UNIVERSITAT DE BARCELONA

Investigació en biomarcadors de l'estat de la dopamina i la serotonina en pacients neuropediàtrics

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Memòria presentada per
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Doctora
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A la mare
Al pare
Al Joan
A la iaia

Agraïments

Als pacients i a les famílies.

A les persones del laboratori de Sant Joan de Déu, especialment als metabòlics: Aroa, Cristina, Juanito, Judit, Mercedes, Montserrat, Raquel, Rosa, Tania i Yahel.

Al Rafa, el director de tesi encobert. Per la seva paciència, per la seva predisposició i per guiar-me i acompanyar-me a trobar el meu camí.

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A totes i a tots. Moltes gràcies!

The force will be with you... always

(Obi-Wan Kenobi to Luke Skywalker. STAR WARS, episode IV, A New Hope)

Resum

Els errors congènits del metabolisme són malalties d'origen genètic amb una baixa prevalença dins de la població general. Aquestes malalties solen estar causades per mutacions en gens que provoquen un mal funcionament de diversos enzims o cofactors que controlen alguna ruta metabòlica de l'organisme. Moltes d'aquestes malalties poden presentar símptomes neurològics que poden comprometre el correcte desenvolupament del sistema nerviós central (SNC), particularment durant el període neonatal i la infància. Tot i la seva baixa prevalença, cada any es descriuen nous desordres i fenotips, motiu pel qual cal seguir investigant en nous biomarcadors.

Les monoamines, com la dopamina i la serotonina, són neurotransmissors que intervenen en el correcte desenvolupament de les funcions motores, perceptives, cognitives i emocionals del SNC. Diverses condicions genètiques i ambientals han demostrat que afecten de manera primària el metabolisme d'ambdós neurotransmissors o bé d'aquells cofactors essencials per la correcta biosíntesis de la dopamina i serotonina. La detecció quantitativa de metabòlits de la dopamina i serotonina (àcid homovaníl·lic (HVA), i àcid 5-hidroxiindolacètic (5-HIAA), respectivament), així com dels seus cofactors (vitamina B6 i tetrahidrobiopterina), en líquid cefaloraquidi (LCR) es considera una important prova bioquímica pel diagnòstic i seguiment de les malalties mencionades anteriorment.

El LCR és un fluid biològic que s'obté mitjançant una punció lumbar, intervenció invasiva que ha de realitzar personal especialitzat i seguint un estricte protocol estandarditzat. Els pacients amb dèficit de dopamina i serotonina d'origen genètic reben tractament amb precursors d'aquests neurotransmissors. El correcte seguiment del tractament es fa per puncions lumbars successives fent que els pacients s'hagin de sotmetre varies vegades a una punció lumbar. Seria de gran utilitat poder trobar un biomarcador relacionat amb la dopamina i/o serotonina, que no estigués influenciat per factors externs, que fos un reflex del que succeeix al SNC i que pogués determinar-se en d'altres fluids biològics menys invasius, com l'orina. Per això vam avaluar la utilitat de la melatonina en orina, una hormona sintetitzada per la glàndula pineal (localitzada al cervell) a partir de la serotonina cerebral. Una vegada és sintetitzada, és

alliberada a la sang perquè pugui ser metabolitzada i posteriorment eliminada per orina com a 6-sulfatoximelatonina (aMT6s).

D'altra banda, tot i que l'obtenció del LCR es realitza mitjançant un procediment protocol·litzat, pot està subjecte a alteracions preanalítiques, com ara la contaminació hemàtica per punció traumàtica. Està descrit que aquesta contaminació hemàtica té efectes sobre la concentració de glucosa i de proteïnes en el LCR, però no hi ha estudis on es descrigui l'efecte en d'altres biomarcadors que s'analitzen en LCR per l'estudi de patologies neurometabòliques.

Tenint en compte aquests antecedents en aquesta tesi hem desenvolupat 3 objectius:

1. Avaluar l'efecte de la contaminació hemàtica en mostres de LCR a l'hora de realitzar i interpretar les anàlisis que realitzem al laboratori: aminoàcids, metabòlits dels neurotransmissors, 5-metiltetrahidrofolat, vitamina B_6, pterines (neopterina i biopterina) i tiamina. Els resultats es van publicar a la revista "Fluids and Barriers of CNS" sota el títol *Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins (2019).*
2. Establir valors de referència de l'aMT6s (metabòlit de la melatonina) en orina en població sana, per tal d'avaluar si pot ser un bon biomarcador perifèric de l'estat de la serotonina cerebral en pacients amb mutacions patològiques de gens relacionats en la biosíntesis de la serotonina. Els resultats d'aquest estudi es van publicar a la revista "Scientific Reports" sota el títol *Urinary sulphatoxymelatonin as a biomarker of serotonin status in biogenic amine-deficient patients (2017).*
3. Avaluar retrospectivament el fenotip clínic i els perfils bioquímics d'amines biògenes en LCR de pacients diagnosticats genèticament d'una malaltia mitocondrial. Els resultats d'aquest estudi es van publicar a la revista "Journal of Inherited Metabolism Diseases" sota el títol C*erebrospinal fluid monoamines, pterins, and folate in patients with mitochondrial diseases: systematic review and hospital experience (2018).*

Paral·lelament a això vam poder publicar un protocol estandarditzat de l'anàlisi de les monoamines i els seus cofactors en LCR per HPLC a la revista "Nature Protocols" (*Analysis of human cerebrospinal fluid monoamines and related cofactors by HPLC.* 2017).

ABREVIATURES

3OMD	3-ortometildopa
5HIAA	Àcid 5-hidroxiindolacètic
5HTP	5-hidroxitriptòfan
5MTHF	5-metiltetrahidrofolat
AADC	l-aminoàcid aromàtic descarboxilasa
adGTPCH	GTPCH autosòmica dominant
ADN	Àcid desoxiribonucleic
ALDH7A1	α-aminoadípic-semialdehid deshidrogenasa
aMT6	Sulfatoximelatonina
arGTPCH	GTPCH autosòmica recessiva
ATP	Adenosina trifosfat
BH2	Dihidrobiopterina
BH4	Tetrahidrobiopterina
BHE	Barrera hematoencefàlica
COMT	Catecol-O-metiltransferasa
DAT1	Transportador de dopamina tipus 1
DBH	Dopamina-β-hidroxilasa
DFC	Dèficiència de folat cerebral
DHFR	Dihidrofolat reductasa
DHPR	Dihidropteridina reductasa
ECM	Errors congènits del metabolisme
ED	Detecció electroquímica
FD	Detecció de fluorescència
FOLR1	Receptor 1 del folat
FRα	Receptor alfa del folat
GABA	Àcid gamma-aminobutíric
GDH	Complexe de la glicina descarboxilasa
GTP	Guanosina trifosfat
GTPCH-I	GTPciclohidrolasa-I
HIOMT	5-hidroxiindol-O-metil transferasa
HPLC	Cromatografia líquida d'alta resolució

HVA	Àcid homovanílic
IMAO	Inhibidors de la MAO
ISRS	Inhibidors de la recaptació de serotonina
KSS	Síndrome de Kearns Sayre
LCR	Líquid cefalorraquidi
L-DOPA	Dihidroxifenilalanina
MAO-A	Monoaminooxidasa A
MHPG	Metoxihidroxifenilglicol
mtDNA	ADN mitocondrial
MTHFR	Metilè-tetrahidrofolat reductasa
NGS	Seqüenciació massiva de nova generació
OAT3	Transportador orgànic aniònic tipus 3
P6C	Piperideïna-6-carboxilat
PCD	Pterina 4-alfa carbinolamina deshidratasa
PCFT	Transportador del folat acoblat a protons
PKU	Fenilcetonúria
PLK	Piridoxal quinasa
PLP	Piridoxal fosfat
PNPO	Piridox(am)ine 5'-fosfat oxidasa
PTPS	6-piruvoyltetrahidropterina sintasa
SAM	S-adenosilmetionina
SNAT	Serotonina-N-acetil transferasa
SNC	Sistema nerviós central
SR	Sepiapterina reductasa
TDP	Tiamina difosfat
TH	Tirosina hidroxilasa
TMP	Tiamina monofosfat
TPH	Triptofan hidroxilasa
UHPLC-MS/MS	Ultra-HPLC acoblada a espectrometria de masses en tàndem
VMAT2	Transportador de monoamina vesicular tipus 2
VR	Valors de referència

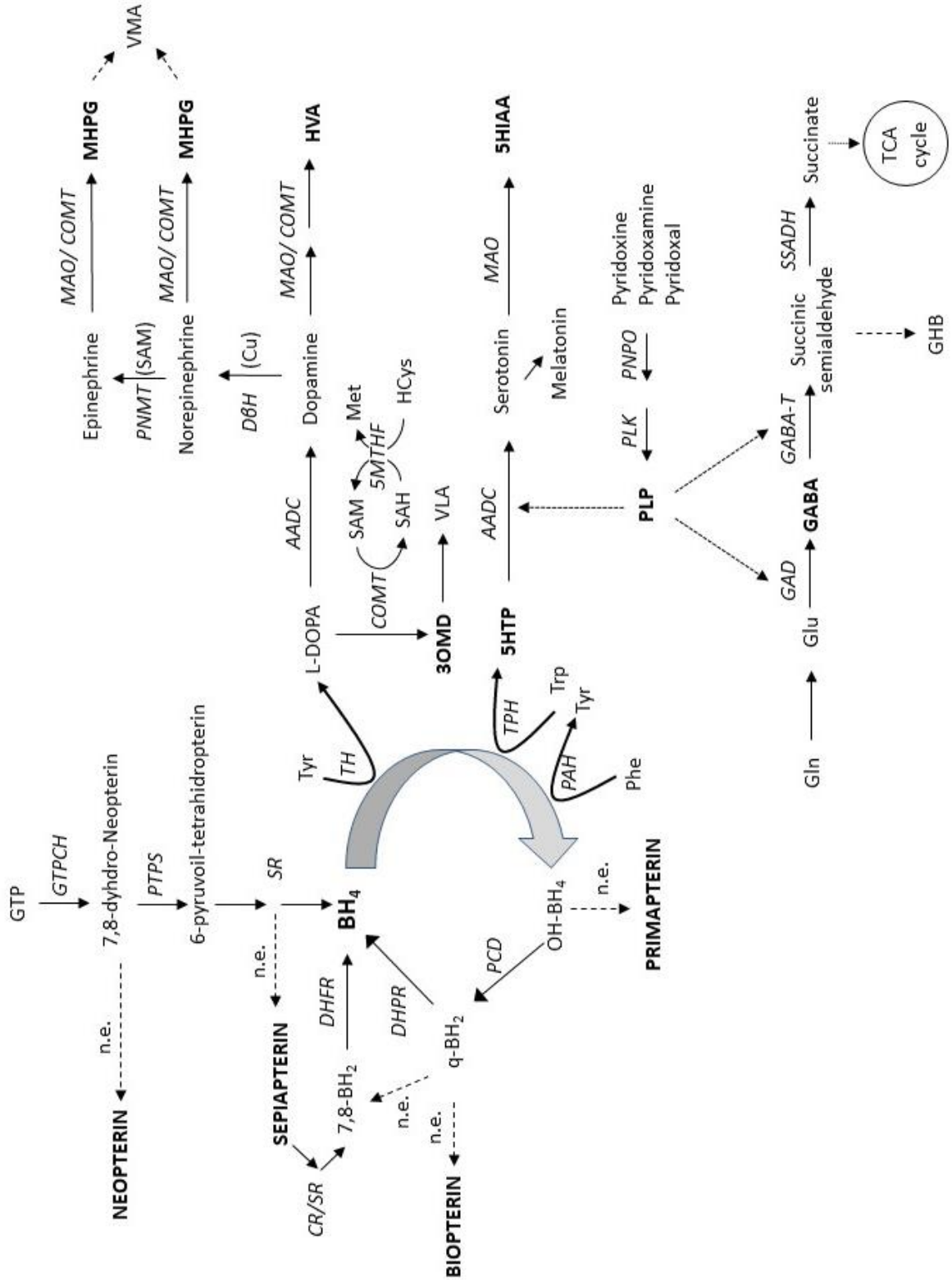
INTRODUCCIÓ

Les monoamines, dopamina i serotonina, són neurotransmissors amb un paper fonamental en les funcions cerebrals motores, perceptives, cognitives i emocionals, i en altres com ara la regulació del to vascular, la temperatura, part de la funció endocrina i la deglució (Ng J et al., 2015). Les vies metabòliques per a la seva síntesi i les malalties associades a defectes genètics es mostren a la figura 1 i a la taula 1.

S'ha demostrat que diverses condicions genètiques i ambientals afecten el metabolisme de la dopamina i la serotonina o als seus cofactors (pterines i piridoxal fosfat (PLP)) que són essencials per a la biosíntesi adequada d'aquests neurotransmissors (Ng J et al., 2015). Les deficiències dels enzims l-aminoàcid aromàtic descarboxilasa (AADC) i de tirosina hidroxilasa (TH) són dues malalties genètiques ben conegudes causants de deficiències de dopamina i serotonina (Figura 1 i Taula 1). La detecció quantitativa de monoamines i els seus cofactors en líquid cefalorraquidi (LCR) es pot utilitzar com a indicador de la biosíntesi de dopamina i serotonina i del seu metabolisme cerebral, i es considera un marcador diagnòstic important per a algunes de les afeccions que descriurem a continuació.

L'estat dels sistemes de dopamina i serotonina i, el seu metabolisme al sistema nerviós central (SNC), es pot estudiar mesurant els nivells d'àcid homovanílic (HVA) i àcid 5-hidroxiindoleacètic (5-HIAA), respectivament (Heales SJ et al., 2008; Blau N & Beat T, 2008). La detecció d'altres metabòlits de dopamina i serotonina es pot fer en el mateix cromatograma, cosa que permet una investigació més completa de l'estat de les monoamines en una sola anàlisi (Heales SJ et al 2008; Blau N & Beat T, 2008). Aquests metabòlits són la 3-ortometildopa (3-OMD) i el metoxihidroxifenilglicol (MHPG) per a la via de la dopamina, i el 5-hidroxitriptòfan (5HTP) per a la via de la serotonina (Figura 1).

Figura 1. Vies metabòliques dels neurotransmissors, pterines, GABA i vitamina B_6 (Figura extreta de Batllori M et al., 2016. Veure Annex 1).



A més, l'avaluació quantitativa de les concentracions en LCR de cofactors que són essencials per a la síntesi de monoamines pot proporcionar informació diagnòstica important. Les pterines (neopterina, biopterina, dihidrobiopterina (BH_2) i tetrahidrobiopterina (BH_4)) són, per exemple, biomarcadors útils per a la investigació de trastorns genètics associats al metabolisme de la BH_4. L'anàlisi de la neopterina és, a més a més, un molt bon biomarcador per identificar les afeccions inflamatòries i immunes del SNC (Molero-Luis M et al., 2013). La determinació de la concentració de PLP en LCR proporciona informació sobre les diverses condicions genètiques i ambientals que condueixen a una deficiència de PLP cerebral (Footitt EJ et al., 2011) que, en conseqüència, afecta la biosíntesi de dopamina i serotonina, i d'altres neurotransmissors com el GABA.

