, MS/MS detection is less sensitive than FD for pterin quantification. For PLP, an UHPLC-MS/MS approach has been published [23,24], which allows the identification of seven vitamin B_6 vitamers in human CSF samples, though this procedure is not routinely used for PLP quantification in biological fluids. Recently, a simultaneous measurement of monoamine metabolites and, also 5-methyltetrahdyrofolate, in CSF by HPLC-FD has been developed[25].

Taking together, the methodological information stated in this work may be very helpful for both clinical and basic research laboratories.

Limitations

Practical limitations in sample preparation are worth of consideration. First, sometimes the sample volume is not sufficient for doing all of the investigations required. Moreover, CSF contamination by blood after traumatic lumbar puncture may cause autoxidation of some compounds (such as monoamines[26]). Regarding CSF matrix effects, they are less important than those of blood or other tissues, and they have been reported mainly associated with mass spectrometry procedures. The main limitations of the present protocol are related to the HPLC-ED system. While HPLC-FD is robust and highly sensitive, HPLC-ED needs an optimum equilibration and preparation of the system and requires specifically trained operators and so may be relatively time-consuming. These issues and other ones will be described in the present protocol. The

establishment of accurate reference values is mandatory for proper data interpretation. The CSF samples from "healthy" paediatric controls are needed, but these samples are difficult to collect outside the Hospital setting. Because there is a strong correlation between the monoamine, pterin and PLP levels and age, a large CSF sample size is required for the the establishment of reference values, since several intervals must be defined. We took advantage of the lumbar punctures collected in our Hospitals for viral and bacterial meningitis diagnoses. Once these diseases were ruled out, we used the remaining sample as control samples to determine biogenic amines, pterins and PLP.

Level of expertise

These analytical procedures can be performed by competent professionals. The most complicated procedure involves ED, and special training for those managing this detector is advisable. However, the critical step involves the data interpretation because a high degree of experience is required to identify the genetic conditions mentioned in **Supplementary Table 1** and to interpret the numerous secondary neurotransmitter, pterin, and PLP disturbances. Therefore, the data interpretation should be performed within the context of the clinical information provided by expert clinician in the field.

Experimental Design

The optimization for the biogenic amine, pterin and PLP measurements is divided into two stages as follows: (1) pre-analytical factors (sample collection and storage protocol) and (2) analytical factors (optimization of the HPLC-ED and FD settings and data interpretation).

Pre-analytical factors: CSF is the ideal biological sample for diagnosing most biogenic amine disorders. A protocolized lumbar puncture collection is required for a reliable analysis of the monoamines, pterins and PLP (**Table 3**). Because there is a rostrocaudal gradient (i.e., the concentration of some metabolites is higher in the final CSF fractions than in the initial fractions), it is important to compare a patient's values to his own reference values, which are established using the same CSF fraction[6,7]. Because red blood cell lysis causes oxidation of amine metabolites, blood-contaminated samples must immediately be centrifuged, and the clear CSF supernatant must be transferred to a new tube. To measure neopterin, BH_2 and BH_4, the CSF sample must be protected from light and stored with stabilizing agents because the reduced forms are extremely light- and oxygen-sensitive3. Because CSF samples have less interfering compounds when compared with other biological matrixes, such as blood, plasma, tissue extracts or urine, no extensive purification or extraction procedures are required, and only single dilution and filtration steps are needed prior to HPLC analysis[7]. However, guard column and graphite filters have to be regularly replaced to assure a good analytical performance. Sample storage at –70ºC with protection from light is mandatory (**Table 3**).

Analytical factors for monoamine quantification: HPLC-ED is the most commonly used procedure for monoamine quantification. This approach is based on ion-pair chromatography separation, with a mobile phase consisting of citrate-acetate buffer at

an acidic pH that is supplemented with an ion-pair agent (heptanosulfonic acid or equivalent)[6,7]. The ion-pair agent has an ionic head group and a non-polar tail. The non-polar tail interacts strongly with the C18-column, while the ionic head group is projected into the mobile phase. This column modification generates an attraction between ion-pair agent and ionized compounds allowing chromatographic retention and elution. Critical separation conditions that can dramatically change a compound's elution time include the pH value of the mobile phase and the ion pair agent[27]. The detection of monoamines is mediated by on coulometric electrochemical detection (ED). In this approach, the electrochemical detector is used to determine the current generated between two electrodes placed in the detection cell. These electrodes are the working electrode (E1), where the current is generated, and the auxiliary (E2, or counter) electrode, where the complementary electrolytic reaction takes place. The potential between the electrodes is held constant, resulting in the complete oxidation of the analysed compounds of interest. Another electrode called reference electrode, is used to keep the potential stable throughout the measurements. The appropriate ED settings are very important to achieve complete oxidation of the analyte. The appropriate potential for ED can be determined by measuring the oxidative current of the analyte (at a constant concentration) over a range of working electrode potentials. The plot of current generated vs. applied potential is called the hydrodynamic voltammogram. We advise collecting a hydrodynamic volammogram plot regularly for every compound since different variables may influence the results, including factors like the mobile phase composition, the type and age of electrodes used, the deterioration of the graphite filter with the time, and others[28]. The applied potential at +450 mV is normally enough for the proper electrochemical oxidation of monoamines. This oxidation results in a current that can be sensed by the detector and is directly proportional to the monoamine concentration. , An example of a hydrodynamic voltammogram is shown for 5HIAA and HVA in **Figure 2**. Ideally, the lowest current that produces the highest analytical response to the electrode should be chosen since this lead to a lower background signal. Regarding the composition of electrodes, we used porous graphite electrodes since they have a large surface area. This permits the eluent to flow through the electrodes and thereby maximizes the contact area with electroactive compounds, increasing the sensitivity of the analysis. Special precautions should be taken for ED cell stabilization, maintenance, and cleaning, which are described in ED equipment setup and Box 1.

Analytical factors for pterin quantification: HPLC with in-line electrochemical and fluorescence detectors is the method of choice for measuring CSF neopterin, BH_2 and BH_4 in the same chromatogram as pterin3. Neopterin, BH_2 and BH_4 are separated using reverse-phase HPLC. BH_4 is measured by ED, where it is oxidized by electrode 1 (E1) to quinonoid dihydrobiopterin and reduced back to BH_4 at electrode 2 (E2). The detector then uses the current generated by this reduction reaction to determine the concentration of BH_4. Total neopterin (dihydroneopterin and neopterin) and BH_2 can be measured in the same injection by fluorescence detection. A post-column oxidation of dihydroneopterin and BH_2, using a conditioning cell at the optimal potential, oxidizes

dihydroneopterin to neopterin and BH_2 to biopterin. Because dihydroneopterin and neopterin co-elute, they are detected as a single peak by the fluorimeter (excitation 360 nm: emission 440 nm), which provides a total neopterin measurement. BH_2 is detected and measured in the same chromatogram. A schematic representation of the ED cells is shown in **Figure 3.**

Analytical factors for PLP quantification: CSF PLP quantification is performed by HPLC with fluorescence detection, as previously reported[8,9]. The mobile phase consists of a phosphate buffer. PLP is derivatized with sodium cyanide under alkaline conditions. The analytical details of these procedures are summarized in **Table 2**, and typical chromatograms of the different procedures are shown in **Figure 4-6**.

Standards and controls: For protocol validation and application, we use different materials: standards (or calibrators) and internal and external quality control materials. For calibration purposes, the standard concentrations are close to those observed in human CSF samples. We use the standards to calibrate the procedure, to establish a relation between the concentration and the analytical response (area under the peak). The control materials are used to assess the matrix effects, since they are based on human biological matrixes. Since 2014, we have participated in an external quality control scheme (ERNDIM program) for monoamines and pterins. The ERNDIM control program consists of 8 different samples which are analysed throughout the year and the results of the 29 participants are compared. At the end of the program and according to fixed criteria the performance for accuracy, precision, linearity and recovery is graded for every participant.