Després d'una avaluació clínica i l'establiment d'hipòtesis diagnòstiques inicials, la investigació de la majoria de les afeccions neurometabòliques que es descriuen en aquesta tesi depenen de l'anàlisi de biomarcadors específics en LCR. En general, els biomarcadors dels fluids biològics perifèrics (sang i orina) no són adequats per al diagnòstic de moltes d'aquestes afeccions. La Taula 1 presenta els biomarcadors en LCR més rellevants i les seves concentracions esperades en fluids biològics segons les diferents malalties revisades.

Taula 1. Biomarcadors en LCR, malalties associades i els seus perfils bioquímics esperats en diferents fluids biològics. (Adaptada de Batllori M et al., 2016. Veure Annex 1).

CSF marker	Associated disease *OMIM*/gene*	Biochemical profile
Glucose	GLUT1 deficiency 138140/*SLC2A1*	CSF: ↓ glucose, ↓ or normal lactate ↓ CSF/plasma glucose ratio
Serine	Serine deficiency 606879/*PHGDH* 610936/*PSAT1* 172480/*PSPH*	CSF: ↓ Ser and Gly Plasma: ↓ Ser (with or without ↓ Gly)
Glycine	Classic NKH 238300/ *GLDC* 238310/AMT 238330/ GCSH	CSF: ↑↑ Gly Plasma: ↑↑ Gly ↑ CSF/plasma Gly ratio Urine: ↑↑ Gly
	Variant NKH 607031/*LIAS* 609588/*GLRX5* 613183/*BOLA3* 608100/*NFU1*	CSF: ↑ Gly, lactate and Ala Plasma: ↑ Gly Urine: ↑ Gly

	615316/*IBA57*	
Biogenic amines (HVA and 5HIAA)	TH deficiency 191290/*TH*	CSF: ↓ HVA and HVA/5HIAA ratio
	AADC deficiency 107930/*DDC*	CSF: ↓↓ HVA and 5HIAA. ↑↑ 3OMD and 5HTP Urine: ↑ vanillactate
	MAO A deficiency 309850/*MAO-A*	CSF: ↓↓ 5HIAA and HVA
	DBH deficiency 609312/*DBH*	CSF: ↑ HVA, HVA/5HIAA ratio, ↓ MHPG* Urine: ↓↓ norepinephrine and epinephrine. ↑ Dopamine
	DAT1 deficiency 126455/*SLC6A3*	CSF: ↑ HVA and HVA/5HIAA ratio
Pterins (NP, BP and BH_4)	Dominant GTPCH deficiency 600225/*GCH*	CSF: ↓ NP, BP, BH_4, HVA and 5HIAA
	Recessive GTPCH deficiency 600225/*GCH*	CSF: ↓↓ NP, BP, BH_4, HVA and 5HIAA Plasma: ↑ Phe Urine: ↓ BP and NP
	PTPS deficiency 612719/*PTS*	CSF: ↑ NP. ↓↓ BP, BH_4, HVA and 5HIAA Plasma: ↑ Phe Urine: ↓ BP. ↑ NP
	SR deficiency 182125/*SRD*	CSF: ↑ BP and SP. Normal NP. ↓ BH_4, HVA and 5HIAA Urine: ↑ SP
	PCD deficiency 126090/*PCBD1*	CSF: ↑ Primapterin. ↓ BH_4, HVA and 5HIAA Plasma: ↑ Phe Urine: ↑ Primapterin
	DHPR deficiency 612676/*QDPR*	CSF: ↑ BP. Normal NP. ↓ BH_4, HVA and 5HIAA Plasma: ↑ Phe Dried blood spot: ↓ DHPR activity
Free-GABA	GABA-T deficiency 137150/*ABAT*	CSF: ↑↑ Free-GABA, homocarnosine and beta-alanine
	SSADH deficiency 610045/*ALDH5A1*	CSF: ↑ Free-GABA Plasma and urine: ↑↑ GHB
5-MTHF	Folate receptor-alpha deficiency 136430/*FOLR1*	CSF: ↓↓ 5MTHF and total folate ↓↓ CSF 5MTHF/Plasma folate ratio Plasma: Normal total folate
	PCFT deficiency 611672/*SLC46A1*	CSF: ↓↓ 5MTHF and total folate ↓ CSF 5MTHF/plasma folate ratio Plasma: ↓↓ total folate.
	MTHFR deficiency 607093/*MTHFR*	CSF: ↓↓ 5MTHF. Normal or ↑ total folate Normal CSF 5MTHF/Plasma folate ratio Plasma: Normal total folate. Severe hyperhomocysteinemia
	DHFR deficiency 126060/*DHFR*	CSF: ↓↓ 5MTHF, Normal CSF 5MTHF/plasma folate ratio Plasma: Normal total folate and Hcys
PLP	PNPO deficiency 603287/*PNPO*	CSF: ↓ PLP, 5HIAA and HVA. ↑ 3OMD and 5HTP. ↑ Gly, Ser and Thr Normal CSF/plasma PLP ratio

		Plasma: ↑ Gly, Ser and Thr Urine: ↑ vanillactate
	Antiquitin deficiency 107323/*ALDH7A1*	CSF: ↓ PLP, 5HIAA and HVA. ↑ 3OMD, 5HTP and X Compound. ↓ GABA. ↑ Gly, Ser and Thr. ↑ AASA and pipecolic acid ↓ CSF/ plasma PLP ratio Plasma: ↑ AASA and pipecolic acid Urine: ↑ AASA and pipecolic acid
Thiamine isoforms	hTHTR2 deficiency 606152/*SLC19A3*	CSF: ↓↓ free-thiamine. ↑ lactic acid Plasma: ↑ branched amino acids Urine: ↑ 2–oxoglutarate
	TPK1 deficiency 606370/*TPK1*	CSF*: ↑ free-thiamine and ↓ TDP Blood, muscle and fibroblasts: ↑ free-thiamine and ↓ TDP

*Resultats esperats. ↑: concentracions augmentades respecte els valors de referència estratificats per edat; ↓: concentració disminuïda respecte els valors de referència estratificats per edat.

Actualment hi ha molts biomarcadors que es poden analitzar en mostres de LCR, però en comparació amb altres matrius, com la sang i l'orina, hi ha poques indicacions per realitzar la recollida i anàlisi de mostres de LCR. Per tant, no sempre és necessari analitzar de forma rutinària el LCR perquè diverses afeccions neurometabòliques es poden diagnosticar en fluids perifèrics. En aquesta tesi, revisarem diferents biomarcadors (metabòlits, vitamines i cofactors) en LCR i destacarem punts crítics en referència a l'anàlisi de laboratori i la interpretació de dades.

En aquests tipus d'estudis, els aspectes biomèdics, preanalítics i analítics poden ser crucials per poder realitzar una correcta interpretació dels perfils. A continuació revisarem l'estat actual d'aquests factors:

1. Aspectes biomèdics

S'ha demostrat que les investigacions metabolòmiques del LCR són una eina important per estudiar vies neurometabòliques específiques, les malalties relacionades i per explorar el transport metabòlic de la sang al SNC (Batllori M et al., 2016). Les deficiències dels enzims relacionats amb aquestes vies causen diferents malalties neurogenètiques. La llista d'aquestes malalties i dels metabòlits que es poden analitzar pel seu diagnòstic bioquímic l'hem detallada anteriorment a la taula 1. A continuació ens centrarem en descriure aquells defectes relacionats amb el metabolisme de les amines biògenes i pterines, el folat cerebral i la vitamina B_6.

1.1. Amines biògenes i pterines

Les amines biògenes comprenen un grup de compostos químicament relacionats que estan estretament relacionats amb els neurotransmissors dopamina i serotonina. La Figura 1 mostra les vies metabòliques de la biosíntesi de dopamina i serotonina i el seu cofactor BH_4. Els aminoàcids tirosina i triptòfan són els precursors de la dopamina i la serotonina, respectivament. Després d'un pas enzimàtic limitant, que és catalitzat per dues hidroxilases dependents de BH_4, se sintetitzen la dihidroxifenilalanina (L-DOPA) i 5HTP. A continuació i, després d'un pas comú de descarboxilació, catalitzat per la AADC (el cofactor del qual és la forma activa de la vitamina B_6), es formen els neurotransmissors dopamina i serotonina. Finalment, diversos passos catabòlics condueixen a la generació dels metabòlits estables finals, l'HVA i l'5HIAA, que constitueixen els biomarcadors més útils pel diagnòstic de trastorns relacionats amb la dopamina i la serotonina.

La BH_4 se sintetitza a partir de guanosina trifosfat (GTP) en una via metabòlica que implica els enzims GTPciclohidrolasa-I (GTPCH-I), 6-piruvoyltetrahidropterina sintasa (PTPS) i sepiapterin reductasa (SR). La via de recuperació de la BH_4 també és important i inclou els enzims pterina 4-alfa carbinolamina deshidratasa (PCD) i dihidropteridina reductasa (DHPR) (Figura 1). L'anàlisi de pterines en LCR es pot utilitzar per identificar les diferents condicions genètiques que afecten tant la síntesi com el reciclatge del cofactor actiu BH_4.

Malalties associades

Es descriuran deu trastorns del metabolisme de les amines biògenes i pterines més dos trastorns relacionats amb el transport (Ng J et al., 2015) de la dopamina i serotonina.

Respecte a les malalties genètiques relacionades amb el metabolisme de les amines biògenes, dos trastorns afecten específicament la biosíntesi de dopamina i altres catecolamines:

- la deficiència de tirosina hidroxilasa (TH; OMIM * 191290) afecta la síntesi de L-DOPA, provocant un dèficit de dopamina i una malaltia neurològica amb signes extrapiramidals (Lüdecke B et al., 1996);
- la deficiència de dopamina-β-hidroxilasa (DBH; OMIM * 609312) afecta la biosíntesi de catecolamines (adrenalina i noradrenalina), i es manifesta amb

hipotensió ortostàtica severa i una insuficiència del sistema nerviós simpàtic (Mann in't Veld AJ et al., 1987).

Uns altres dos trastorns del metabolisme de les amines biògenes impliquen tant el metabolisme de la dopamina com el de la serotonina:

- la deficiència de l-aminoàcid aromàtic descarboxilasa (AADC; OMIM * 107930) provoca una deficiència combinada de dopamina i serotonina, que condueix a un quadre clínic complex que inclou hipotonia muscular, crisis oculogíries, trastorns del moviment i trets autònoms (sudoració excessiva i inestabilitat de la temperatura) (Hyland K et al; 1992);
- la deficiència de monoaminooxidasa A lligada al cromosoma X (MAO-A; OMIM * 309850), és l'únic defecte del catabolisme de les monoamines, que provoca trastorns greus i aïllats del comportament (Brunner HG et al., 1993).

Abans de mencionar les malalties associades a la BH_4, cal remarcar que aquest cofactor és necessari al fetge per a la conversió de fenilalanina en tirosina en una reacció catalitzada per la fenilalanina hidroxilasa. Dels sis defectes de la biosíntesi de la BH_4, quatre cursen amb valors elevats de fenilalanina en sang i, per tant, són malalties cribrades en el programa de detecció neonatal en mostra de sang seca del taló del nounat. Aquestes quatre entitats són les deficiències dels enzims trifosfat de guanosina ciclohidrolasa 1 (GTPCH-I; OMIM * 600225), 6-piruvoyltetrahidropterina sintasa (PTPS; OMIM * 612719), pterina 4-alfa carbinolamina deshidratasa (PCD; OMIM * 126090) i dihidropteridina reductasa (DHPR; OMIM * 612676), heretades recessivament (Ng J et al., 2015; Korman SH et al., 2002). Aquests defectes també presenten alteracions en l'estat de les amines biògenes al SNC, ja que, la TH i la TPH depenen de la BH_4. Aquestes malalties no es tractaran en aquesta tesi. D'altra banda, les dues entitats que cursen amb valors normals de fenilalanina i que, per tant, no són detectables en els programes de cribratge neonatal, són la forma dominant del dèficit de l'enzim trifosfat de guanosina ciclohidrolasa 1 (GTPCH-I; OMIM * 600225) (Ichinose H et al; 1994) i la forma recessiva de la deficiència de sepiapterina reductasa (SR; OMIM * 182125) (Friedman J et al., 2012). Ambdues malalties causen principalment trastorns del moviment a causa de la deficiència de dopamina, amb diferents graus d'alteracions clíniques relacionades amb la serotonina.

Recentment s'han descrit dues afeccions genètiques que afecten el funcionament del transport sinàptic: la deficiència del transportador de dopamina 1 (DAT1; OMIM * 126455) (Kurian MA et al., 2009) i el defecte del transportador de monoamina vesicular tipus 2 (VMAT2; OMIM * 193001) (Rilstonne JJ et al., 2013), que causen una distonia Parkinsoniana precoç. El DAT1 elimina la dopamina de l'espai sinàptic, la qual cosa finalitza la seva acció sobre els receptors postsinàptics i presinàptics. VMAT2 trasllada la dopamina i la serotonina a vesícules sinàptiques i és essencial per al seu alliberament a la fissura sinàptica.

La identificació precoç d'aquestes malalties pot ser fonamental perquè algunes presenten bons resultats clínics després del tractament amb precursors de neurotransmissors (L-DOPA i 5HTP) o agonistes de neurotransmissors.

Interpretació de resultats

En general, l'anàlisi d'amines biògenes en fluids perifèrics com l'orina no és adequat pel diagnòstic d'aquests trastorns genètics i només es pot detectar un perfil anormal d'àcids orgànics (augment de l'àcid vanil·làctic) en alguns pacients amb deficiències d'AADC i vitamina B_6. El LCR ha estat identificat com la mostra biològica més adequada per al diagnòstic de la majoria dels trastorns d'amines biògenes i dels dos defectes de pterines que no presenten fenilcetonúria (PKU).

La Taula 1 indica el valor diagnòstic de les amines biògenes i pterines en LCR per a les diferents condicions genètiques comentades anteriorment.

Cal una recollida protocol·litzada del LCR quan es duu a terme una punció lumbar per a poder realitzar una anàlisi fiable de les amines biògenes. Això és degut al fet que la concentració d'alguns metabòlits és més alta en les darreres fraccions del LCR en comparació amb les mostres inicialment recollides (gradient rostrocaudal) i és important comparar els valors del pacient amb els valors de referència que li corresponguin per edat i que han estat establerts amb la mateixa fracció de LCR. A més, la lisi d'eritròcits provoca l'oxidació de metabòlits; per tant, les mostres contaminades amb sang han de ser centrifugades immediatament i el sobrenedant transparent de LCR s'ha de transferir a un nou tub. L'emmagatzematge de mostres ha de ser a –80 °C fins a la realització de la tècnica analítica.

Les condicions preanalítiques per a l'anàlisi de pterines també s'han de tenir molt presents. Per a la mesura de neopterina, biopterina i sepiapterina, la pràctica més

habitual inclou l'estabilització de mostres de LCR amb diòxid de manganès o iode i protegir la mostra de la llum (Burlina A et al., 2014). S'han descrit diversos protocols per a la investigació d'aquests factors preanalítics (Ormazabal A et al., 2005 i Hyland K et al., 1993).

Les concentracions en LCR d'HVA i 5HIAA es poden utilitzar com a marcadors indirectes del funcionament de les vies metabòliques de la dopamina i la serotonina al SNC, respectivament. L'anàlisi de 3OMD i MHPG com a metabòlits de dopamina i noradrenalina i el 5HTP com a metabòlit precursor de la serotonina (Figura 1), permeten el diagnòstic diferencial de trastorns de les amines biògenes (Taula 1). Els defectes en la biosíntesi d'amines biògenes indiquen una disminució d'HVA en LCR i/o de 5HIAA (deficiència de TH, AADC i BH_4 (Taula 1)), així com un augment substancial dels precursors (3OMD i 5HTP) en la deficiència d'AADC. Un defecte de DBH acostuma a presentar valors d'HVA elevats (Figura 1), mentre que un defecte de MAO-A presenta nivells de HVA i 5HIAA en LCR marcadament baixos. La deficiència de DAT1 també presenta concentracions augmentades d'HVA en LCR a causa d'una degradació accelerada de la dopamina a l'espai sinàptic, mentre que un defecte de VMAT2 no presenta un perfil d'amines biògenes clarament alterat en LCR. Per tant, les variacions dels valors d'HVA no sempre són predictives de la deficiència de dopamina perquè els valors d'HVA baixos i alts (per exemple, en les deficiències de TH i DAT1) són indicatius de neurotransmissió amb deficiència de dopamina. En canvi, els valors extremadament baixos de HVA i 5HIAA en LCR poden indicar "intoxicació" per dopamina i serotonina com a conseqüència d'un deteriorament del catabolisme de les amines biogèniques, com ara la deficiència de MAO-A (Figura 1).

L'anàlisi de les pterines en orina es realitza en tots els pacients amb nivells augmentats de fenilalanina en sang detectats a través dels programes de cribratge neonatal per confirmar el diagnòstic diferencial de PKU; aquesta anàlisi inclou la determinació de neopterina, biopterina i primapterina (Wijemanne S et al., 2015 i Opladen T et al., 2012). Pel que fa al LCR, es poden determinar la neopterina, la biopterina i la sepiapterina que són biomarcadors útils per al diagnòstic diferencial de trastorns genètics relacionats amb el metabolisme de BH_4 en pacients que presenten valors normals de fenilalanina en sang (SR i la forma dominant de defectes GTPCH-I) (Figura 1). La deficiència de GTPCH-I presenta una disminució parcial dels valors de neopterina

i biopterina, mentre que la deficiència de SR presenta un augment de la biopterina en LCR secundari a l'augment de la degradació de BH_4 i a l'augment de la concentració de sepiapterina. Els pacients amb dèficit de SR no presenten PKU perquè a nivell perifèric, a través de dues reductases diferents i de la dihidrofolat reductasa (DHFR) (Figura 1), poden seguir una via alternativa per sintetitzar la BH_4 necessària a nivell hepàtic.

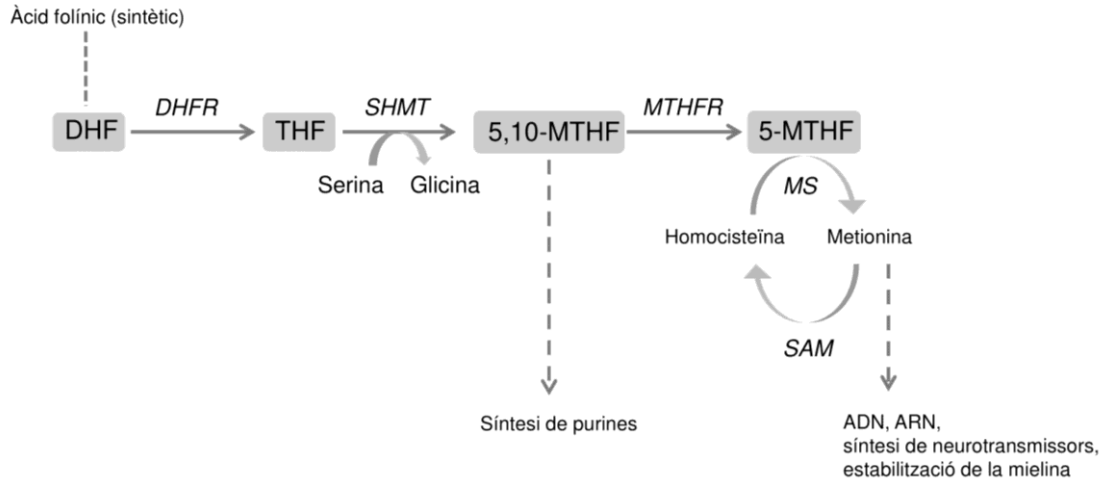
De manera similar a la resta de biomarcadors, les alteracions de les concentracions d'amines biògenes i pterines en LCR són causades principalment per esdeveniments secundaris, ja que les malalties genètiques primàries que condueixen a deficiències de dopamina i serotonina són extremadament rares. Per tant, les concentracions alterades de HVA i 5HIAA (augment o disminució de les concentracions en LCR) s'han associat de forma secundària amb diversos trastorns neurològics (genètics o ambientals) (De Grandis E et al., 2010 i Molero-Luis M et al., 2013). S'ha descrit un augment de la concentració de neopterina en LCR secundari a un increment en la seva síntesi provocat per l'activació de citocines, com l'interferó-alfa. Aquest fet és una eina important a l'hora d'identificar malalties inflamatòries o autoimmunes que afecten el SNC (Molero-Luis M et al., 2013) i s'ha descrit la neopterina com a biomarcador d'aquestes malalties (Molero-Luis M et al., 2020).

La confirmació diagnòstica de les condicions genètiques que condueixen a deficiències de dopamina i serotonina s'aconsegueix mitjançant les anàlisis genètiques dels gens candidats. També es poden analitzar les activitats enzimàtiques, que donen suport al diagnòstic inicial, especialment quan les dades de les anàlisis moleculars no són concloents. En alguns casos, si la sospita diagnòstica va molt encaminada a una malaltia concreta, l'estudi genètic es fa de manera individual, mitjançant seqüenciació de Sanger. Però en la majoria de casos la sospita diagnòstica no és tant acurada i és més eficient estudiar tots els gens implicats en una ruta metabòlica per seqüenciació massiva de nova generació (NGS), ja que permet un diagnòstic més ràpid. Utilitzant l'exoma sencer i la seqüenciació del genoma, s'han identificat nous gens que causen una alteració de l'estat de les monoamines (Hovarth GA et al., 2016). L'enorme complexitat de la transmissió sinàptica, en què participen centenars de proteïnes amb diferents funcions, suggereix fermament que la seqüenciació massiva de grans sèries de pacients amb malalties neurològiques no diagnosticades conduirà al descobriment de nous gens implicats en la neurotransmissió i de noves malalties.

1.2. Folat

El folat és una vitamina B hidrosoluble que es troba principalment al SNC com a 5MTHF. El 5MTHF és el donant de metils més important de l'organisme i participa en més de 100 reaccions de metilació a través de la S-adenosilmetionina (SAM). Entre aquestes reaccions, el folat és fonamental per a l'estabilitat de la mielina, el recanvi de dopamina i serotonina, la síntesi de purines i el metabolisme dels aminoàcids (com ara l'homocisteïna, la metionina, la serina i la glicina) (Ormazabal A et al; 2015 i Watkins D et al; 2012).

Figura 2. Representació esquemàtica del metabolisme del folat i del seu transport a través de la BHE. (Adaptada de Batllori et al., 2016. Veure annex 1).



DHF, dihidrofolat; DHFR, dihidrofolatreductasa; THF, tetrahidrofolat; SHMT, serina-hidroximetiltransferasa; MTHF, metiltetrahidrofolat; MTHFR, MTHF reductasa; MS, metioninasintasa; SAM, S-adenosilmetionina.

Els requeriments de folat que necessita l'organisme s'han d'obtenir íntegrament de fonts dietètiques, ja que, les cèl·lules dels mamífers no poden sintetitzar-lo *de novo*. La deficiència perifèrica de folat s'ha relacionat amb anèmia megaloblàstica, retard de creixement, defectes congènits al naixement, complicacions de l'embaràs, osteoporosi, càncer i diverses malalties neurodegeneratives i psiquiàtriques (Hyland K et al., 2010).

Malalties associades

La deficiència de folat cerebral (DFC) és una malaltia descrita relativament recent, que es caracteritza per una baixa concentració de 5MTHF al LCR amb un metabolisme del folat perifèric normal. Aquesta afecció presenta diversos símptomes neurològics, inclosos trastorns de la substància blanca, retard en el desenvolupament, epilèpsia,

irritabilitat, hipotonia, discinèsia, autisme, espasticitat, atàxia, distonia, esquizofrènia catatònica, demència o mioclonus (Watkins D et al., 2012 i Gordon N., 2009). La DFC s'ha associat tant amb condicions genètiques primàries (defectes del transport de folat a través de la BHE i defectes de la via metabòlica del folat) com secundàries.

- Defectes associats a condicions genètiques primàries:

Pel que fa als defectes del transport del 5MTHF cap al SNC es produeix principalment en les cèl·lules epitelials del plexe coroide a través del receptor alfa del folat d'alta afinitat (FRα) en un procés dependent de l'adenosina trifosfat (ATP). Aquest procés condueix a un augment de la concentració del 5MTHF en LCR de 1.5 vegades superior respecte a la que podem trobar en plasma (Watkins D et al., 2012). Les mutacions en el gen del receptor 1 del folat (FOLR1), que codifica pel receptor alfa del folat (OMIM * 136430), causen deficiències en el transport del folat cerebral (Steinfeld R et al; 2009 i Pérez-Dueñas B et al., 2010). L'altra condició genètica que afecta el transport de folat en diversos teixits, inclòs el cervell, és la deficiència del transportador de folat acoblat a protons (PCFT; OMIM * 611672). Aquesta condició genètica dóna lloc a una malaltia d'aparició precoç que provoca una malabsorció hereditària de folat donant lloc a una deficiència de folat tant a nivell perifèric com central (Serrano M et al., 2012). Ambdues condicions provoquen que dins del SNC hi hagi, freqüentment, desmielinització i calcificacions intracranials, combinades amb retard en el desenvolupament, retard mental, convulsions i alteracions motores. Bioquímicament, el tret distintiu d'aquestes dues patologies és que la ratio de folat LCR/sèrum està baixa independentment de què hi hagi presència o absència de deficiència de folat perifèric. El tractament d'aquests pacients amb àcid folínic pot millorar parcialment el quadre clínic (Pérez-Dueñas B et al., 2010).

Entre les alteracions del metabolisme del folat, les deficiències més profundes de 5MTHF en LCR es produeixen en la forma greu de deficiència de metilè-tetrahidrofolat reductasa (MTHFR; OMIM * 607093) i en la deficiència de dihidrofolat reductasa (DHFR; OMIM * 126060). En totes dues patologies, bioquímicament, els pacients presenten concentracions normals de folat en sèrum associades a una deficiència severa de 5MTHF cerebral, similar a la que s'observa en les alteracions genètiques que afecten els transportadors de folat (Pérez-Dueñas B et al., 2010).

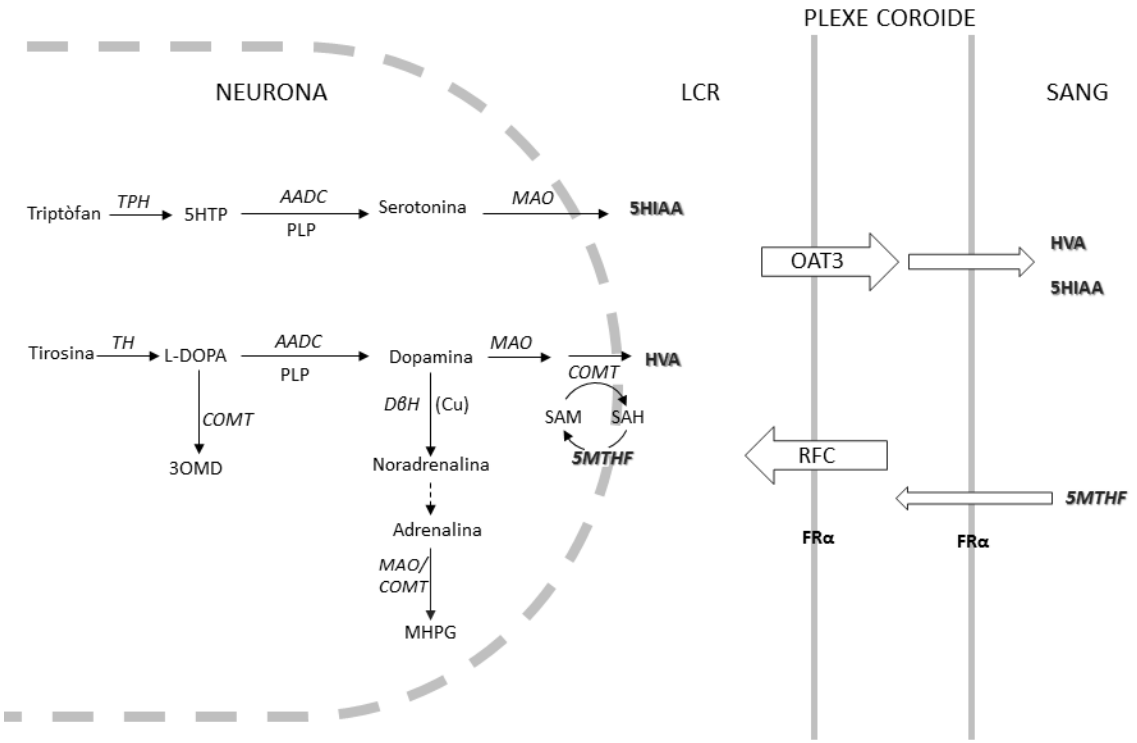
- Defectes associats a condicions adquirides i genètiques secundàries:

L'alteració del transport de folat cerebral també és la causa d'alguns casos de DFC. S'ha descrit que la presència d'autoanticossos contra el receptor d'alta afinitat del folat és una de les principals causes d'aquesta deficiència (Ramaekers VT et al., 2002). En la síndrome de Kearn Sayre (KSS; OMIM # 530000), l'acumulació de còpies d'ADN mitocondrial mutades a l'epiteli del plexe coroide s'ha descrit com la causa d'una alteració del transport de folat (Casado M et al., 2014; Pineda M et al., 2006; Serrano M et al., 2010 i Garcia-Cazorla A et al., 2008). Altres afeccions associades al DFC inclouen la deficiència de DHPR, defectes en la biosíntesi de serina, síndrome de Rett, síndrome d'Aicardi-Goutiere's, altres malalties mitocondrials, síndrome d'hipomielinització amb atròfia dels ganglis basals i esquizofrènia catatònica (Watkins D et al., 2012 i Pineda M et al., 2006). En molts casos de DFC, l'etiologia subjacent és desconeguda (Pérez-Dueñas B et al., 2011).