MATERIALS

REAGENTS

!CAUTION Handle all toxic and flammable chemicals in a fume hood.
!CAUTION All corrosive, flammable and poisonous reagents must be stored in appropriate laboratory storage systems as follows: 90-minute EN fire-resistant safety cabinet, 144470-1, Asecos, MPA Dresden, and a chemical cabinet for acids and alkalis, Asecos, MPA Dresden.
▲CRITICAL Avoid using detergents to clean the glassware for HPLC-ED. This will cause a substantial increase in the signal background. Glassware is rinsed with miliQ or ultrapure water and dried with methanol. Use glassware instead of plastic ware for the mobile phase and standard solution preparations. Transfer the solvents using glassware.
Human CSF samples.
▲CRITICAL All reagents should be freshly prepared for each use in volumetric glassware, unless suitable storage conditions are specified. When water is indicated, ultrapure water (18 MΩ) should be used. Mili-Q water treatment consists on UV lamp with two wavelengths to assure the degradation of organic molecules by photoxydation. Then, another ultrafiltration system (Quantum®) eliminates ionic and organic contaminants to very low trace concentrations. Finally, additional specific filters can be chosen according the needs of the laboratory (ultrapure water without particle matter, bacteria, lipopolisaccharide, nucleases and proteases).

Cerebrospinal fluid (CSF): See Reagent Setup on information on sample collection and **Table 3** for information on the required quantities.
!CAUTION Our protocol was developed at two different institutions, The National Hospital for Neurology and Neurosurgery, United Kindom (UK) and Hospital Sant Joan de Déu , Spain. In the UK, the Royal College of Pathologist's guidelines (2012) that refer back to the Human Tissue Act, allow for residual anonymised patient material to be used for method performance assessment and quality control purposes. At Hospital Sant Joan de Déu, the Ehics Committee approved the use of residual and anonymised CSF samples for the same purposes. Users of the protocol should obtain appropriate institutional approval and conform to national regulations.
!CAUTION For the collection of CSF into the clinical diagnostic workflow, informed consent was always collected as for other medical interventions in both institutions. All samples from the patients were obtained in accordance with the 2013 revised Helsinki Declaration of 1964.
!CAUTION Adhere to all guidelines and regulations associated with the collection and use of human CSF. All personnel working with such samples should be informed of the risks associated with borne pathogens and be trained in the use of correct sample-handling techniques. Some infectious meningoencephahylitis cases can mimic genetic diseases and may be unrecognized prior to the HPLC analysis. Use appropriate personal protective equipment.

Monoamine analysis

- Ultrapure water (Type I), 18.2 MΩ·cm; Milli-Q® Integral ZRXQ003WW.
- Methanol (HPLC grade, ≥99.8%; Merck KGaA, cat. No. 1060018). **!CAUTION** Methanol is toxic and highly flammable, and it should be handled in the fume hood.
- Citric acid (ACS reagent, ≥99.5%; Sigma-Aldrich, cat. No. 251275).
- Sodium acetate trihydrate (BioXtra, ≥99.0%; Sigma-Aldrich, cat.no. S-7670).
- Ethylenediaminetetraacetic acid dipotassium salt dihydrate (≥ 98%; Sigma-Aldrich, cat. No. ED2P).
- Ion pair agent: 1-Heptanesulfonic acid sodium salt (BioXtra, Sigma-Aldrich, cat. no. H8901).

Monoamine Standards

- L-3-O-Methyl-DOPA (3-OMD) (Powder, ≥98%; Sigma-Aldrich, cat. No. M4255).
- 4-Hydroxy-3-methoxyphenylglycol sulfate potassium salt (MHPG) (≥98%; Powder, Sigma-Aldrich, cat. no. H8759).
- 5-Hydroxy-L-tryptophan (5HTP) (>98% Powder, Sigma-Aldrich, cat. no. H9772).
- 5-Hydroxyindole-3-acetic acid (dicyclohexylammonium) salt (5HIAA) (Crystalline, ≥98% (TLC); Sigma-Aldrich, cat. no. H2255).
- Homovanillic acid (HVA) (Powder ≥98%; Sigma-Aldrich, cat. no. H1252).
- Internal quality control. Special Assays Urine, SKML, cat.no. SAU-01.

▲**CRITICAL**: Store all standards and controls at -70ºC.

Pterin analysis

- Ultrapure water (18 MΩ; Main Lab).
- Sodium acetate AnalaR (VWR, cat. no. 27652.232).
- Citric acid (BDH HiPersolv, cat. no. 153144M).
- Ethylenediaminetetraacetic acid (EDTA) disodium salt (for electrophoresis and molecular biology, 99.0-101.0% (titration); Sigma-Aldrich, cat. no. E5134).
- 1,4-Dithioerythritol (DTE) (≥99.0%; Sigma-Aldrich, cat. no. D8255).
- Diethylenetriaminepentaacetic Acid (DETAPAC) (≥99%; Sigma-Aldrich, cat. no. D6518). **!CAUTION** DETAPAC can cause respiratory problems, and it should be handled in the fume hood.
- Methanol (HPLC grade) (VWR HiperSolv for HPLC, cat. no. 152506X). **! CAUTION** Methanol is toxic and highly flammable, and it should be handled in the fume hood.
- Sodium hydroxide, pellets (Sigma, cat. no. 221465).

Pterin Standards

- D-Neopterin (Sigma-Aldrich, cat. no. N3386). ▲**CRITICAL** Store in the dark at -70ºC.
- (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (Sigma, cat. no. T4425) ▲**CRITICAL** Store in the dark at -70ºC.

- 7,8-Dihydro-l-biopterin (Sigma, cat. no. 37272). ▲**CRITICAL** Store in the dark at -70ºC.
- Internal quality control. This is generated by pooling the clear CSF samples from previous patients with no evidence of pterin disorders. Mix thoroughly, aliquot and immediately freeze in labelled Eppendorf tubes at –70˚C.

Pyridoxal phosphate analysis

- Ultrapure water (Type I), 18.2 MΩ·cm; Milli-Q® Integral ZRXQ003WW.
- Trichloroacetic acid (ACS grade, ≥99.5%; Merck KGaA, cat. no. 100807). **!CAUTION** Trichloroacetic acid is corrosive and should be handled in the fume hood.
- Potassium phosphate dibasic K_2HPO_4 (ACS reagent, ≥98%; Sigma-Aldrich, cat. no. P3786).
- Sodium cyanide (ACS reagent, ≥95%; Sigma-Aldrich, cat. no. 205222). **!CAUTION** Sodium cyanide is corrosive with acute toxicity and a high health hazard (respiratory sensitization, mutagenicity and carcinogenicity) and should be handled in the fume hood.
- Acetonitrile (HPLC grade ≥99%, Merck KGaA, cat. no. 100030). **!CAUTION** Acetonitrile is toxic and highly flammable and should be handled in the fume hood.
- Orthophosphoric acid (ACS reagent, 85%; Merck KGaA, cat. no. 100573). **!CAUTION** Orthophosphoric acid is corrosive and should be handled in the fume hood.
- Semicarbazide hydrochloride (≥95%; Merck KGaA, cat. no. 107722).
- Potassium phosphate monobasic KH_2PO_4 (ACS grade, ≥99.0%; Sigma-Aldrich, cat. no. P5379).

Pyridoxal phosphate Standards

- Pyridoxal phosphate plasma/serum calibration standard, lyophilized, 5x1 mL; Chromasystems, Teknokroma, cat. no. 36005.
- Pyridoxal phosphate internal quality control, lyophilized, 5x2 mL; Chromasystems, Teknokroma, cat. no. 0039.

EQUIPMENT

HPLC systems

Two HPLC systems coupled to electrochemical and fluorescence detectors are used. Please refer to **Table 2** for detailed information on the different systems.

Monoamines and pyridoxal phosphate

- HPLC binary pump (Waters, model 1525. Milford, MA 01757, USA).
- Refrigerated autosampler injection system (Waters, model 717 plus). ▲**CRITICAL** We advise the use of a refrigerated autosampler, especially if a large number samples will be analysed.
- In-line degasification system, AF 186001273, Waters. ▲**CRITICAL** If dissolved

gases reaches the ED or FD, HPLC pressure oscillations and high-noise background signals will appear (as described in *Coulochem II. Operating Manual* ESA Inc (USA, 1997)).