Centrant-nos una mica més en les malalties mitocondrials, aquestes, conformen un grup heterogeni de trastorns genètics resultants de mutacions en gens mitocondrials (mtDNA) o gens nuclears, que condueixen a una disfunció del procés de fosforilació oxidativa mitocondrial i altres vies relacionades amb el metabolisme energètic. S'ha suggerit que els defectes del metabolisme energètic poden afectar el sistema dels neurotransmissors (Garcia-Cazorla A et al., 2008a, b) perquè les sinapsis requereixen grans quantitats d'energia mitocòndria-dependents (Ly CV i Verstreken P, 2006). Se sap que la sinapsi implica un alt consum d'energia i que els mitocondris proporcionen fins al 90% de l'ATP necessari per mantenir el potencial de la membrana sinàptica (Ly CV i Verstreken P, 2006). Aquest mateix sistema també sembla alimentar nombrosos passos del cicle que segueixen les vesícules, com ara escissió, pèrdua del recobriment, recàrrega de neurotransmissors i transport vesicular al terminal sinàptic (Murthy VN i De Camilli P, 2003). Per tant, sembla plausible que es pugui observar un estat de neurotransmissors alterats en aquest grup de malalties. Alguns pacients amb malalties mitocondrials han presentat trets clínics de parkinsonisme i altres trastorns del moviment (Garcia-Cazorla A et al., 2008a, b; Ghaoui R i Sue CM, 2018; Kuster A et al., 2018; Tzoulis C et al., 2016). Més consistent és l'associació entre la deficiència de folat cerebral i els trastorns mitocondrials, sent el plexe coroide l'òrgan diana d'aquesta associació (Tanji K et al., 2000).

El 5-metiltetrahidrofolat (5MTHF) és un cofactor essencial per a les reaccions de metilació al cervell i altres òrgans mitjançant la SAM. Participa, per exemple, en el catabolisme de la dopamina fins al seu metabòlit final HVA. La deficiència de folat cerebral (definida com a valors baixos de 5MTHF en LCR tenint el folat perifèric normal) s'ha descrit associada a diferents condicions neurogenètiques i ambientals (Pérez-Dueñas et al. 2011) i específicament a alguns trastorns mitocondrials (Garcia-Cazorla A et al., 2008a) tals com la KSS.

Figura 3. Representació esquemàtica de les vies relacionades amb el metabolisme de la dopamina i la serotonina juntament amb els sistemes de transport a través del plexe coroide d'aquestes i del folat.



A la figura anterior, es mostren el transportador del folat del plexe coroide (receptor alfa del folat; FRα) i el transportador de monoamines (transportador orgànic aniònic tipus 3; OAT3). Per controlar l'homeòstasi del LCR i garantir una correcta nutrició i protecció del SNC, s'ha descrit un sistema de transport molt complex entre els costats apical i basolateral de les cèl·lules epitelials del plexe coroide (Spector R, 2010; Spector R i Johanson CE, 2010). El plexe coroide controla la quantitat i la composició del LCR secretant molècules cap al LCR i excretant-ne d'altres des del LCR cap a la circulació sanguínia (Orešković D et al., 2017). Cal destacar dues característiques addicionals: (1)

el plexe coroide està enriquit en mitocondris perquè els sistemes de transport actius que consumeixen ATP són els responsables de l'entrada de folat cap al LCR i de l'excreció de monoamines des del LCR cap a la sang (Alebouyeh M et al., 2003; Mori S et al., 2003), i (2) s'ha suggerit que l'acumulació de mitocondris mutats al plexe coroide dels pacients amb KSS provoca un fracàs en l'absorció i transport i/o secreció de proteïnes, folats i monoamines (Tanji K et al., 2000). Per tant, la manca d'ATP a causa de mitocondris mutats no pot proporcionar l'energia necessària per transportar molècules contra gradient al LCR. Així i tot, aquesta teoria sobre la manca d'energia no explica plenament el dèficit de folat observat en pacients amb KSS ni en els dèficits que s'observen en altres malalties mitocondrials (Garcia-Cazorla A et al., 2008a, b). D'altra banda, es creu que l'acumulació d'aquests mitocondris mutats al plexe coroide pot contribuir a una destrucció anatòmica que impedeix el transport correcte de molècules a través del plexe. S'ha descrit que, en pacients amb KSS, hi ha una correlació entre les concentracions de 5MTHF i els nivells d'expressió de FRα (Grapp M et al., 2013). Altres mecanismes com l'augment de la generació d'espècies reactives d'oxigen i/o la pèrdua d'antioxidants del LCR poden ser factors addicionals a tenir en compte pel que fa al desenvolupament d'una deficiència central de 5MTHF en trastorns mitocondrials (Aylett SB et al., 2013).

Interpretació de resultats

El diagnòstic dels DFC requereix la determinació de la concentració de 5MTHF en LCR i una avaluació del folat perifèric i els seus metabòlits relacionats (metionina, homocisteïna, serina, glicina i vitamina B_{12}). En el cas del folat no hi ha requeriments especials referents a l'obtenció de la mostra. L'anàlisi del 5MTHF es realitza normalment en laboratoris especialitzats mitjançant HPLC amb detector de fluorescència o electroquímic o bé, per cromatografia líquida acoblada a espectrometria de masses en tàndem (LC-MS o LC-MS/MS) (Verbeek MM et al., 2008 i Akiyama T et al., 2015). Tanmateix, també és important i ha resultat d'utilitat en el cribratge de DFC, la determinació del folat total (que inclou 5MTHF) en el LCR mitjançant analitzadors automatitzats disponibles en els laboratoris clínics de rutina (Verbeek MM et al., 2008 i Ormazabal A et al., 2011). A més, la major part del folat de l'organisme es troba en forma de 5MTHF; per tant, aquest enfocament és una bona

eina per a una investigació de primera línia en la detecció de problemes en el transport de folat al SNC, que posteriorment es pot confirmar mitjançant l'anàlisi específica de 5MTHF. Per tant, la possibilitat d'utilitzar un procediment ràpid i automatitzat per accelerar aquest procés diagnòstic és molt útil, ja que, en alguns casos, aquestes malalties són tractables.

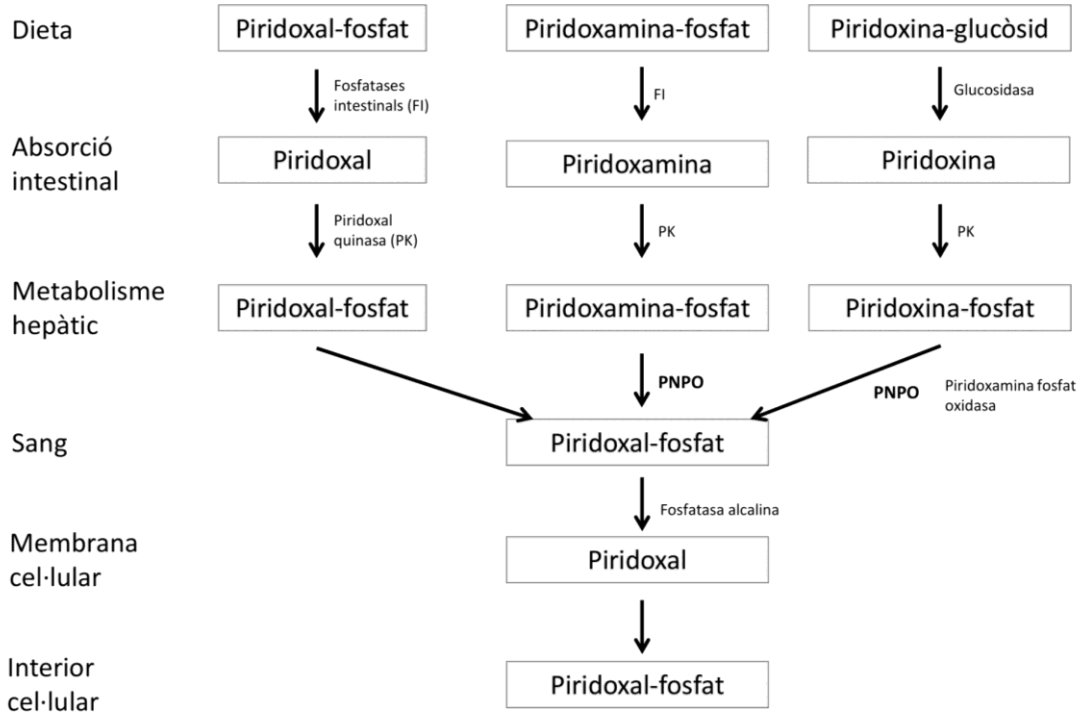
Les deficiències profundes de 5MTHF en LCR (menys de 10 nmol/L) les trobem en pacients que pateixen malalties genètiques que provoquen una alteració en el transport a través de la BHE o també en les alteracions genètiques del metabolisme del folat. Per altra banda, en les altres formes secundàries de DFC, les concentracions de 5MTHF, en general, es troben més moderadament disminuïdes. Pel que fa als pacients amb defectes en el transport, també presenten una deficiència profunda del folat total en LCR, cosa que suggereix que el transport alterat afecta a totes les formes de folat (Akiyama T et al., 2015). Respecte a les alteracions metabòliques del cicle del folat, com ara la deficiència de MTHFR, les concentracions totals de folat en LCR poden ser elevades o normals, cosa que reflecteix una biosíntesi i captura de folat en altres formes metabòliques-inactives d'aquesta vitamina. Pel que fa als pacients amb un dèficit lleu de folat cerebral, s'ha suggerit que pot ser un esdeveniment secundari i una troballa recurrent en molts pacients neuropediàtrics. En aquest sentit, la determinació simultània del folat en sèrum és més que recomanable per a una millor interpretació del DFC, ja que, un estat de folat sanguini subòptim podrà explicar la major part dels casos que presenten concentracions parcialment baixes de 5MTHF en LCR (Verbeek MM et al., 2008 i Akiyama T et al., 2015). L'anàlisi genètica confirmarà l'etiologia del DFC. Una investigació clínica exhaustiva dels pacients és molt important perquè hi ha altres condicions genètiques no relacionades, tals com, els trastorns mitocondrials abans esmentat i altres d'ambientals que poden provocar DFC.

1.3. Vitamin B_6

La vitamina B_6 és present en una àmplia varietat d'aliments, com ara carn, productes lactis, patates, mongetes, fruits secs i diverses fruites i verdures (Bender DA, 2005). La forma biològicament activa de la vitamina B_6 és el piridoxal fosfat (PLP), que actua com a coenzim en més de cent passos enzimàtics diferents. Està estretament relacionat

amb la biosíntesi de dopamina, serotonina i GABA (Figura 1). La piridoxal quinasa (PLK) converteix la vitamina B_6 en els corresponents vitàmers fosforilats, mentre que la piridox(am)ine 5'-fosfat oxidasa (PNPO) la converteix en PLP. Per entrar a les cèl·lules i travessar la BHE, les vitamines B_6 han de ser desfosforilades per una fosfatasa alcalina associada a la membrana i posteriorment tornar a ser fosforilades a l'interior de les cèl·lules (Clayton P, 2006); Di Salvo ML et al., 2011).

Figura 4. Absorció i metabolisme de la vitamina B_6. Figura modificada de Clayton P, 2006.



Malalties associades

Les formes clàssiques de deficiència de vitamina B_6 que causen convulsions sensibles a piridoxina o PLP, s'han associat amb dues malalties genètiques: deficiències de PNPO (OMIM * 603287) i dèficit d'antiquitina (OMIM * 107323). En la deficiència de PNPO (Figura 1), l'alteració de la biosíntesi de PLP condueix a una disminució dels nivells de PLP tant en plasma com en LCR en la majoria dels casos, constituint el principal biomarcador de la malaltia. Es caracteritza per una bona resposta terapèutica a l'administració de dosis farmacològiques de PLP (Baxter P 2001). La deficiència d'antiquitina s'ha associat a l'epilèpsia clàssica dependent de piridoxina i és causada

per una deficiència en l'α-aminoadípic-semialdehid deshidrogenasa (ALDH7A1). L'antiquitina funciona com una deshidrogenasa de la via de degradació de la lisina i la seva deficiència provoca l'acumulació d'àcid pipecòlic, semialdehid α-aminoadípic i piperideïna-6-carboxilat (P6C). Aquest últim es condensa químicament amb el PLP, fet que provoca un segrest de la vitamina i una deficiència funcional de PLP (Shin YS et al., 1984).

A part d'aquestes dues formes clàssiques també s'han descrit trastorns que afecten la importació de PLP al cervell (deficiència de fosfatasa alcalina i els defectes en la síntesi d'ancoratge de glicosilfosfatidilinositol), un trastorn d'una proteïna intracel·lular d'unió al PLP (PLPBP, anteriorment anomenada PROSC) i la hiperprolinèmia tipus II, un altre trastorn on s'acumulen metabòlits que inactiven el PLP. Els pacients amb aquestes malalties poden respondre ràpidament i controlar les convulsions després de l'administració de piridoxina i/o PLP, tot i que dependran de per vida de l'administració de dosis suprafisiològiques de vitamina B_6 (Wilson MP et al., 2019).

Interpretació de resultats

El PLP és el metabòlit que s'hauria d'analitzar en lloc de determinar la vitamina B_6 total. No hi ha factors preanalítics rellevants respecte a la recollida del LCR més enllà de l'emmagatzematge de mostres a -80 °C. El PLP és cofactor dels enzims implicats en el metabolisme de diversos neurotransmissors, com la dopamina, la serotonina i el GABA. Un d'aquests enzims és l'AADC, que converteix L-DOPA i 5HTP en dopamina i serotonina respectivament (Figura 1). Per tant, en els trastorns de piridoxina, el perfil metabòlic dels neurotransmissors en LCR és similar al que observem en la deficiència primària d'AADC amb una disminució de HVA i 5HIAA i augment de 3OMD (Rodan LH et al., 2015). A més, i a través del mateix mecanisme, es preveu que les concentracions de GABA en LCR siguin baixes quan hi ha un estat deficient de PLP. Els aminoàcids en LCR també poden presentar augments de glicina, serina i treonina perquè els enzims serina i treonina deshidratasa i el complex de la glicina descarboxilasa (GDC) són dependents de PLP. En la deficiència de PNPO, aquestes alteracions analítiques també es poden identificar en mostres de plasma (Shin YS et al., 1984).

Els pacients amb deficiència d'antiquitina normalment no presenten evidències bioquímiques de deficiència de PLP en sang; no obstant això, poden presentar una

reducció de les concentracions de PLP en LCR. Això implica que el PLP sembla que s'exhaureix abans al SNC i, conseqüentment, la proporció de PLP entre el LCR i el plasma pot representar un marcador adjuvant en el diagnòstic (Shin YS et al., 1984). Per tant, es pot utilitzar la determinació simultània de PLP en plasma i LCR per distingir la deficiència de PNPO de la d'antiquitina. A més, en la deficiència d'antiquitina, s'identifica habitualment la presència d'un compost desconegut (compost X) que apareix si es realitza en paral·lel una anàlisi d'amines biògenes en LCR mitjançant HPLC. En el perfil d'aminoàcids d'aquests pacients s'observa també un augment de la concentració d'àcid pipecòlic tant en LCR com en plasma i orina.

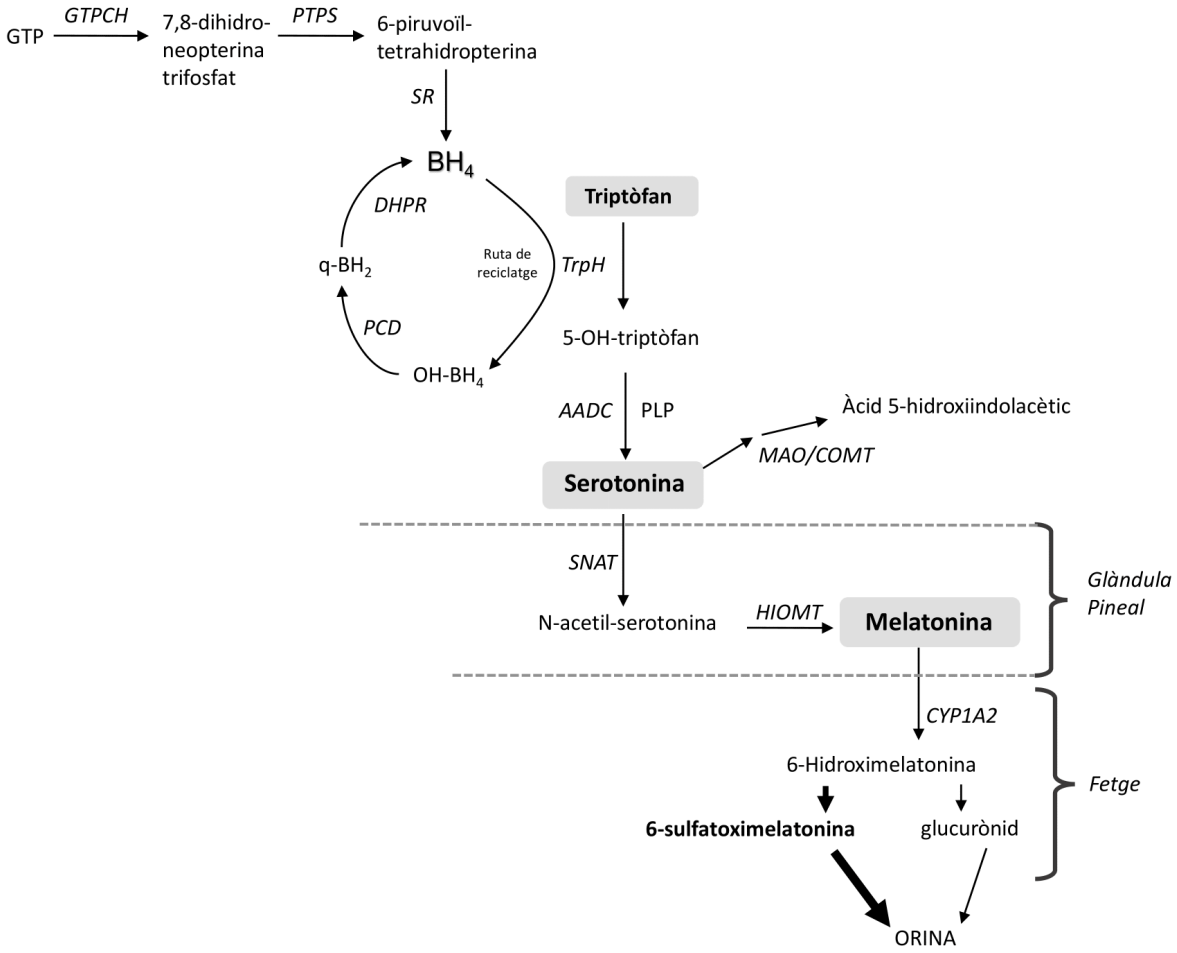
De manera similar a altres trastorns relacionats amb les vitamines, la deficiència secundària de PLP pot associar-se a altres afeccions, com la hiperprolinèmia tipus II (OMIM * 606811), la mucopolisacaridosi tipus III (OMIM * 607664, * 6097001, * 605270 i * 610453) i altres malalties. Les proves genètiques són necessàries per confirmar la base molecular de les diferents malalties potencialment associades a la deficiència de PLP.

1.4. Biomarcadors perifèrics

Tal com s'ha comentat anteriorment, hi ha una carència de biomarcadors perifèrics que permetin la monitorització del tractament d'aquestes malalties sense necessitat de fer puncions lumbars successives. Un dels marcadors secundaris que ens van semblar interessant va ser la melatonina en orina, que ha estat prèviament utilitzada per a l'estudi d'altres malalties.

La melatonina (5-metoxi-N-acetiltriptamina) és secretada per la glàndula pineal i es sintetitza a partir de la serotonina. La síntesi de melatonina està regulada per dos enzims específics: la serotonina-N-acetil-transferasa (SNAT, EC 2.3.1.5), enzim limitant, i la 5-hidroxiindol-O-metil transferasa (HIOMT, EC 2.1.1.4), que transfereix un grup metil de SAM al 2-hidroxil de N-acetilserotonina (Figura 5). Posteriorment, la melatonina s'allibera de la glàndula pineal i entra a la circulació. Altres fonts de melatonina són la retina, l'intestí, la pell, les plaquetes i la medul·la òssia, però la seva contribució a la melatonina en circulació és menys rellevant que la de la glàndula pineal (Claustrat B i Leston J, 2015).

Figura 5. Via de síntesi i degradació de la melatonina



La melatonina es metabolitza al fetge a 6-hidroximelatonina mitjançant el citocrom CYP1A2 (EC 1.14.14.1) i s'excreta en orina com a sulfatoximelatonina (aMT6) i, en menor mesura, com a glucurònid conjugat (Claustrat B i Leston J, 2015). L'excreció en orina de aMT6 es correlaciona estretament amb el perfil de melatonina plasmàtica (Claustrat B i Leston J, 2015; Arendt J et al., 1985) i és un bon indicador de la secreció de melatonina de la glàndula pineal (Pääkkönen T et al., 2006). Per tant, s'ha suggerit que la determinació de aMT6 en orina pot ser un bon biomarcador de l'estat de la serotonina cerebral. Yano et al. van descriure que les concentracions de melatonina en sang i de aMT6 en orina poden servir com a biomarcadors que reflecteixen la síntesi de serotonina cerebral en pacients amb PKU.

Tal com ja s'ha descrit anteriorment, hi ha diverses alteracions genètiques que afecten la biosíntesi de la serotonina i la dopamina en el SNC i que es troben representades en la Figura 1. La presentació clínica d'aquests trastorns inclou símptomes relacionats

amb disfunció autonòmica, que es manifesten com sudoració, desregulació de la temperatura, hipersalivació, congestió nasal i signes psiquiàtrics (Ng J et al., 2015), com ara mal comportament i trets autistes. Els trastorns del moviment són causats principalment per la desregulació de la dopamina, que inclou trastorns de la marxa, distonia, discinèsia, parkinsonisme, tremolors, crisis oculogíries, ptosi palpebral i hipotonia axial (Pearl PL et al., 2005 i Assmann B et al., 2003). No obstant això, els símptomes que es creu que estan relacionats amb la deficiència de serotonina són més difícils d'avaluar clínicament.

Per poder determinar la deficiència de serotonina en aquestes condicions, l'anàlisi de l'àcid 5-HIAA en LCR és el marcador més adequat (Hyland K, 2008). A més, la majoria dels pacients que presenten aquests trastorns acostumen a seguir diferents protocols de tractament per restaurar els nivells de serotonina (i dopamina) cerebrals. Per avaluar la resposta bioquímica al tractament, els pacients sovint se sotmeten a una punció lumbar per determinar l'estat de la serotonina cerebral mitjançant la quantificació de 5HIAA.

2. ***Aspectes preanalítics***

El LCR és un fluid biològic produït principalment pel plexe coroide, que constitueix la interfície entre els vasos sanguinis i el LCR (Hladky SB et al., 2016; Spector R, 2010). La composició del LCR també està controlada per la BHE, que separa la sang del parènquima cerebral (Redzic Z, 2011). Ambdues estructures proporcionen substrats per al metabolisme de les cèl·lules cerebrals i eliminen els residus corresponents (Hladky SB et al., 2016; Nedergaard M., 2013; Akaishi T et al., 2019). En general, la BHE restringeix considerablement l'afluència de la majoria de molècules, inclosos els aminoàcids i altres compostos (Mann GE et al., 2003).

Els aminoàcids, amb poques excepcions (per exemple, la glutamina), mostren valors més baixos en el LCR en comparació amb els del plasma (Kornhuber ME et al., 1986; Duran M, 2008). Per a altres metabòlits, les vies biosintètiques estan compartimentades al cervell i es poden observar concentracions similars en LCR i en sang, ja que no hi ha transport d'aquestes substàncies des de la sang fins al LCR; aquest és el cas de les amines biògenes i pterines (Coppus AW et al., 2007; Mori S et al.,

2003). En canvi, algunes vitamines han de ser transportades al cervell a través de barreres del SNC per transportadors específics i hi ha diferències importants entre les concentracions de vitamines en el LCR respecte al plasma (Steinfeld R et al., 2009; Ortigoza-Escobar D et al., 2016). Mentre que el folat és una de les poques molècules més concentrades en el LCR en comparació amb el plasma, altres vitamines com la tiamina (vitamina B_1) i la piridoxina presenten la situació inversa, valors més baixos en el LCR respecte al plasma (Ortigoza-Escobar JD et al., 2016; Footitt EJ et al., 2011).

El primer pas crític cap a l'estudi dels compostos esmentats és el procediment de recollida de mostres de LCR. Per a la majoria de les malalties que es mostren a la taula 1, les anàlisis de mostres de sang o d'orina no són fiables per determinar l'estat de la dopamina i la serotonina cerebrals, ja que les concentracions en aquests fluids poden reflectir una síntesi perifèrica emmascarant així, la central. La recollida de mostres de LCR ha de ser realitzada per un metge expert i és obligatòria l'estricta adhesió a un protocol preanalític per obtenir resultats fiables (Ormazabal A et al., 2005; Hyland K et al., 1993).

En les darreres dècades, s'han utilitzat enfocaments metabolòmics dirigits per a l'estudi d'aquestes afeccions neurogenètiques (Ormazabal A et al., 2017). A causa de les importants diferències en les concentracions de metabòlits entre la sang i el LCR, la contaminació del LCR amb sang pot causar efectes dramàtics en les concentracions mesurades de la majoria dels metabòlits esmentats (Akiyama T et al., 2014; MacNeil AL et al., 2018; Srinivasan L et al., 2013; McFarlin KE et al., 1990). El LCR es recull mitjançant un mètode invasiu que consisteix a fer una punció lumbar. Atès que la contaminació sanguínia/plasmàtica es pot observar amb freqüència per diferents causes (punxades lumbars traumàtiques, alteració de la permeabilitat de la BHE o sagnat intraventricular) (Tan R et al., 2017; Krueger M et al., 2019; Klebe D et al., 2019), s'ha de minimitzar al màxim una interpretació errònia dels perfils metabòlics per evitar una nova punció lumbar o donar un diagnòstic erroni.

Hi ha múltiples factors que influeixen en la concentració de neurotransmissors en LCR i que han de ser controlats. Per aquest motiu, l'obtenció de les mostres de LCR s'ha de realitzar seguint un protocol estandarditzat (Ormazabal A et al., 2005).

3. Aspectes analítics

3.1. Visió general dels procediments

Per a la separació cromatogràfica dels biomarcadors que es presenten aquí, s'utilitzen diferents mètodes d'HPLC: la separació cromatogràfica de parell iònic es basa en la modificació d'una columna de fase inversa per poder separar els compostos ionitzats. L'HPLC en fase inversa permet la separació de compostos per interaccions hidrofòbiques amb la fase estacionària de la columna. Pel que fa a la detecció, s'utilitza la detecció electroquímica (ED) per quantificar els compostos redox actius aplicant un potencial i mesurant el corrent elèctric produït, que és proporcional a la concentració de l'anàlit. La detecció de fluorescència (FD) s'utilitza per a la quantificació de compostos que presenten fluorescència nativa (anàlisi de pterines) i també d'altres que no la presenten i només són quantificables després de sotmetre's a un procés de derivatització (anàlisi de PLP). En la FD, el compost s'excita a una longitud d'ona (excitació) i es mesura a una altra longitud d'ona (emissió).

Per a la quantificació de monoamines en LCR, el *gold standard* és l'HPLC de parell iònic amb detecció electroquímica (HPLC-ED), perquè permet una separació i quantificació altament sensibles d'aquests compostos (Heales SJ, 2008; Hyland K et al., 1993).

Per a l'anàlisi de pterines en LCR, l'HPLC amb FD (HPLC-FD) permet una quantificació ràpida de les pterines en les seves formes oxidades (neopterina, biopterina, primapterina i sepiapterina) i també és molt útil per a l'estimació de la biosíntesi i metabolisme de la pterina activa (BH_4) (Blau N i Beat T, 2008). L'HPLC-ED-FD en sèrie permet fer la quantificació de la neopterina total, BH_4 i el seu precursor, BH_2. Aquest mètode en sèrie permet la quantificació simultània de compostos electroactius (BH_4) i fluorescents (BH_2 i neopterina).

I per últim, per l'anàlisi de PLP, l'HPLC-FD ofereix una aproximació d'alta sensibilitat (Bates CJ et al., 1999; Ormazabal A et al., 2008).

3.2. Aplicacions dels mètodes

Les aplicacions més habituals dels mètodes esmentats anteriorment inclouen el

diagnòstic de malalties genètiques primàries que donen lloc a deficiències de neurotransmissors i altres malalties neurològiques greus, d'inici precoç que poden estar associades a alteracions en les vies metabòliques de la dopamina i la serotonina cerebrals (Molero-Luis M et al., 2013; García-Cazorla A et al., 2007). Les tècniques descrites també poden ser adequades per a la quantificació de monoamines, com la dopamina i la serotonina i compostos relacionats, en models animals i models cel·lulars experimentals després de fer una extracció i purificació d'aquelles mostres d'interès. Una manera és la precipitació d'àcid sobre gel, centrifugació i filtració de sobrenedant, tal com s'ha descrit en varis treballs (Fukushima T i Nixon JC 1980., Soblosky JS et al., 1998; Allen GF et al., 2013). Els procediments de microdiàlisi cerebral també permeten determinar els nivells de monoamines i altres molècules en el líquid de teixits intersticials (Chefer VI et al., 2009). Fukushima et al. han publicat diversos treballs on estudien la BH4 i els seus metabòlits per HPLC amb FD en diferents teixits de rata (pineal, fetge, suprarenal, cervell i sang), demostrant que la mateixa metodologia també és útil per a mostres humanes (sang i orina) (García-Cazorla A et a.l, 2007).

Respecte a les aplicacions de les pterines, a banda de la identificació de causes genètiques primàries, la quantificació de neopterina en LCR és de gran utilitat per l'estudi de processos immuno-inflamatoris cerebrals (Molero et al., 2013). L'anàlisi de PLP pot detectar un estat subòptim de vitamina B_6 ja sigui degut a deficiències nutricionals, augment del reciclatge, augment de segrest de B_6 per la formació d'adductes químics o altres condicions ambientals i genètiques que promouen la seva deficiència (Footitt EJ et al., 2011; Stockler S et al., 2011; Ruiz A et al., 2008)

3.3. Comparació amb altres mètodes d'ús més freqüent

Els mètodes descrits aquí s'han utilitzat habitualment per estudiar les amines biògenes i les pterines en LCR. Tanmateix, els mètodes d'ultra-HPLC acoblats a espectrometria de masses en tàndem (UHPLC-MS/MS) s'utilitzen cada cop més en investigacions bioanalítiques, per la seva sensibilitat, especificitat i eficàcia en diferents materials biològics (Kovac A et al., 2014).