- Interface module for equipment communication (SAT/IN, Waters).
- Integration programme, Breeze (Version 3.0, Waters).
- Monoamine column: Reverse-phase HPLC column, nucleosil C18 (octadecylsilane; ODS, 5-µm, 250 × 5 mm column) cat. no. 720041.46, Macherey-Nagel. Guard ODS column (Teknokroma cat. no. TR-C-160-1).
- Pyridoxal phosphate column: HPLC column C8 (octylsilane; OS, 5-µm, 250 × 4.6 mm column) Symmetry shic, cat. no. WAT 200661. Guard C8 column (Teknokroma cat. no. TR-C-160-3).

  ▲CRITICAL Columns are used only for the indicated applications. Average numer of samples analyzed with the same column were 650 for monoamines and 1450 for PLP.

Pterins

- TSP (Thermo separation products) Spectra series P100 pump.
- Jasco AS-2057 plus a refrigerated autosampler.
- Kontron instruments DEG 103 degasser.
- Jones chromatography column heater.
- Azur data capture system, PC and software.
- Columns. Reverse-phase HPLC column, C18 (octadecylsilane; ODS, 5 µm, 250 x 4.6 mm column), ODS pre-column cartridge, Teknokroma cat. no. TR-015326.

  ▲CRITICAL The column is used only for the indicated application. Average number of samples analyzed with the same column was 825.

Common HPLC accessories

- PEEK tubing 1/16, 3 m Teknokroma cat. no. 25067. **▲CRITICAL** Due to the sensitivity of the chromatographic separation, the internal diameter of the tubing should be consistently maintained between the HPLC and the column and between the column and the ED and FD.
- Reagent bottles with caps (250 mL, 500 mL, 1 L, and 5 L), blue PP screw cap and pouring ring; Duran laboratory. cat. no. Z305189**,** Z305197, and Z305200.

Electrochemical detector

- Electrochemical detector Coulochem II; Hucoa-Erlöss.
- Electrochemical detector cell; ESA model 5011 analytical cell and ESA model 5021 conditioning cell.
- In-line graphite filters (Thermofisher, cat. no. 70-0898) placed between the column and the ED cell. **▲CRITICAL** This is necessary to remove electroactive impurities prior to ED. It should be regularly replaced.

Fluorescence detector

- Pterins. Jasco model FP-920, intelligent fluorescence detector.
- Pyridoxal phosphate. Fluorescence detector (Waters, model 2475 Multi-fluorescence detector).

Accessories for sample and mobile phase preparation

- pH-meter GLP21, Crison. **▲CRITICAL** Calibration of the pH meter by an external accredited company is needed.
- Precision scale Mettler Toledo New Classic MF model 104S. **▲CRITICAL** Calibration of the precision scale by an external accredited company is needed.
- Freezer at -70°C, Nuaire, model NU6580E.
- Refrigerated centrifuge, HERAEUS, Labofuge 400R.
- Microcentrifuge, cat. no.. 175508, Nirco (for pterins and PLP sample preparation).
- Magnetic stirrer with ceramic plate, H20 LBX, 20 L; Labbox, cat. no. STIH-020-001. Stirrer bar; Labbox, cat. no. MAGC-020-005.
- Ductless Fume Cabinet; Ascent Max; Inflow: 187 L/s or 0.40 mL/s ± 0.025. ESCO cat. no. ADC-6C1. **!CAUTION** Electrical Hazard. Wear appropriate chemical-resistant protective attire.
- Vacuum pump KNF lab. Laboport and solvent filtration unit, Teknokroma cat. no. K-953855-1047.
- Automatic pipettes 10-100 µL, 20-200 µL and 100-1000 µL, Nichipet EX, Nichiryo. **▲CRITICAL** Pipette calibrations by an external accredited company are required.
- Glassware. Flasks and bottles: 5, 4, 2, and 1 L ± 0.4; 500 mL ± 0.250; and 10 mL ± 0.02; Graduated cylinders: 100 ± 0.5 mL and 1000 ± 5 mL. Labbox; Glass pipettes: 5 ± 0.045 mL and 2 ± 0.015 mL, Daslab, Nirco. **▲CRITICAL** Glassware calibrations by an external accredited company are essential for the preparation of the calibration solutions.
- 3 mL polypropylene round bottom tube (11 x 55 mm) Daslab, Nirco cat. no. 175594.
- Filtering membrane filters: nylon, 0.45-µm pore size and 47-mm diameter, pk/50, Teknokroma cat. no. NY 504700, for mobile phase filtering. 4-mm syringe filter (for 2.5 mL plastic syringes, Ico Plus3, Novico Medica cat. no. N15649) with a hydrophilic PVDF membrane (0.22-µm pore size) (Millex-GV; Merck Millipore cat. no. SLGVR04NL) and 10kDa centrifugal filters (Nanosep 10K omega, Pall Life Sciences cat. no. OD010c34) for non-sterile filtration of the samples.
- HPLC vials: a 1-mL amber glass shell vial with a polyethylene snap cap, Waters cat. no. 025053C. Vial insert: 150-µL glass insert with polymer feet, Agilent cat. no. 5183-2088.
- Heat cabinet, Conterm Poupinel; Selecta cat. no. 2000200 for PLP sample preparation.

REAGENT SETUP

CSF Sample collection: An overview of the CSF samples we collect and the required volumes is summarized in **Table 3.** Additional biomarkers, such as amino acids, can be determined in the CSF samples, and it is important to utilize this advantage. CSF samples are collected in conventional plastic tubes (10-ml round-bottom polystyrene tube) without preservatives, except for the pterins. For the pterin analysis, the sample must be collected into a microcentrifuge tube that contains dithioerythritol (1 mg) and diethylenetriaminepentaacetic acid (1 mg) (antioxidants). The sample volume estimation by drop number is useful for the clinician in charge of the CSF collection because the liquid leaves the spine drop by drop. Critical factors for consideration when collecting CSF samples include the rostrocaudal gradient, time of sample collection, protection from light and blood contamination. Immediately after collection, tubes (particularly tubes 2 - 4) must be placed on dry ice and frozen immediately at – 70ºC in the dark. If CSF is contaminated with blood, centrifuge the CSF samples (1500 g x 10 minutes at 4ºC), and store the clear supernatant. Patient CSF samples can be stored for at least 10 years at -70°C. **!CAUTION** The lumbar puncture is an invasive procedure, and critical factors should be considered.

Monoamine "Mobile phase (buffer acetate citrate)". This buffer contains 0.1 M sodium acetate, 0.1 M citric acid, 1.2 mmol/L EDTA, 1.2 mmol/L 1-heptanosulphonic acid, and 75 mL methanol, and it is adjusted to pH = 4. Weigh 13.61 ± 0.01 g of sodium acetate into a 1-L flask, and add water up to 1 L. Weigh 9.60 ± 0.01 g of citric acid into a 500- mL flask, and fill the flask to 500 mL with water. Adjust the pH of the sodium acetate with the citric acid to pH = 4. Separately weigh 0.44 ± 0.01 g of EDTA and 0.242 ± 0.01 g of 1-heptanosulphonic acid, and add them into a 1-L glass bottle with a stir bar. In this bottle, add 75 mL of methanol and mix with the acetate-citrate buffer (pH = 4) up to 1 L. Filter the mobile phase through a solvent filtration unit with a 0.45-µm nylon membrane. **▲CRITICAL** This buffer should be freshly prepared on the day of the analysis.

Monoamine "3-OMD Standard": Stock solution (401 µM). Weigh 2.30 ± 0.04 mg of 3-OMD and dissolve in a 25-mL volumetric flask with water. This can be stored for up to one year at -70°C.

Monoamine "MHPG Standard": Stock solution (546 µM). Weigh 3.10 ± 0.04 mg of MHPG and dissolve in a 25-mL volumetric flask with water. This can be stored for up to one year at -70°C.

Monoamine "5-HTP Standard": Stock solution (270 µM). Weigh 11.01 ± 0.04 mg of 5-HTP and dissolve in a 200-mL volumetric flask with water. This can be stored for up to one year at -70°C.