L'estudi metabòlic dirigit és una bona aproximació a tenir en compte en les estratègies diagnòstiques basades en HPLC, i en un futur, s'espera que apareguin noves aplicacions pel diagnòstic humà. Kovac A et al., van desenvolupar un mètode de LC-MS/MS per avaluar 5-hidroxitriptamina, 5HIAA, HVA, noradrenalina, adrenalina, dopamina, glutamat, àcid γ-aminobutíric, àcid 3,4-dihidroxifenilacètic i histamina en el LCR d'un model de rata. Santos-Fandila et al., van descriure una anàlisi de neurotransmissors, metabòlits i derivats en microdialitzats de rates per UHPLC-MS/MS. La tecnologia de LC-MS/MS també s'ha utilitzat per a l'anàlisi de pterines i permet la detecció simultània de BH_4, BH_2 i biopterina en mostres d'orina, extractes cel·lulars i cervell de rata (Kim HR et al., 2012; Fismen L et al., 2012; Jiménez Girón A et al., 2012). No obstant això, segons els autors abans esmentats, per a la quantificació de pterines, la detecció per MS/MS és menys sensible que la FD. Per a la detecció de PLP, s'han publicat mètodes per UHPLC-MS/MS (Van der Ham M et al., 2012; Footitt EJ et al., 2013) que permeten la identificació de set isoformes diferents de la vitamina B_6 en mostres de LCR humà, encara que aquest procediment no s'usa de manera rutinària per a la quantificació de PLP en fluids biològics. Recentment, s'ha desenvolupat un mètode per HPLC-FD (Akiyama T et al., 2016) per a la mesura simultània de metabòlits de monoamines, i de 5-metiltetrahidrofolat (5MTHF, la forma activa del folat), en LCR.
En conjunt, la informació metodològica presentada en aquest treball pot ser de gran ajuda per a laboratoris tant de recerca clínica com de recerca bàsica.

JUSTIFICACIÓ DE LA UNITAT TEMÀTICA

Els errors congènits del metabolisme (ECM) són malalties d'origen genètic amb una baixa prevalença dins de la població general. Moltes d'aquestes malalties poden presentar símptomes neurològics (malalties neurometabòliques) que poden comprometre el correcte desenvolupament del SNC. La dopamina i la serotonina són neurotransmissors que intervenen en el correcte desenvolupament del SNC i, per tant, la detecció quantitativa en LCR dels seus metabòlits àcids homovaníl·lic (HVA) i 5-hidroxiindolacètic (5HIAA) i dels seus cofactors (piridoxal fosfat (vitamina B_6) i tetrahidrobiopterina (BH_4)) es considera una important prova bioquímica pel diagnòstic i seguiment d'aquestes malalties.

En aquesta tesi hem investigat el procés complet de l'aplicació de biomarcadors en malalties neuropediàtriques. Primer ens hem centrat a estudiar la influència de diferents variables preanalítiques en la interpretació de resultats i en els aspectes merament analítics de la determinació quantitativa de les monoamines i els seus cofactors. També hem desenvolupat un component més biomèdic que va consistir en la validació de la melatonina com biomarcador per a l'estudi en fluids perifèrics del metabolisme central de la serotonina i l'estudi de biomarcadors en pacients amb malalties del metabolisme energètic mitocondrial.

HIPÒTESI

L'estudi dels defectes de la neurotransmissió en pacients neuropediàtrics és una eina fonamental pel diagnòstic de certes malalties neurometabòliques. Tot i que aquests estudis s'han anat realitzant en dècades passades en laboratoris especialitzats, hem cregut rellevant efectuar una anàlisi profunda de les condicions preanalítiques, analítiques i postanalítiques per una correcta interpretació dels resultats. Aquesta activitat d'estandardització ens aportarà nous coneixements sobre les condicions òptimes per l'anàlisi i interpretació d'aquestes biomolècules.

En relació amb els aspectes biomèdics d'aquest treball la nostra posició com a centre de referència per l'estudi d'aquestes malalties ens permetrà validar nous biomarcadors en pacients amb defectes genètics de la neurotransmissió i en altres malalties neuropediàtriques.

OBJECTIUS

L'objectiu principal d'aquesta tesi doctoral és aprofundir en les tècniques d'anàlisi i en la investigació de nous biomarcadors de l'estat de la dopamina i la serotonina en pacients neuropediàtrics amb malalties neurometabòliques.

Els objectius específics són els següents:

1. Estudi preanalític per avaluar la influència de la contaminació hemàtica en les mostres de líquid cefalorraquidi a l'hora de realitzar estudis metabolòmics dirigit.
2. Descriure els protocols analítics basats en cromatografia líquida d'alta resolució (HPLC) per a la quantificació d'amines biògenes i els seus cofactors en líquid cefalorraquidi.
3. Avaluar la melatonina com a biomarcador perifèric de l'estat de la serotonina central en pacients amb defectes enzimàtics de la síntesi d'amines biògenes.
4. Avaluar retrospectivament el fenotip clínic i els perfils bioquímics d'amines biògenes i cofactors relacionats en líquid cefalorraquidi de pacients diagnosticats genèticament d'una malaltia mitocondrial.

PACIENTS, MATERIALS I MÈTODES

1. Pacients i controls: mostres biològiques

1.1 Mostres per a l'estudi preanalític de contaminació hemática

Per iniciar aquest estudi primer vam obtenir un pool de LCR de mostres romanents i anonimitzades. Per realitzar aquest pool van utilitzar-se mostres de LCR de pacients que havien vingut a urgències de l'hospital per sospita de meningitis i, després d'excloure aquesta malaltia o qualsevol altra d'origen central, es va utilitzar l'excedent del LCR. D'aquest pool de líquid se'n fan diferents alíquotes i aquestes es contaminaran amb quantitats creixents de sang (interferent) de manera que obtindrem diverses alíquotes de LCR contaminades amb concentracions creixents de sang. Els volums d'interferent es mostren a la taula següent:

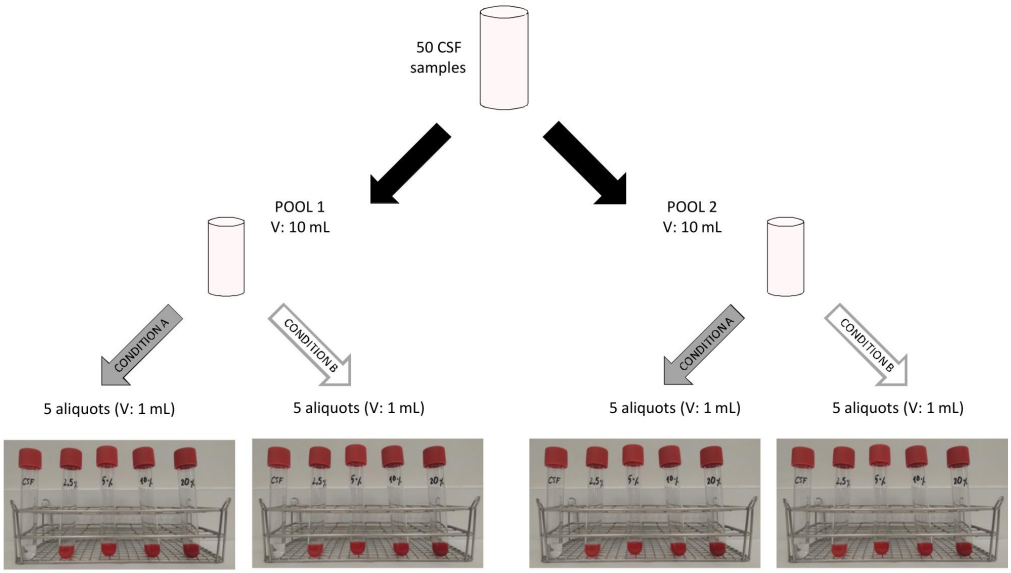
Sang total (µL)	200	100	50	25
Líquid cefalorraquidi (µL)	800	900	950	975
% de contaminació amb sang	20%	10%	5%	2.5%
Concentració d'albúmina (g/L)	9.2	4.6	2.3	1.15
Concentració d'hemoglobina (g/dL)	2.9	1.45	0.72	0.35

La sang s'extreu d'un voluntari/a sa en el moment de realitzar el protocol d'anàlisi. A la mostra de sang que s'utilitza com a interferent se li realitza un hemograma per conèixer la concentració d'hemoglobina. D'aquesta manera podrem calcular la concentració d'hemoglobina teòrica de cada alíquota de LCR contaminada.

Un cop contaminades les alíquotes de LCR amb la quantitat de sang corresponent, les mostres les tractarem de dues maneres diferents:

Condició A: mostres de LCR contaminades amb sang que es congelen a -80°C durant 1 hora i es descongelen per provocar la lisi dels hematies. Un cop descongelades se'n fan noves alíquotes que es tornaran a congelar fins el dia l'anàlisi.

Condició B: mostres de LCR contaminades amb sang que centrifuguem a 1500xg durant 10 min. En separem el sobrenedant, en fem alíquotes i el congelem fins el dia de l'anàlisi.



1.2 Mostres per a la validació tècnica dels protocols d'anàlisi de neurotransmissors, pterines i PLP en LCR

En la redacció del segon treball d'aquesta tesi no s'han utilitzat mostres de pacients, ni tampoc de població control. Les mostres de pacients i els valors de referència ja varen ser publicades prèviament pel nostre grup (Ormazabal A et al., 2005; Ormazabal A et al., 2006 i Molero-Luis M et al., 2013).

1.3 Població d'estudi per a l'anàlisi de la melatonina com a biomarcador perifèric de l'estat de la serotonina central

- <u>Població control</u>

  La població control va constar de 65 individus (36 dones i 29 homes) d'edats compreses entre els 2-61 anys (mitjana aritmètica 10.0 anys). Tots van dormir en un ambient controlat d'il·luminació (foscor) i entre 8 i 11 hores.

  Criteris d'exclusió:

  - Tractaments farmacològics en el moment de l'estudi que poguessin afectar l'estat de la serotonina.
  - Evidència d'abús de drogues o alcohol.
  - Haver realitzat feines que impliquessin canvis de torns de treball.

- Haver fet algun viatge a través de 2 o més zones horàries en el mes precedent a l'estudi.

- Mostres recollides fora de protocol (orina que no era de primera micció del matí, pèrdues durant la nit)

- Població d'estudi amb malalties genètiques

Es van estudiar 28 pacients amb defectes genètics que afectaven la via metabòlica de la serotonina (edats compreses entre 2 i 55 anys; mitjana aritmètica de 13.0 anys). Aquests 28 pacients es van classificar en dos grups:

Grup 1: 18 pacients que no rebien cap tractament serotoninèrgic, o bé prenien L-dopa/carbidopa (18 pacients), que és un precursor de la via de síntesis de dopamina i, per tant, un fàrmac que teòricament no afecta l'eix serotoninèrgic.

El diagnòstic dels pacients d'aquest grup va ser: 14 pacients amb deficiència autosòmica dominant o recessiva de guanosina trifosfat ciclohidrolasa-I; 3 pacients amb deficiència de sepiapterina reductasa; i 1 cas amb deficiència de la descarboxilasa dels aminoàcids aromàtics.

Grup 2: 11 pacients que rebien tractament serotoninèrgic amb precursors de la serotonina (5-hidroxitriptòfan, (5HTP)), inhibidors de la monoamino oxidasa (IMAOs), inhibidors selectius de la recaptació de serotonina (ISRS), cofactor que promouen la síntesi de serotonina/melatonina (BH_4, PLP i àcid folínic) o en tractament amb melatonina.

El diagnòstic dels 11 pacients va ser: 5 casos amb deficiència de la descarboxilasa dels aminoàcids aromàtics; 1 pacient amb deficiència de sepiapterina reductasa; 3 pacients amb deficiència de dihidropteridina reductasa; i 2 casos amb deficiència de 6-piruvoyltetrahidropterin sintasa.

Cal mencionar que la suma dels pacients dels dos grups d'estudi no és 28 (18 pacients al grup 1 i 11 pacients al grup 2) ja que el pacient 18 va poder ser estudiat al moment del diagnòstic (grup 1 com a pacient 18) i dos mesos després de ser instaurat el tractament serotoninèrigc amb IMAO (grup 2 com a pacient 18 bis).

- Mostres: tant per controls com per pacients es va recollir l'orina de primera hora del matí, es va centrifugar i es va guardar a -80 °C fins que es van analitzar.

En alguns pacients (en 13 dels 28) també disposem de les concentracions de 5HIAA en LCR. Alguns d'aquests resultats representen el valor al moment del diagnòstic, però la majoria varen ser realitzats per monitoritzar el tractament.

1.4 Població de pacients diagnosticats genèticament d'una malaltia mitocondrial

- Pacients

En els últims dotze anys, s'han reclutat 29 pacients (edats compreses entre els 3 mesos i els 34 anys) afectats d'una malaltia mitocondrial confirmada genèticament. Totes les mutacions dels pacients (tant les nuclears com les d'ADN mitocondrial) estan publicades en el HGMD (human genome mutation database). En la taula següent es mostra l'edat, sexe i mutació genètica de cadascun d'ells:

Patient	Age / sex	Disease (OMIM) *Gene(OMIM)*
1	9y / F	KEARNS-SAYRE SYNDROME (#530000) mtDNA deletion
2	4y / F	Mitochondrial DNA depletion syndrome 4A (Alpers type) (#203700) *POLG* (*174763)
3	7y / M	KEARNS-SAYRE SYNDROME (#530000). mtDNA deletion
4	3y / M	Leigh syndrome (#256000). *MTTV* (*590105)
5	3y / F	Leigh syndrome (#256000). *MTATP6* (*516060)
6	12y / M	KEARNS-SAYRE SYNDROME (#530000). mtDNA deletion
7	22y / M	KEARNS-SAYRE SYNDROME (#530000). mtDNA deletion
8	34y / F	KEARNS-SAYRE SYNDROME (#530000). mtDNA deletion
9	1y / F	Multiple mitochondrial dysfunctions syndrome 1 (#605711). *NFU1* (*608100)
10	8y / M	Leigh syndrome (#256000)/NARP SYNDROME MTATP6 (*516060)
11	16y / M	Thiamine metabolism dysfunction syndrome (#607483). *SLC19A3* (*606152)
12	11y / M	MERRF syndrome (#545000). *MTTK* (*590060)
13	13y / M	CARDIOMYOPATHY WITH SKELETAL MYOPATHY. *MTTL1* (*590050)
14	2mo / F	Fatal infantile lactic acidosis. *KARS* (*601421)
15	3mo / F	Combined oxidative phosphorylation deficiency 1 (#609060). *GFM1*(*606639)
16	1y / M	Mitochondrial DNA depletion syndrome 4A (Alpers type)(#203700) *POLG* (*174763)
17	1y / F	Mitochondrial DNA depletion syndrome 5 (encephalomyopathic with methylmalonic aciduria) (#612073). *SUCLA2* (*603921)
18	5mo / M	Mitochondrial complex I deficiency (#252010) / Cockayne syndrome, type A (#216400). *NDUFAF2*(*609653) / *ERCC8* (*649412)
19	2mo / F	Leigh syndrome (#256000). *MTATP6* (*516060)
20	1y / M	Thiamine metabolism dysfunction syndrome (#607483). *SLC19A3* (*606152)

21	11mo/ F	Epileptic encephalopathy. 5q14.3 deletion (*612881)
22	7y / M	Pyruvate dehydrogenase E1-alpha deficiency (#312170). *PDHA1* (*300502)
23	1y / M	PEARSON MARROW-PANCREAS SYNDROME (#557000). mtDNA deletion
24	13y / F	MELAS SYNDROME (#540000). *MTTL1* (*590050)
25	7y / F	Pyruvate dehydrogenase E1-alpha deficiency (#312170). *PDHA1* (*300502)
26	13y / F	Leigh syndrome(#256000)/NARP SYNDROME. MTATP6 (*516060)
27	10y / M	Pyruvate dehydrogenase E1-alpha deficiency (#312170). *PDHA1* (*300502)
28	13y / F	Mitochondrial myopathy. *MTTA* (*590000)
29	11y /M	Pyruvate dehydrogenase E1-alpha deficiency (#312170). *PDHA1* (*300502)

Tanmateix, a tots els pacients se'ls han estudiat els neurotransmissors i el 5MTHF en LCR en el moment del diagnòstic. Les dades genètiques, bioquímiques, clíniques i radiològiques dels 29 pacients estan recollides en el tercer article d'aquesta tesi.

- Mostres: es van recollir mostres de sang, orina i LCR i, quan procedia, es van fer biòpsies de pell/múscul per poder establir el diagnòstic genètic.

Les històries clíniques dels pacients procedents de l'HSJD van ser revisades i es va establir l'edat d'inici, el fenotip relacionat amb els gens i els signes i símptomes que determinen una neurotransmissió alterada: (1) deficiència de dopamina: parkinsonisme-distonia, discinèsia, hipotonia axial/ crisi oculogírica, ptosis, hipersalivació. (2) deficiència de serotonina: insomni, depressió, inestabilitat de la temperatura, motilitat gastrointestinal anormal. Aquesta classificació es va fer amb l'ajut d'un neuropediatre.

Per a l'exploració neuroradiològica es van realitzar estudis d'imatge i d'espectroscòpia de ressonància magnètica, classificant als pacients en 3 grups: (1) resultats normals; (2) trastorns de ganglis basals (la majoria es presenten com a síndrome de Leigh); (3) altres característiques (lesions en la substància blanca i cos callós i lesions isquèmiques). Aquesta classificació es va fer amb l'ajut d'un neuropediatre.

1.5 Aspectes ètics

L'estudi de pacients i controls es va realitzar d'acord amb la Declaració d'Helsinki de 1964, revisada l'any 2013. Tant pels estudis bioquímics com genètics es va sol·licitar el consentiment informat dels pacients o dels tutors legals. El comitè d'ètica de l'Hospital

Sant Joan de Déu i, quan pertocava, dels altres hospitals participants, van aprovar els estudis realitzats.

2. Mètodes analítics

2.1 Mètodes analítics per l'estudi de la contaminació hemàtica

a) Obtenció de la mostra. Criteris d'exclusió.

Hi ha múltiples factors que influeixen en la concentració de neurotransmissors en LCR i que han de ser controlats. Per aquest motiu, l'obtenció de les mostres de LCR es va realitzar seguint un protocol estandarditzat (Ormazabal et al. 2005).

La punció lumbar s'ha de realitzar de 7 h a 9 h del matí després de 8-10 hores de dejú del pacient. En pediatria s'estableix un dejú fisiològic (temps en què un nen pot estar sense menjar) de 3 a 4 hores en nounats, de 5 a 7 hores en lactants i de 8 hores en nens més grans. Si el pacient rep alguna medicació, aquesta serà administrada després de la punció lumbar.

Si, a part de LCR, s'ha d'extreure també sang, aquesta s'ha d'extreure uns 30 minuts abans de fer la punció lumbar per evitar la hiperglucèmia postpunció.

En el LCR existeix un gradient rostrocaudal Per tant, és important seguir un ordre a l'hora de recollir la mostra en els diferents tubs per tal que els resultats seguin comparables:

Primer tub (5 gotes): bioquímica (proteïnes, glucosa, hematies, leucòcits i lactat).

Segon tub (10 gotes): amines biògenes (l'HVA i el 5HIAA representen respectivament el metabolisme de la dopamina i la serotonina) i tiamina.

Tercer tub (10 gotes): pterines, piridoxal fosfat i 5-metiltetrahidrofolat (5MTHF). Important recobrir el tub amb paper d'alumini perquè les pterines s'oxiden ràpidament).

Quart tub (10 gotes): aminoàcids i GABA.

Un cop obtinguda la mostra, els tubs s'introdueixen en gel i són immediatament traslladats al laboratori per ser emmagatzemats a -80 °C fins al moment d'analitzar-los. Les mostres de LCR que estiguin contaminades de sang per punció traumàtica, se centrifuguen i se separa el sobrenedant abans de congelar-les. Com que les mostres de LCR es consideren mostres de difícil obtenció i de gran valor diagnòstic, les puncions

hemàtiques s'aprofiten per a aquesta finalitat i només es demana nova mostra si els resultats no es poden interpretar. Si la contaminació hemàtica és franca, es recomana repetir la prova al cap d'uns dies. En qualsevol cas, per aquesta tesi, s'han descartat totes les puncions hemàtiques o no recollides segons el protocol esmentat.

b) Anàlisis automatitzades en LCR (glucosa, proteïnes, làctic i albúmina)

La determinació de glucosa, proteïnes, àcid làctic i albúmina en LCR, es van realitzar per espectrofotometria en l'analitzador automàtic Arquitect C8000 (Abbott Laboratories, IL, USA).

c) Anàlisi de neurotransmissors, pterines i piridoxal fosfat en LCR.

La metodologia d'anàlisi de neurotransmissors (cromatografia líquida d'alta resolució (HPLC) amb detector electroquímic), pterines (HPLC amb detector electroquímic i de fluorescència) i piridoxal fosfat (HPLC amb detector de fluorescència) estan descrits de forma exhaustiva en el segon treball d'aquesta tesi. Per aquest motiu la seva metodologia no es detalla aquí sinó que es descriu en l'article.

d) Anàlisi d'aminoàcids, folat i tiamina en LCR

- **Anàlisi d'aminoàcids**

L'anàlisi d'aminoàcids en LCR es va realitzar per cromatografia líquida amb detector espectrofotométric de masses en tàndem (UPLC-MS/MS) utilitzant el kit de derivatització AccQ-Tag Derivatization Kit (Waters ref. 186003836) i fent la separació cromatogràfica en una columna CORTECS C18 2.1 × 150, 1.6 µm (Waters). Aquest mètode ha estat publicat pel nostre grup (Casado M et al., 2017. Metabolomics veure annex 2)

- **Anàlisi de 5MTHF**

La determinació de 5MTHF en LCR es va realitzar per cromatografia líquida en fase reversa amb detector de fluorescència (Waters, MA, USA) utilitzant una longitud d'ona excitació i emissió de 295 i 355 nm, respectivament i fent la separació cromatogràfica en una columna Nucleosil 120 C18 de 5 µm 15x0.4 (Teknokroma). El mètode ha estat publicat pel nostre grup (Ormazabal A et al., 2006).

- **Anàlisi de tiamina**

L'anàlisi de tiamina en LCR es va realitzar per HPLC amb detector de fluorescència (Waters, MA, USA), derivatitzant (ferricianur potàssic 10 mM en NaOH al 15%) i columna cromatogràfica Nucleosil 100 C18 5 µm, 25 x 0.4 mm (Teknokroma) (Ortigoza-Escobar JD & Molero-Luis M et al., 2016)

2.2 Mètodes analítics del protocol d'anàlisi dels neurotransmissors

El segon article de la tesi és un article purament metodològic i és allà on es detalla profundament i exhaustiva la metodologia de l'anàlisi dels neurotransmissors en LCR.

2.3 Mètodes per a l'anàlisi de la melatonina en orina

L'anàlisi de l'aMT6s (metabòlit de la melatonina) en orina es va realitzar per duplicat mitjançant un kit d'ELISA competitiu (IBL; ref RE54021) i la densitat òptica es va mesurar amb un fotòmetre (ATOM S.A. Barcelona, Spain) a 450 nm i a 630 nm. La concentració de creatinina es va determinar a l'analitzador Arquitect C8000 (Abbott Laboratories, IL, USA) per espectrofotometria automatitzada. Els resultats es van expressar en µmol aMT6s/mol creatinina.

Per determinar l'aMT6s en orina prèviament es van diluir les mostres d'orina, els calibradors i els controls en tampó d'assaig (1:50) i es va preparar el tampó de rentat (dilució en aigua (1:20)).

Procediment analític:

- Carregar a la placa d'ELISA 50 µL de les dilucions de les mostres, els calibradors i els controls.
- Preparar l'enzim conjugat (dilució 1:41 en tampó d'assaig) i pipetejar-ne 50 µL a cada pou de la placa d'ELISA.
- Pipetejar 50 µL de l'antisèrum de sulfat de melatonina.
- Cobrir la placa amb un adhesiu protector i incubar 2 hores a temperatura ambient i en agitació suau.
- Descartar el líquid dels pouets i fer 4 rentats de la placa amb el tampó de rentat.
- Afegir 100 µL de substrat a cada pouet i incubar 30 minuts a temperatura ambient i en agitació suau.

- Aturar la reacció afegint 100 µL de la solució de STOP.
- Mesurar la densitat òptica a 450 nm i a 630 nm.

a) Estudis preanalítics:

- Per estudiar els canvis d'excreció urinària de la melatonina relacionats amb el temps de mostreig, es van analitzar les concentracions de l'aMT6s en mostres d'orina de primera i segona micció del matí en 10 persones sanes (interval d'edat: 4-33 anys).
- Per estudiar les variacions intraindividuals de l'excreció de l'aMT6s urinari, es va analitzar la seva concentració en tres voluntaris sans durant 7 dies consecutius.
- Per avaluar la imprecisió de l'assaig (intraassaig i interassaig), es van calcular els coeficients de variació (CV = desviació estàndard / valor mitjà aMT6s * 100) en 10 replicats.

Després d'aquests estudis metrològics, es van establir els valors de referència utilitzant l'orina de primera micció del matí, tal com havien fet altres grups prèviament descrits en la literatura.

Els resultats es refereixen a la creatinina per evitar errors de dilució/concentració de la mostra i per tal que els resultats puguin ser comparables entres sí. S'entreguen com a µmol aMT6s/mol creatinina.

b) Avaluació clínica dels pacients

Els pacients van ser avaluats al servei de Neurologia de l'HSJD. Els responsables d'aquestes avaluacions van ser neuropediatres experts en aquestes malalties.

Les avaluacions van iniciar-se per una anamnesi seguida d'una exploració clínica, principalment enfocada a l'estat neurològic del pacient. Les dades clíniques enregistrades per dur a terme l'estudi van ser l'estudi de neuroimatge per ressonància magnètica (RM) amb les seves múltiples opcions (T1, T2, Flair, difusió de masses i espectroscòpia quan va ser procedent) per mitjà d'un aparell d'1-5 Tesles (Signa EchoSpeed System, GE Medical Systems).

Amb totes aquestes dades i amb l'anàlisi de la història clínica de tots els pacients es va poder dirigir millor l'estudi genètic i arribar així al seu diagnòstic.

2.4 Estudis moleculars pel diagnòstic genètic de malalties mitocondrials

Els pacients mitocondrials s'han anat reclutant al llarg de dotze anys i, per aquest motiu, s'han utilitzat diferents aproximacions clíniques i tècniques per establir el diagnòstic genètic. Les mutacions de l'ADN mitocondrial (mtDNA) es van estudiar mitjançant procediments específics prèviament descrits (O'Callaghan MM et al., 2015; Montiel-Sosa JF et al., 2013) tals com, Sanger, Southern-blot i PCR a temps real. L'estudi de les mutacions de l'ADN nuclear es va realitzar per Sanger, en funció de quin gen dirigia la sospita diagnòstica o, en els últims 4 anys, per seqüenciació de nova generació (NGS), mitjançant panells personalitzats (Yubero D et al., 2016) o comercials (panell de seqüenciació TruSight One, Illumina) en els seqüenciadors MiSeq o NextSeq500 (Illumina). Es van realitzar estudis de progenitors per avaluar el model d'herència i establir el diagnòstic molecular.

3. Mètodes estadístics

3.1. Validació dels procediments analítics i valors de referència

Els diferents procediments analítics utilitzats en aquesta tesi van ser validats i estandarditzats segons els requeriments de garantia de la qualitat del laboratori. Els diferents procediments, així com els valors de referència establerts per cada metodologia, han estat publicats en diverses revistes científiques (Ormazabal A et al., 2005, 2006 i 2008, Moyano D et al., 1998, Casado M et al., 2017). Tanmateix, totes les proves metodològiques esmentades a les seccions anteriors han estat certificades per AENOR (Associació Espanyola de Normalització i Certificació). Les anàlisis de neurotransmissors, pterines, 5MTHF i aminoàcids han estat acreditades per ENAC (Entitat Nacional d'Acreditació) seguint els criteris de les normes ISO 15189. Aquesta acreditació suposa el màxim grau de competència tècnica dels laboratoris de la nostra regió.

3.2. Anàlisi estadística

Totes les variables clíniques i bioquímiques han estat recollides en una base de dades (Microsoft Excel).

Pels estudis estadístics s'han aplicat bàsicament les proves següents:

- Per a l'estudi de distribució de dades: prova de Kolmogorov-Smirnov.

- Per a la comparació de variables qualitatives: prova de Chi-quadrat.
- Per a la comparació de variables quantitatives:

  Variables paramètriques:

  - Prova T de Student per a la comparació de mitjanes.

  Variables no paramètriques:

  - Prova de Wilcoxon per a dades aparellades.
  - Prova U de Mann-Whitney per a dades no aparellades.
- Proves de correlació simple:

  - Prova de Pearson per a variables paramètriques.
  - Prova de Spearman per a variables no paramètriques.

Els detalls de cada una d'aquestes proves figuren en cada un dels articles publicats.
Els càlculs estadístics es van realitzar amb el programa SPSS 23.0. Es va considerar significatiu un valor de $p<0.05$.

RESULTATS

INFORME DE LES DIRECTORES

La memòria de la Tesi Doctoral "Investigació en biomarcadors de l'estat de la dopamina i la serotonina en pacients neuropediàtrics" presentada per Marta Batllori Tragant, es presenta com un compendi de 4 publicacions. La doctoranda Marta Batllori ha participat de forma activa en la part experimental, en la interpretació de dades i en la redacció de tots els articles derivats d'aquesta tesi. Totes les publicacions que consten aquí formen part del nucli de la present tesi, i no seran utilitzades en altres tesis doctorals.

PUBLICACIÓ 1. *Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins*. Batllori M, Casado M, Sierra C, Salgado MDC, Marti-Sanchez L, Maynou J, Fernandez G, Garcia-Cazorla A, Ormazabal A, Molero-Luis M, Artuch R. Fluids Barriers CNS. 2019 Nov 14;16(1):34.