Monoamine "5HIAA and HVA Standard": Stock solution (247 µM 5HIAA; 241 µM HVA). Weigh 4.60 ± 0.04 mg of 5HIAA and 2.20 ± 0.04 mg of HVA, and dissolve both in a 50-mL volumetric flask with water. This can be stored for up to one year at -70ºC.

Monoamine "Calibration working solution": 40.1 nM 3-OMD, 54.6 nM MHPG, 27 nM 5-HTP, 494 nM 5HIAA and 482 nM HVA. In a 100-mL volumetric flask, add 10 µL of each 3-OMD, MHPG and 5-HTP stock solution and 200 µL of each 5HIAA and HVA stock solution. Add 120 µL of 37% HCL, and fill the volumetric flask up to 100 mL with water. ▲**CRITICAL** The working calibration solution should be freshly prepared.

Monoamine "Internal quality control": Add 5 mL water to the lyophilized commercial vial. Mix gently and incubate for 15 min at room temperature. It is analysed for every run to check for data accuracy and to accept or reject the results of the analysis. ▲**CRITICAL** Store in separate aliquots (110 µL) at -70°C. It is stable until the commercial expiry date.

Monoamine Neurotransmitter external quality control scheme (ERNDIM programme): Add 500 µL water to the lyophilized sample. Mix by vortexing. The sample is ready to analyse. This can be stored for up to one year at -70°C.For details of this external quality control scheme, visit (www.erndimqa.nl). It is blindly analysed eight times per year to validate the accuracy of the procedure. The results are available upon request.

Monoamine HPLC system wash solution: Filter 500 mL of water with 7.5% methanol (vol/vol). This can be stored for up to 1 month at room temperature (20-25ºC).

Monoamine HPLC injector wash solution: water with 10 % methanol (vol/vol). This can be stored for up to 1 month at room temparature. ▲**CRITICAL**: The column and injector washing procedures for all methods must contain organic solvents to avoid contaminations during storage.

Pterin "Mobile phase": Dissolve 27.20 ± 0.01 g of sodium acetate (final concentration 50 nM), 4.20 ± 0.01 g of citric acid (final concentration 5 mM), 80.00 ± 0.04 mg of EDTA (final concentration 48 µM), and 98.81 ± 0.04 mg of DTE (final concentration 160 µM) in 1.5 L of ultrapure water in a 2-L beaker. Bring the volume to 4 L with water in a volumetric flask. Thoroughly mix and transfer the buffer into the 5-L reagent bottle. ▲**CRITICAL** All reagents should be freshly prepared for use with volumetric glassware, unless suitable storage conditions are specified. When water is indicated, ultrapure water (18 MΩ) should be used.

Pterin "BH_4 Standard"(500 µM): In a 100- mL volumetric flask, add 100 ± 0.1 mg of DTE and DETAPAC to 80 mL of water and sonicate for 5 minutes to ensure that the DETAPAC has completely dissolved. Dissolve 15.7 ± 0.1 mg of BH_4 and bring the final

volume to 100 mL with water. Then, mix on ice for 30 minutes with a stir bar and a magnetic stirrer (in the dark). ▲**CRITICAL** The 0.5-mL aliquots should be transferred to Eppendorf tubes and immediately frozen at -70ºC. The aliquots can be stored for up to 1 year.

Pterin "D-Neopterin Standard"(500 µM): Prepare a 10 M NaOH solution by dissolving 10.0 ± 0.1 g of NaOH in 25 mL of water. Slowly add NaOH while cooling on ice. Dissolve 10 µL of 10 M NaOH and 12.70 ± 0.04 mg of D-neopterin in 80 mL of water in a foil-wrapped volumetric flask. Bring the volume to 100 mL and mix on ice for 30 minutes using a magnetic stirrer (in the dark). ▲**CRITICAL** The 0.5-mL aliquots should be transferred to Eppendorf tubes and immediately frozen at -70ºC. The frozen aliquots can be stored for up to 1 year. Due to their light and temperature sensitivities, the 500 µM stock solutions for BH_4 and D-neopterin should be prepared in foil-wrapped volumetric flasks and stored on ice.

Pterin "BH_2 Standard"(500 µM): Dissolve 80 µL of 10 M NaOH and 11.96 ± 0.04 mg of BH_2 in 80 mL of H_2O in a foil-wrapped volumetric flask. Bring the volume to 100 mL with water and mix for 30 min on ice using a magnetic stirrer (in the dark). ▲**CRITICAL** The 0.5-mL aliquots should be transferred to Eppendorf tubes and immediately frozen at -70ºC. The aliquots can be stored for up to one year.

Pterin "Standard diluent": Dissolve 25 ± 0.04 mg of DTE and 2.5 ± 0.04 mg of DETAPAC in 25 mL of water. Sonicate for 5 minutes to ensure DETAPAC dissolution. This can be stored for up to one year -70°C.

Pterin "Working standard": Pour approximately 8 mL of the standard diluent into a 10-mL volumetric flask. Add 100 µL of each BH_4, BH_2 and neopterin stock standard, and bring the volume to 10 mL with the standard diluent. Thoroughly mix by inversion. Fill another 10-mL volumetric flask with approximately 8 mL of the standard diluent, and add 100 µL of the first stock standard dilution. Bring the volume to 10 mL, and thoroughly mix by inversion. ▲**CRITICAL** A single working standard solution that contains 50 nM neopterin, BH_2 and BH_4, should be freshly prepared each day. The solution is stable throughout the day if stored in the dark on ice.

Pterin "external quality control scheme (ERNDIM programme)": Add 1 mL of water to the lyophilized sample. Mix by vortexing. The sample is ready to analyse. This can be stored for up to one year at -70°C.For details regarding this external quality control scheme, visit (www.erndimqa.nl). It is blindly analysed eight times per year to validate the accuracy of the procedure. The results are available upon request.

Pterin "HPLC system wash solution": water and 50 % methanol/water solution (vol/vol). This can be stored for up to one month at room temperature.

Pterin "HPLC injector wash solution": water with 10 % methanol (vol/vol). This can be stored for up one month at room tempearture.

Pyridoxal phosphate "Mobile phase": 50 mM potassium dihydrogen phosphate (KH_2PO_4) with 75 mM semicarbazide. Weigh 6.80 ± 0.01 g of KH_2PO and 8.36 ± 0.01 g of semicarbazide into a 1-L flask, and add miliQ water up to 1 L (pH = 2.85). Filter the mobile phase through a solvent filtration unit with a 0.45-µm nylon membrane. ▲ **CRITICAL** Prepare as needed. The mobile phase acidic pH is required for chromatographic separation, but it may decrease the FL signal.

Pyridoxal phosphate "Trichloroacetic acid (10%)": Weigh 10 ± 0.01 g in a 100-mL flask, and add milliQ water. Store between 4-8ºC. The solution is stable for 6 months.

Pyridoxal phosphate "Dipotassium hydrogen phosphate (3.3 M)": Weigh 14.37 ± 0.04 mg of K_2HPO_4 in a 25-mL flask, and add milliQ water. Store between 4-8ºC. The solution is stable for 1 month.

Pyridoxal phosphate "Sodium cyanide (0.04 M)": Weigh 64.1 ± 0.04 mg of sodium cyanide in a 25-mL flask, and add milliQ water. Store between 4-8ºC. The solution is stable for 1 month. **! CAUTION** Sodium cyanide is extremely poisonous and should be handled in the fume hood with protective clothing and glasses.

Pyridoxal phosphate "Orthophosphoric acid (2.86 M)": Pipette 4.08 mL of 85% orthophosphoric acid (vol/vol) into a 25-mL flask, and add milliQ water. Store between 4 and 8ºC. The solution is stable for 6 months.

Pyridoxal phosphate "PLP Standard": Add 1 mL milliQ water to the lyophilized commercial vial. Mix gently and incubate for 10-15 min at room temperature. Swirl the vial to completely dissolve its contents. This can be stored for up to 3 months at -20ºC

Pyridoxal phosphate "PLP internal quality control": A lyophilized commercial PLP control is available. Add 2 mL of distilled water into the vial. Gently mix and incubate for 10-15 min at room temperature. Swirl the vial to completely dissolve its contents. This can be stored for up to 3months at -20°C.

Pyridoxal phosphate "HPLC system wash solution": milliQ water with 10% acetonitrile (vol/vol). This can be stored for up to one month at room temparature.

EQUIPMENT SETUP

HPLC setup and maintenance The main details of the equipment description and setup are summarized in **Table 2**. HPLC with the ED and FD equipment setup should be performed by the user according to the manufacturer's instructions.

Detailed initial setup and maintenance procedures are provided by the manufacturer for the HPLC and ED/FD systems. The most critical details, which involve maintenance

and troubleshooting of the ED and FD are detailed in **Table 4. Box 1** provides information for Electrochemical cell cleaning procedure.

▲**CRITICAL**. The samples and freshly prepared mobile phases must be filtered (i.e., free of particulate matter). Check that columns are stable with regards the mobile phase and the overall system is clean. The in-line graphite filters should be placed before the ED cell. Flush the cells with ultrapure water and methanol when the system is shut down. The tubing end must be capped to avoid drying the ED cell.

PROCEDURES

1. This protocol describes the procedures for characterization of monoamines (**Option A**), Pterins (**Option B**), and Pyridoxal phosphate (**Option C**) levels from human CSF. Each section contains instructions for the preparation of standards, controls and samples, together with specific instructions for HPLC-setup and data-acquisition for each compound.

A) Monoamine analysis TIMING 40 min for Standard, control and sample preparation, 3 hours for HPLC-ED setup and 35 min for data acquisition and analysis

i. ***Standard, control, and sample preparation (Steps i-vii):*** Take the monoamine calibration stock solution, the internal quality control and CSF samples (see Reagent Setup) from the -70 ºC storage, and thaw the samples at room temperature. ▲**CRITICAL STEP** The CSF samples, calibration solution and internal controls should be processed in the same way in all subsequent steps.

ii. Prepare the calibration working solution as described in the Reagent Setup.

iii. Thaw an internal quality control aliquote and dilute it 1:100 (vol/vol) in miliQ water: prepare 1:100 dilution by mixing 20 µL of internal quality control with 1980 µL of miliQ water Prepare the external quality control (see Reagent Setup) according to ERNIDM schedule, 8 times per year)

iv. Process the previously prepared CSF samples, calibration solution and internal and external controls by diluting the samples 1:2 (vol/vol) mixing 125 µL of sample with 125 µL of chromatographic mobile phase. ▲**CRITICAL STEP**: For the ED, dilution with the mobile phase is required to reduce the sample matrix effects.

v. Centrifuge the samples at 1500 x g for 10 min at 4ºC.

vi. Filter the samples through 0.22-µm nylon filters to remove major contaminants and impurities.

vii. Aliquot 200 µL of each sample into chromatographic labeled vials and put them in the rack.

viii. ***HPLC-ED setup (Steps viii-xviii):*** Before running the standard, controls and CSF samples, the user has to connect the HPLC circuit**.** First, connect the graphite in-line filter before the ED cell. (see problem and solution in Table 4, **TROUBLESHOOTING**).

▲**CRITICAL STEP** The acquisition software (Breeze) should be operated by the user according to the manufacturer's instructions (Waters).

▲**CRITICAL STEP** Ensure not to overtighten the graphite filter, since it can become scratched if overtightened, thereby damaging the ED cell (Table 4, **TROUBLESHOOTING**).

ix. Connect the mobile phase to the HPLC and purge all lines. ▲**CRITICAL STEP** Before connecting the column, confirm that all incompatible solvents from the previous sample runs have been fully displaced.

x. Place the column. Columns are used only for the indicated applications (Table 4, **TROUBLESHOOTING**).

xi. Check for fluid leaks before equilibrating the system (Table 4, **TROUBLESHOOTING**).

xii. Turn on the computer, and load the Breeze programme to capture and process the data from the ED.

xiii. Transfer the inlet line to the mobile phase.

xiv. Purge the pump with the mobile phase for 5 minutes using the Breeze programme (Table 4, **TROUBLESHOOTING**) .

xv. Purge the injector and the detector with the mobile phase for 10 minutes using the Breeze programme.

xvi. Equilibrate the system at 1.3 mL/min for at least 2 hours ▲**CRITICAL STEP** The flow rate can be reduced to 0.3 mL/min if the system is allowed to run overnight. However, it is usually necessary to stabilize the system for at least 1 hour after the flow rate is restored to 1.3 mL/min the following day.

xvii. During the equilibration stage, programme the electrode parameters (E1 = +50 mV; E2 = +450 mV, R2 = 200 nA) on the ED. Allow equilibration to proceed for at least 1 hour until the cell signal is stabilized (i.e., baseline signal is below 500 nA) (Table 4, **TROUBLESHOOTING**) .

xviii. Programme the autosampler in the Breeze programme to inject 30 μL of each sample from its position in the rack and set the time for 30 minutes. Upload the monoamine method stored in the Breeze program. ED autozero function is applied after each sample analysis.

xix. ***Data acquisition (Steps xix-xxi):*** To run the sample, click the "run sample" icon in the Breeze programme. The data capture will begin once an injection signal has been received from the autosampler.

xx. Check the standard and internal quality control. If they are well-separated and the values of calibration working solution (see Reagent Setup step, monoamine "calibration working solution") and internal quality control (according to SKML commercial sheet) are within the assigned limits (usually within two standard deviations of the assay mean), the CSF samples can be run. Review the chromatograms and calculate the concentrations using the Breeze program. To calculate the results, load the chromatogram file and integrate the peaks of interest. If no CSF samples have to be reinjected, the ED can be turned off.

xxi. Wash the system with 1 mL/min 7.5% (vol/vol) methanol (the same percentage as the mobile phase) for 2 hours.

B) Pterin ● TIMING 40 min for Standard, control and sample preparation, 3 hours for HPLC-ED-FD setup and 25 min for data acquisition and analysis.

i. ***Standard, control and sample preparation (Steps i-ii):*** Take the pterin calibration stock solution, the internal quality control, or CSF samples (see Reagent Setup) from the -70 ºC storage. Thaw at room temperature and thoroughly mix. Keep at 2 – 8°C in the dark until the analysis.

▲**CRITICAL STEP** The CSF samples, calibration solution and internal controls should be equally processed in all subsequent steps. It is not strictly necessary to filter samples for the pterins method. However, some samples do require filtering (high protein or coloured CSFs). Filter CSF samples through 10kDa centrifugal filters to remove major contaminants and impurities.

ii. Aliquot 200 µL into amber capped, labelled autosampler vials and position them in the rack

iii. ***HPLC-ED-FD setup (Steps iii-xii):*** Before running the standard, controls and CSF samples, the user has to connect the HPLC circuit**.** First, connect the graphite in-line filter before the ED cell Table 4, **TROUBLESHOOTING**).

▲**CRITICAL STEP** The acquisition software should be operated by the user according to the manufacturer's instructions. Refer to the equipment setup procedures.

▲**CRITICAL STEP** Ensure not to overtighten the graphite filter, since it can become scratched if overtightened, thereby damaging the ED cell. The guard cell is placed after the ED and before the fluorescence detector.

iv. Connect the mobile phase to the HPLC and purge all lines. ▲**CRITICAL STEP** Before connecting the column, confirm that all incompatible solvents from the previous sample runs have been fully displaced.

v. Place the column. Columns are used only for the indicated applications (Table 4, **TROUBLESHOOTING**) .

vi. Check for fluid leaks before equilibrating the system (Table 4, **TROUBLESHOOTING**).

vii. Purge the pump and ash the system with 50% methanol at a flow rate of 1 mL/min for 2-3 hours (Table 4, **TROUBLESHOOTING**). ▲**CRITICAL STEP** Because there is no methanol in the mobile phase, it is necessary to wash the system again with ultrapure water at 1 mL/min for 2-3 hours.

viii. Transfer the inlet line to the mobile phase and allow the system to equilibrate at 1.3 mL/min for at least 2 hours. ▲**CRITICAL STEP** The flow rate can be reduced to 0.3 mL/min if the system is allowed to run overnight. However, it is usually necessary to stabilize the system for at least 1 hour after the flow rate is restored to 1.3 mL/min the following day.

ix. After 30 min, turn on the ED and fluorescence detector. Programme the electrode parameters and wavelengths (Table 2).

x. Check the optimal potentials from the previous run, and check the voltammograms to ensure that the optimal potentials are used for the analysis.

E1 should be adjusted so that the current is +0.5uA and E2 should be adjusted so that the current is -0.05uA – these currents give the optimal peak area for tetrahydrobiopterin. The voltages applied may vary but usually are: E1=+200 mV; E2 = -500 mV; conditioning cell = +1100 mV (**Table 2**). Measure the area of the peak. Modify the potentials of electrodes E1 and E2 as appropriate and repeat until the necessary potential/current is established for the maximum peak area. The peak area for the tetrahydrobiopterin standard should be consistent between runs (Table 4, **TROUBLESHOOTING**) .

▲CRITICAL STEP Ensure that the green light is functioning for the cell's in/out and run/stop keys, and leave the ED to equilibrate for at least 1 hour.

xi. Turn on the computer and load Azur Data Capture to capture and process the data from the electrochemical and fluorescence detectors.

xii. Programme the autosampler to inject 50 µL of the sample, control or standard onto the HPLC and set the run time to 20 minutes. Upload the pterins method stored in Azur program. ED autozero function is applied after each sample analysis.

xiii. ***Data acquisition (Steps xiii-xv):*** To run the sample, click the "run sample" icon. The data capture will begin once an injection signal has been received from the autosampler.

xiv. Check the standard and internal quality control. If they are well-separated and the values of calibration working solution (see Reagent Setup step) and quality control are correct, run the CSF samples. Review the chromatograms and calculate the concentrations using the Azur program. To calculate the results, load the chromatogram file and integrate the peaks of interest. If any CSF sample has not to be reinjected, turn off the ED and fluorescence detector.

xv. Wash the system with ultrapure water at a flow rate of 1 mL/min for at least 1-2 hours. Wash with 50% methanol/water (vol/vol) at a flow rate of 1 mL/min for 2 hours or overnight.

C) Pyridoxal phosphate analysis ● TIMING 40 min for Standard, control and sample preparation, 2 hours for HPLC-FD setup and 30min for data acquisition and analysis

i. ***Standard, control and sample preparation (Steps i-vii):*** Take the PLP calibration stock solution, the internal quality control, and CSF samples (see Reagent Setup) from the -70 ºC storage, and thaw them at room temperature.
▲CRITICAL STEP The CSF samples, calibration solution and internal controls should be equally processed in all subsequent steps.

ii. Pipette 125 µL of each sample and 125 µL of 10% TCA into an Eppendorf vial, and mix by vortexing. Incubate at 50ºC in the dark for 5 min to completely release the protein-bound vitamin.

iii. Centrifuge at 12.000 x g (10 min, 4ºC).

iv. Pipette 200 µL of the resulting supernatant into an Eppendorf vial. Add 70 µL of 3.3 M K_2HPO_4 and 20 µL of 0.04 M sodium cyanide in the chromatographic

vial to the supernatant and mix by vortexing (pH=7.4). Incubate at 50ºC in the dark for 25 min. **▲CRITICAL STEP**. In alkaline conditions, sodium cyanide oxidizes PLP to pyridoxic-5-phosphate, which can be measured by fluorescence detection.

v. Add 25 µL of 2.86 M orthophosphoric acid to the samples to achieve an acidic medium (pH 2-4).

vi. Filter the samples through 0.22-µm nylon filters to remove major contaminants and impurities. **!CAUTION** Cyanide/orthophosphoric acid can create toxic hydrogen cyanide and samples must be prepared and capped in the fume hood.

vii. Transfer the filtered samples to chromatographic vials. The samples are now ready to inject.

viii. ***HPLC-FD setup (Steps viii-xvii):*** Before running the standard, controls and CSF samples, the user has to connect the HPLC circuit**.** First, connect the FD to the system.

▲CRITICAL STEP The acquisition software (Breeze) should be operated by the user according to the manufacturer's instructions (Waters).

ix. Connect the mobile phase to the HPLC and purge all lines. **▲CRITICAL STEP** Before connecting the column, confirm that all incompatible solvents from the previous sample runs have been fully displaced.

x. Place the column. Columns are used only for the indicated applications.

xi. Check for fluid leaks before equilibrating the system.

xii. Turn on the computer, and load the Breeze programme to capture and process the data from the fluorescence detector.

xiii. Purge the pump with the mobile phase for 5 minutes using Breeze programme.

xiv. Purge the injector and the detector with the mobile phase for 10 minutes using Breeze programme.

xv. Equilibrate the system at 1.5 mL/min for at least 30 min with the mobile phase.

xvi. During the equilibration turn on the fluorescence detector and check that the signal is stable. The typical signal value should be close to zero (-0.06 – 0.03 FD signal) (Table 4, **TROUBLESHOOTING**) .

xvii. Program the autosampler to inject 50 µL of each sample and set the time for 25 minutes. Upload the PLP method stored in the Breeze program. FD autozero function is applied after each sample analysis.

xviii. ***Data acquisition and analysis (Steps xviii-xx):*** To run sample, click the "run sample" icon. The data capture will begin once an injection signal has been received from the autosampler.

xix. Check the standard and internal quality control. If they are well-separated and the values of PLP calibration and internal quality control (according to Teknokroma commercial sheet) are within the assigned limits (usually within two standard deviations of the assay mean), the CSF samples can be run. Review the chromatograms and calculate the concentrations using the Breeze program. To calculate the results, load the chromatogram file and integrate the peaks of interest. If no CSF samples have to be reinjected, the FD can be turned off.

xx. Wash the system with ultrapure water/acetonitrile 90/10 (vol/vol), for 1 hour.

● TIMING

Option A: Monoamine analysis

Steps i-vii: Standard, control and sample preparation. 40 min.

Steps viii-xviii: HPLC-ED setup: 3 hours (1 hour to prepare the HPLC system and 2 hours to equilibrate the system). Overnight equilibration is advisable (flow rate 0.3 mL/min). The next day, restore the flow rate to 1.3 mL/min at least one hour to stabilize the HPLC system.

Steps xix-xxi: Data acquisition and analysis: 35 min per sample: 30 min for running the sample and 5 min for chromatographic data analysis.

Option B: Pterin analysis

Steps i-ii: Standard, control and sample preparation. 40 min.

Steps iii-xii: HPLC-ED-FD setup. 3 hours (1 hour to prepare the HPLC system and 2 hours to equilibrate the system). Overnight equilibration is advisable (flow rate 0.3 mL/min). The next day, restore the flow rate to 1.3 mL/min at least one hour to stabilize the HPLC system.

Steps xiii-xv: Data acquisition and analysis. 25 min per sample (20 min for running the sample and 5 min chromatographic data analysis

Option C: Pyridoxal phosphate analysis

Steps i-vii: Standard, control and sample preparation. 40 min.

Steps viii-xvii: HPLC-FD setup: 2 hours (30 minutes to prepare the HPLC system and 1.5 hours to equilibrate the system) .

Steps xviii-xx: Data acquisition and analysis: 30 min per sample: 25 min for running the sample and 5 min for chromatographic data analysis.

TROUBLESHOOTING

ED performance problems HPLC coulometric ED is a high sensitivity procedure since the analysed compounds are expected to be 100% oxidized. Analytical troubles may be remarkable if ED cell cleaning and maintenance protocols are not strictly followed-up. In the procedures described in this protocol, the most common performance problems

are usually caused by the ED and not by the HPLC separation procedures. These issues usually occur when the ion pair agent and the pH values are strictly controlled. Dissolved gases and electroactive compound adsorption to the porous graphite electrode, the presence of electroactive compounds in the mobile phase or sample, or poor electrode maintenance can lead to low electrochemical signals and unacceptably high background currents with a noisy baseline (as described in Coulochem II. Operating Manual ESA Inc (USA, 1997)). When the ED is flushed with water-methanol-water (a described in Box 1), the analytical performance remarkably improves. This cleaning procedure is easy to perform and consist on flush the cells with water during 30 minutes (4 mL/min), then 30 minutes with methanol (4 mL/min) and finally 30 minutes with water (4 mL/min). It is especially useful as a first procedure to be done when the ED analytical parameters are not good. If the ED cell does not work properly, refer to **Table 4** for complete troubleshooting information. Additional troubleshooting information for the rest of the procedures can also be found in **Table 4**.

ANTICIPATED RESULTS

The procedures described in this protocol will provide suitable methods to determine the concentration of monoamines and their cofactors (the pterins and PLP) in CSF samples.

Typical chromatograms of human CSF monoamines, are shown together with those for the calibration mixtures in **Figure 4**. A rapid identification of primary genetic or secondary conditions that lead to dopamine and serotonin disturbances is expected, as previously reported[4]. Four additional chromatograms depicting the neopterin, BH_4, and BH_2 (**Figure 5**) results in calibration mixtures and human CSF samples are shown. A chromatogram displaying the PLP calibration and a human CSF sample for the PLP analysis is also presented (**Figure 6**).

An experiment that required troubleshooting to improve their analytical qualities is presented in **Figure 7**.

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Author contributions: M.B., M.M-L., A.O., M.C., and C.S. and. performed the experiments and collected all technical data regarding the monoamine and PLP determinations. S.P. managed the technical aspects of the pterin analysis and prepared the quality control scheme. A.G-C. and M.K. directed the sample collection and the management and establishment of the pre-analytical protocols and clinical discussions. S.H. and R.A. designed the methods and planned the strategies for further methodological developments. M.B., M.M-L., A.O., and R.A. wrote the manuscript. All authors critically reviewed the content of the manuscript.

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BOX 1 Electrochemical cell cleaning procedure

Disconnect the cell from the control module and from the HPLC system.

Flush the cell using a flushing syringe with the solutions indicated below (5-10 mL of each).

1) For general cleaning, flush with deionized water/methanol/deionized water (up to pH > 5).
2) For lipophilic materials, flush with deionized water/methanol/acetonitrile/THF/acetonitrile/methanol/deionized water.
   !CAUTION: NaOH, organic solvents and nitric acid can cause personal injury. Use eye protection and protective clothing in a fume hood.
3) For organic materials, flush the system with deionized water/DMSO/deionized water.
4) Silica from the column is flushed with deionized water/2 M NaOH (leave in cell for 10-30 minutes)/deionized water (until pH < 7).
5) Oxidation/reduction cycle cleaning. Replace the mobile phase with fresh phosphate buffer 50 mM pH=3 flowing at 1 mL/minute. Apply a potential of +1000 mV to the electrode for 10 minutes with phosphate buffer flowing. Do not recycle the phosphate buffer during the treatment. After that apply a potential of -400 mV for 10 minutes and then apply again a potential of +1000 mV for 30 minutes. Reset the potentials to the assay's working potentials and test the response.
6) If these procedures do not work, clean the cell with nitric acid as a last resort. Deionized water/6 M nitric acid (leave in the cell for 15-30 minutes)/flush the cell with deionized water until pH > 5. After this procedure it will be necessary to determine the optimum oxidation potential of the compounds of interest by obtaining a new hydrodynamic voltammogram.

- End of BOX 1 -

TABLES

Table 1: Overview of the procedures described in this Protocol

	CSF marker	Approach	Option (steps)
Biogenic amines	3-OMD	HPLC-ED	A (steps i and ii)
	MHPG	HPLC-ED	A (steps i and ii)
	5-HTP	HPLC-ED	A (steps i and ii)
	5HIAA	HPLC-ED	A (steps i and ii)
	HVA	HPLC-ED	A (steps i and ii)
Pterins	NP	HPLC-ED-FD	B (steps i and ii)
	BH_2	HPLC-ED-FD	B (steps i and ii)
	BH_4	HPLC-ED-FD	B (steps i and ii)
Pyridoxal phosphate	PLP	HPLC-FD	C (steps i and ii)

Table 2. Summary of the equipment description and setup. HPLC systems are coupled to ED and FL detectors.

Parameters		**Monoamines Option A**	**Pterins, Option B**	**PLP, Option C**
Detector type		Electrochemical (ESA)	Electrochemical (ESA) Fluorescence (Jasco)	Fluorescence (Waters)
Electrochemical detector conditions	E1	+50 mV	+ 200 mV	
	E2	+450 mV	- 500 mV	
	Gain	200 nA	1 µA	
	Filter	10 sec	10 sec	
	Output	+1 V	-1 V	
	Offset	10%	0%	
Conditioning cell potential			+1100 mV	
Fluorescence detector conditions	Excitation		360 nm	325 nm
	Emission		440 nm	418 nm
	Gain		1	1

	EUFS		1000	1000
Column	Stationary phase	ODS (C-18)	ODS (C-18)	OS (C-8)
	Large and diameter	250 × 5 mm	250 x 4.6 mm	250 × 4.6 mm
	Particle size	5-µm	5-µm	5-µm
Flow-rate (mL/min)		1.3	1.3	1.5
Sample injection volume (µl)		30	50	50
Pump program		Isocratic	Isocratic	Isocratic
Running time (min)		30	20	25
Column wash 1	Solvent	Water/methanol 92.5/7.5 (vol/vol)	Water/methanol 50/50 (vol/vol),	Water/acetonitrile 90/10 (vol/vol),
	Flow-rate, time	1 mL/min, 2 h	1 mL/min, 2 h	1 mL/min 1 h
Column wash 2	Solvent		Water,	
	Flow-rate, time		1 mL/min, 2 h	
Column storage		Water/methanol 80/20 (vol/vol)	Water/methanol 50/50 (vol/vol)	Water/acetonitrile 80/20 (vol/vol)

Table 3. Pre-analytical protocol for CSF sample collection, management and storage.

Tube	volum	Biomarkers analysed
1st tube	5-10 drops (200-400 µL)	Glucose, proteins, cells and lactate
2nd tube	10 drops	Monoamines and PLP/other vitamins
3rd tube *	10 drops	Pterins
4th tube	10 drops	Amino acids

*The sample must be collected into a microcentrifuge tube with the following antioxidants: dithioerythritol (1 mg) and diethylenetriaminpentaacetic acid (1 mg).

Table 4. Troubleshooting, causes and solutions for a reliable ED and FL detection performance.

*Columns should stand under mobile phase pH values beween 2.5 – 7.5**.**

OPTION (STEP)	PROBLEM	POSSIBLE REASON	SOLUTION
Electrochemical detection			
A (step xiv) **B (step vii)**	The baseline is noisy	There is dissolved gas in the pump head	Degas the mobile phase (under helium) and re-prime the pump.
A (step xvii) **B (step x)**		There is dissolved gas in the detector cell	Remove the cell from the system, increase the flow rate for 30 minutes with water/degassed methanol/water (4 ml/min).
A (step xi) **B (step vi)**		The pump head or system leaks	Check the system for leakage.
A (step xiv) **B (step vii)**		The mobile phase is not properly mixed or degassed	Check the aspect and degas under helium current.
A (step x) **B (step v)**		Contaminants eluting from column	Remove the column from the system and check if the problem persists.
A and B		Electronic problems	Contact maintenance service
A (step xvii) **B (step x)**	High background currents	There are electroactive impurities in the mobile phase	Attach the 2 in-line electrodes to the same potential. If the current for E1 is higher than that for E2, mobile phase impurities are likely. In that case replace the mobile phase.
A (step x) **B (step v)**		Electroactive species eluted from the column	Replace the column. For new columns, allow equilibration with the mobile phase, removing the ED to avoid silica precipitation in the cell.
A (step xvii) **B (step x)**		Adsorption of impurities onto electrode that cause decreased current and increased noise	Reverse the potential or clean the cell (Box 1).
A (step xi)		Leaking cell	Check for leaks. Tighten fittings or

B (step vi)			replace cell.
A (step viii) **B (step iii)**	Increased back pressure	Accumulation of particles from the mobile phase or injected samples	Replace graphite filter. Use freshly prepared and filtered mobile phase and samples.
A (step viii and/or x) **B (step iii and/or v)**		Accumulation of particles from column	Replace graphite filter. Replace column. Be careful with silica columns with mobile phase pH values >7.5 or < 2.5.
A and B		Clogged cell	Clean the cell (see Box 1).
A (step xvii) **B (step x)**	Loss of ED cell response	Unstable compounds	Check stabilities of the analyzed biomarkers (especially 5HIAA and BH_4).
A (step xvii) **B (step (x)**		Decreased ED cell performance	Perform hydrodynamic voltammogram (sigmoidal curve). Small variations in current may lead to large variations in response. Usually voltammogram is performed when the procedure is standardized or when the ED is cleaned or changed or when a significant loss of response is observed.
A and B		Adsorption of impurities onto electrode	Clean the cell (see Box 1)
Fluorescence detection			
B (step x)	Noisy baseline, decreased sample energy levels or calibration failure	Dirty flow cell	Flush the flow cell with ultrapure water/6 N nitric acid/ultrapure water (until pH > 5). If this does not work you should replace the cell.
B (step x) **C (step xvi)**	Failure to ignite at startup, a decrease in sensitivity or noisy baseline	Damaged lamp	Replace the lamp.

FIGURE LEGENDS

Figure 1. Chromatograms with different monoamine related molecules (standadrd mixture).

A) Upper panel (condition 1) shows a chromatogram with seven dopamine and serotonin compounds (L-dopa, nor-epinephrine, epinephrine, DOPAC, dopamine, 3-metoxytyramine and serotonin). Down panel (condition 2) shows a chromatogram with six dopamine and serotonin compounds (DOPAC, dopamine, 5HIAA, HVA, 3-metoxytyramine and serotonin). Condition 1. The mobile phase is already explained in this protocol (Reagent Setup section). It consists in 75 mL methanol (7.5%), 13.61 sodium acetate trihidratate, 9.60 g citric acid monohidratate, 0.44 g EDTA sodium, 0.242 octanoilsulfonic acid and H_2O up to 1 litre. Final pH= 4.00. Injection volum= 30µL. Voltage: E1=50 mV and E2= 450 mV. Flow rate: 1.3mL per minute. The order of the calibrators is L-dopa, nor-epinephrine, epinephrine, DOPAC, dopamine, 3-metoxytyramine and serotonin. Condition 2. The mobile phase consists in 320 mL methanol (16%), 5.44 g sodium acetate trihidratate (20 mM), 5.25 g citric acid monohidratate (12.5 mM), 0.074 g EDTA sodium (0.1 mM), 0.95 g octanoilsulfonic acid (2.2 mM) and H_20 up to 2 litres. Final pH= 3.92. Injection volum= 50 µL. Voltage: E1=20 mV and E2=400 mV. Flow rate: 1.5mL per minute.

B) Chemical structures of the molecules in panel A.

Figure 2. Hydrodynamic voltammogram for 5HIAA and HVA in CSF. A current/voltage plot is generated after injecting a constant sample volume, which is measured after fixing different potentials. The lowest current that produces the highest analytical response to the electrode should be chosen.

Figure 3. A representation of a column, the in-line graphite filter, and two in-line electrochemical detector cells: A) the first one is the analytical cell, which contains electrodes E1 and E2 (the latter is the electrochemical detector). After the sample exits the electrochemical detector cell and is electrochemically detected, it can be oxidized at the conditioning cell for fluorescence detection of neopterin and BH_2.

Figure 4. Chromatograms of monoamines from standard mixture and CSF samples from pediatric patients

A) A calibration mixture. The elution order is 3-OMD, MHPG, 5-OHtrp, 5HIAA and HVA. **B**) CSF sample from 1-month-old patient with increased 3-OMD values. **C**) CSF sample from a 6-years-old patient. **B and C** show a high response in the first 2-4 minutes of the chromatogram due to the presence of interfering compounds in the CSF sample. **D**) Chemical structures of the molecules

Figure 5. Chromatograms of pterins from standard mixture and CSF samples from pediatric patients.

Representative chromatograms of BH_4, neopterin and BH_2 in the standard mixture and in a human CSF sample are presented. **A)** Fluorescence detection: Neopterin and BH_2 chromatograms from standard mixture (left panel) and from a human CSF sample (right panel). The elution order is neopterin and BH_2. **B)** Electrochemical detection: BH_4 from standard mixture (left panel) and from a human CSF sample (right panel).

Figure 6. Chromatograms of Pyridoxal phosphate from standard mixture and CSF samples from pediatric patients.

A) Representative chromatograms of pyridoxal phospate plasma-matrix standard provided by Teknokroma and **B**) Pyridoxal phospate in a human CSF sample. The chemical structure drawn is the derivatized compound.

Figure 7. Experiment that requires troubleshooting to improve their analytical quality. For HPLC-ED, the most common problems usually arise from electrochemical cell contamination or adsorption of electroactive impurities caused by dissolved gases in the system, pump problems, leaks, mobile phase not properly mixed or contaminants leaving from the column. Flushing the cells with water-methanol-water, as detailed in Box 1, is a useful practice when the baseline deviation is higher than 10%. A chromatogram depicting a bad performance is shown in panel **A**. The result after the ED cell cleaning procedure is shown in panel **B**.

Supplementary Figure 1: Metabolic pathways for the synthesis and catabolism of monoamines, pterins and pyridoxal phosphate. The key metabolites for neurotransmitters and pterins are marked in bold and capital letters. Enzymes appear in italics. Tyrosine and tryptophan are the amino acid precursors of dopamine and serotonin, respectively. After a common rate-limiting enzymatic step, catalysed by two tetrahydrobiopterin (BH_4)-dependent hydroxylases, L-dopa and 5-hydroxytryptophan are synthesized. After a common decarboxylation step, catalysed by L-aromatic amino acid decarboxylase (whose cofactor is pyridoxal-phosphate), the active neurotransmitters—dopamine and serotonin—are formed. Finally, several catabolic steps lead to the generation of the main end-stable metabolites, homovanillic acid (HVA) and 5-hydroxyindoleacetic (5HIAA) acid, which are the most useful diagnostic biomarkers. *Modified from Batllori et al 2016[29].

Abbreviations

AADC: aromatic L-amino acid decarboxylase; 7,8-BH2: 7,8-dihydrobiopterin; BH_4: tetrahydrobiopterin; COMT: catechol O-methyltransferase; CR: carbonyl reductase; DHFR: dihydrofolate reductase; DHPR: dihydropteridine reductase; DβH: dopamine betahydroxylase; GABA: Gamma-aminobutyric acid; GABA-T: Gamma-aminobutyric acid-transaminase; GAD: glutamate decarboxylase; GHB: 4-hydroxybutyric; GTP: guanosine triphosphate; Gln: glutamine; Glu: glutamate; GTPCH: GTP cyclohydrolase I; 5HIAA: 5-hydroxyindoleacetic acid; 5HTP: 5-hydroxytryptophan; 5-MTHF: 5-methyltetrahydrofolate; HCys: homocysteine; HVA: homovanillic acid; L-dopa: 3,4-dihydroxyphenylalanine; MAO: monoamine oxidase;

Met: metionine; MHPG: 3-methoxy-4-hydroxyphenylglycol; n.e.: non-enzymatic; 3OMD: 3-O-methyldopa; OH-BH_4: hydroxy-tetrahydrobiopterin; PCD: pterin-4a-carbinolamine dehydratase; PLK: pyridoxl kinase; PLP: pyridoxal phosphate; PNMT: phenylethanolamine N-methyltransferase; PNPO: pyridoxine-5'-phosphate oxidase; PTPS: 6-pyruvoyl-tetrahydropterin synthase; q-BH2: quinoide-dihydrobiopterin; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SR: seapiapterin reductase; SSADH: succinic semialdehyde dehydrogenase; TCA: tricarboxylic acid cicle; TPH: tryprophan-5-hydroxylase; TH: tyrosine 3-hydroxylase; VLA: vanillactic acid; VMA: vanillmandelic acid; vB_6: vitamin B_6.

Supplementary Table 1: Cerebrospinal fluid biomarkers, associated diseases and their expected biochemical profile in the different biological fluids. Thirteen genetic disorders affecting monoamine, pterin and pyridoxal-phosphate metabolism have been recognized. A great variety of other neurological and environmental conditions may affect the status of these biomarkers.

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