La doctoranda, Marta Batllori, ha contribuït en la recollida i preparació de les mostres tenint en compte les diferents condicions preestablertes en el protocol per poder realitzar tota la part experimental, ha contribuït en la ideació del protocol de contaminació de mostres i posteriorment ha participat en la interpretació dels resultats i en l'escriptura del manuscrit.

PUBLICACIÓ 2. *Analysis of human cerebrospinal fluid monoamines and their cofactors by HPLC*. Batllori M, Molero-Luis M, Ormazabal A, Casado M, Sierra C, García-Cazorla A, Kurian M, Pope S, Heales SJ, Artuch R. Nat Protoc. 2017 Nov;12(11):2359-2375.

La doctoranda ha participat en la recopilació d'informació, cromatogrames i elaboració de taules i figures del protocol i ha participat en l'escriptura de tots els protocols descrits en el manuscrit.

PUBLICACIÓ 3. *Urinary sulphatoxymelatonin as a biomarker of serotonin status in biogenic amine-deficient patients*. Batllori M, Molero-Luis M, Arrabal L, Heras JL, Fernandez-Ramos JA, Gutiérrez-Solana LG, Ibáñez-Micó S, Domingo R, Campistol J,

Ormazabal A, Sedel F, Opladen T, Zouvelou B, Pons R, Garcia-Cazorla A, Lopez-Laso E, Artuch R. Sci Rep. 2017 Nov 7;7(1):14675.

La doctoranda ha estat responsable de part del treball experimental. Comparteix coautoria amb la segona signant que és codirectora d'aquesta tesi. Ha contribuït en la fase de reclutament de pacients de centres externs, ha estat responsable dels estudis preanalítics del treball i de la part experimental. Ha participat en la posada a punt i la validació de la tècnica i en l'anàlisi estadístic per a l'establiment de valors de referència. Ha intervingut en la interpretació dels resultats, la realització de taules i figures i en la redacció del manuscrit.

PUBLICACIÓ 4. *Cerebrospinal fluid monoamines, pterins, and folate in patients with mitochondrial diseases: systematic review and hospital experience*. Batllori M, Molero-Luis M, Ormazabal A, Montero R, Sierra C, Ribes A, Montoya J, Ruiz-Pesini E, O'Callaghan M, Pias L, Nascimento A, Palau F, Armstrong J, Yubero D, Ortigoza-Escobar JD, García-Cazorla A, Artuch R. J Inherit Metab Dis. 2018 Nov;41(6):1147-1158.

La doctoranda ha participat en l'elaboració de la base de dades de pacients i ha establert el contacte amb els neuropediatres implicats en aquesta revisió. Ha participat en l'anàlisi estadística i ha realitzat les taules i figures del treball participant activament en la redacció del manuscrit.

Aida Ormazabal Marta Molero

Esplugues de Llobregat, 18 de desembre de 2020

OBJECTIU 1: Avaluació de la influència de la contaminació hemàtica en mostres de líquid cefalorraquidi a l'hora de realitzar estudis metabolòmics dirigits.

PUBLICACIÓ 1:

Títol: Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins

Autors: Marta Batllori, Mercedes Casado, Cristina Sierra, Marta Molero, Maria del Carmen Salgado, Laura Martí, Joan Maynou, Guerau Fernandez, Angels Garcia-Cazorla, Aida Ormazabal, Rafael Artuch.

Referència: *Fluids Barriers CNS*. 2019 Nov 14;16(1):34.

Resum:

La investigació dels defectes de la neurotransmissió es fonamenta en l'anàlisi bioquímica del LCR. Tot i que aquest tipus de mostra biològica, a priori, és de fàcil maneig perquè presenta poques interferències en comparació amb altres tipus de fluids (com el plasma o l'orina), el principal problema que pot presentar és la contaminació hemàtica deguda a puncions traumàtiques, hemorràgia intraventricular o alteracions en la permeabilitat de la barrera hematoencefàlica.

La mostra de LCR s'obté mitjançant una punció lumbar, una prova invasiva que ha de realitzar personal especialitzat i seguint un estricte protocol estandarditzat. Està descrit que la contaminació hemàtica del LCR té efectes sobre la concentració de glucosa i de proteïnes, però no hi ha estudis on es descrigui l'efecte en altres biomarcadors que s'analitzen en LCR per l'estudi de patologies neurometabòliques. En aquest context, l'objectiu principal d'aquest treball ha estat avaluar fins a quin punt la contaminació hemàtica pot afectar a la interpretació dels diferents perfils bioquímics tenint en compte que els metabòlits d'estudi analitzats tenen concentracions en sang molt diferents respecte al LCR.

Aquests biomarcadors són els aminoàcids, metabòlits de la dopamina i la serotonina (HVA i 5-HIAA), metabòlits de la tetrahidrobiopterina (biopterina i neopterina) i cofactors vitamínics (5-metiltetrahidrofolat, vitamina B_6 i tiamina).

Síntesi de resultats:

A les mostres de LCR contaminades amb la màxima concentració de sang utilitzada (20%, dilució 1:5) l'aspartat, el glutamat, la taurina, l'ornitina, la glicina i la citrulina van tenir valors més alts quan es lisaven els eritròcits (condició A) respecte a quan se centrifugava i separava el sobrenedant abans de la congelació (condició B). El PLP, 5-MTHF i tiamina també van seguir aquesta tendència. Contràriament, l'arginina, el 5-HIAA i l'HVA van tenir valors més baixos quan es lisaven els eritròcits en les mostres de LCR, mentre que la resta de metabòlits estudiats van tenir concentracions similars en les dues condicions. La dilució 1:40 de la condició A (eritròcits lisats) va mostrar percentatges de variació moderadament superiors al 10% per a la majoria dels metabòlits. Tanmateix, en la condició B (eritròcits eliminats del LCR), només alguns aminoàcids, la tiamina i el PLP van tenir valors superiors al 10% en comparació amb les concentracions de la mostra de LCR no contaminada. Com era d'esperar, l'albúmina va augmentar en funció del grau de contaminació en les dues condicions. Respecte, a la tiamina difosfat, una forma intracel·lular de tiamina, només es va incrementar en les mostres de la condició A (eritròcits lisats). Mentre que la majoria de metabòlits van mostrar augments en les concentracions quan s'afegia sang a les mostres de LCR, l'arginina, el 5-HIAA i l'HVA van disminuir. Les concentracions de pterines i glutamina no van variar en les diferents condicions de contaminació.

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Fluids and Barriers of the CNS

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Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins

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Abstract

Background: Cerebrospinal fluid (CSF) metabolomic investigations are a powerful tool for studying neurometabolic diseases. We aimed to assess the effect of CSF contamination with blood on the concentrations of selected biomarkers.

Methods: CSF samples were spiked in duplicate with increasing volumes of whole blood under two conditions: (A) pooled CSF spiked with fresh whole blood and frozen to cause red blood cell (RBC) lysis; (B) pooled CSF spiked with fresh blood and centrifuged (the supernatant with no RBCs was frozen until the moment of analysis). CSF concentrations of amino acids, biogenic amines, pterins, and vitamins were analysed by HPLC coupled with tandem mass spectrometry, electrochemical and fluorescence detection.

Results: Aspartate, glutamate, taurine, ornithine, glycine, citrulline, pyridoxal 5´-phosphate, 5-methyltetrahydrofolate, and thiamine showed higher values when RBCs were lysed when compared with those of CSF with no RBC, while arginine, 5-hydroxyindoleacetic and homovanillic acids showed lower values. When RBCs were removed from CSF, only some amino acids, thiamine and pyridoxal 5´-phosphate showed moderately higher values when compared with the non-spiked CSF sample.

Conclusions: CSF-targeted metabolomic analysis is feasible even when substantial RBC contamination of CSF has occurred since CSF centrifugation to remove RBC prior to freezing eliminated most of the interferences observed.

Keywords: Cerebrospinal fluid, Amino acids, Biogenic amines, Pterins, Vitamins, Blood contamination

Background

Cerebrospinal fluid (CSF) is a biological fluid that is mainly produced by the choroid plexus, which constitutes the interface between blood vessels and CSF [1, 2]. The composition of CSF is also controlled by the blood–brain barrier, which separates blood from the brain parenchyma [3]. Both structures deliver substrates for brain cell metabolism and remove the corresponding waste [1, 4, 5]. In general, the blood–brain barrier greatly restricts the influx of most molecules, including amino acids and other compounds [6]. Amino acids, with few exceptions (e.g., glutamine), show lower values in CSF when compared with those of plasma [7, 8]. For other metabolites, biosynthetic pathways are compartmentalized in the brain, and similar concentrations may be observed in CSF and blood since no transport from blood to CSF is expected; this is the case for biogenic amines and pterins [9, 10]. In contrast, some vitamins have to be transported into the brain through central nervous system barriers by specific transporters, and differences between vitamin concentrations in CSF and blood samples are noticeable [11, 12]. While folate is one of the few molecules more concentrated in CSF when compared to plasma, other

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vitamins such as thiamine and pyridoxine display lower values in CSF when compared to those of blood [12, 13].

CSF metabolomic investigations have been demonstrated to be a powerful tool for studying specific neurometabolic pathways and related diseases and for exploring metabolic transport from the blood into the brain [14]. Several neurogenetic conditions are caused by specific disturbances in these processes (Table 1). In recent decades, targeted metabolomic approaches have been used for the study of these neurogenetic conditions [14]. Owing to the important differences in the metabolite concentrations between blood and CSF, contamination of CSF with blood may cause dramatic effects in the measured concentrations of most of the above-mentioned metabolites [15–18]. CSF is collected by lumbar puncture, which is an invasive method. Since blood/plasma contamination can be frequently observed by different causes (traumatic lumbar punctures, impaired blood–brain barrier permeability or intraventricular bleeding) [19–21], a misinterpretation of metabolic profiles is a problem that should be minimized to avoid repeated lumbar puncture procedures and diagnostic errors.

Table 1 CSF biomarkers and their semiological value for different diseases (left columns)

Biomarkers	Diseases and expected value: high or low (↑/↓)	A). Blood/CSF ratio mean value of CSF pools 1 and 2 (standard error of the mean)	B). Plasma/CSF ratio mean value of CSF pools 1 and 2 (standard error of the mean)
Albumin		28 (3.9)	29 (3.2)
Amino acids			
Taurine	–	4.9 (0.2)	2.9 (0.5)
Aspartate	–	15 (8.2)	1.3 (0.07)
Threonine	–	1.9 (0.1)	1.7 (0.2)
Serine	Serine deficiency (↓)	1.6 (0.1)	1.3 (0.1)
Glutamate	–	7.3 (1.7)	1.6 (0.1)
Glutamine	Hyperamoniemias (↑)	1.05 (0.03)	1.1 (0.1)
Glycine	Hyperglycinemias (↑)	3.2 (0.06)	2.2 (0.1)
Alanine	Mitochondrial Dis. (↑)	2.5 (0.3)	2.5 (0.4)
Citrulline	–	2.6 (0.2)	2.1 (0.3)
Valine	BCAA defects (↑/↓)	2.5 (0.3)	2.6 (0.5)
Methionine	–	2.4 (0.3)	2.5 (0.4)
Isoleucine	BCCA defects (↑/↓)	2.3 (0.2)	2.3 (0.3)
Leucine	BCCA defects (↑/↓)	2.3 (0.3)	2.2 (0.4)
Tyrosine	–	2.05 (0.2)	1.8 (0.2)
Phenylalanine	–	1.4 (0.1)	1.6 (0.2)
Ornithine	–	4.6 (0.6)	1.9 (0.02)
Lysine	–	2.2 (0.3)	2.2 (0.3)
Hystidine	–	1.9 (0.01)	1.8 (0.1)
Arginine	–	0.55 (0.1)	1.5 (0.1)
5-HIAA	Serotonin related (↓)	0.6 (0.04)	0.93 (0.04)
HVA	Dopamine (↑/↓)	0.78 (0.01)	0.9 (0.01)
Biopterin	Pterin defects (↑/↓)	1.0 (0.1)	0.9 (0.1)
Neopterin	Pterin defects (↑/↓) Immune events (↑)	1.1 (0.1)	1.1 (0.1)
Thiamine	Transport defects (↓)	3.0 (0.05)	2.2 (0.05)
5-MTHF	Transport/metabolism defects (↓)	1.75 (0.1)	1.0 (0.1)
PLP	Transport/metabolism defects (↓)	3.7 (0.3)	2.5 (0.3)

Blood/CSF ratios for the two experimental conditions: A) Red blood cells (RBC) lysed in CSF and B) RBC removed from CSF. CSF samples were spiked with 20% of whole blood. A total of 20 CSF aliquots coming from 50 CSF samples were analysed (see details in Additional file 2: Figure S1)

When CSF blood contamination occurs, the most critical metabolites for data interpretation can be glycine (the ratio blood/CSF glycine values is very high) and vitamins such as pyridoxine, thiamine and folate: In genetic diseases leading to brain pyridoxine, folate, and thiamine deficiencies, the blood concentrations of these vitamins can be normal, while CSF values may be near undetectable. Thus, blood contamination could mask the CSF vitamin deficiency. The monoamines HVA and 5-HIAA are sensitive to haemoglobin oxidation

BCAA Branched chain amino acids

With this background, we aimed to assess the effect of CSF contamination with blood on the concentrations of selected molecules which are biomarkers for the study of different neurometabolic conditions.

Methods

Samples

CSF samples were collected from patients where lumbar puncture was done to rule out meningoencephalitis, and stored at −80 °C, following a previously reported protocol [22]. The remnants of 50 CSF anonymized samples with no red blood cell (RBC) contamination (assessed by light microscopy as less than 5 RBC per field) were thawed, pooled (25 samples for pool 1 and the other 25 for pool 2), reaching a final volume of 10 mL for each pool. The pooled samples were divided into 1 mL aliquots, which were spiked with different volumes of whole blood (at that moment, a fresh blood sample was withdrawn from a healthy volunteer). Non-spiked CSF samples and four spiking conditions were prepared in duplicate in the 2 CSF pools. The CSF pools were spiked with increasing volumes of whole blood: 2.5%, 5%, 10%, and 20%, in 2 different conditions: (A) CSF samples spiked with fresh whole blood and then frozen at −80 °C to cause RBC lysis. (B). CSF samples spiked with fresh blood, then centrifuged at 1500×*g* 10 min at 4 °C, with the clear supernatant frozen at −80 °C. Details of the protocol are stated in Additional file 1: Table S1 and Additional file 2: Figure S1. The total sample preparation time spent was 45 min (all samples were frozen at the same time). With these conditions we could assess the effect of whole blood interference (RBC can increase metabolite concentrations in CSF and can cause oxidative/catabolic effects on some of the metabolites studied (condition A) when compared with plasma contamination (condition B), where an increase in metabolites which are more concentrated in plasma than CSF is expected).

Initially, to identify a cut-off value at which blood contamination can cause substantial interference in the measurement of the above-mentioned metabolites, CSF was spiked with whole blood volume range from 1 to 0.02%. No relevant effects were detected in most metabolite concentrations studied under these conditions (data not shown).

Methods

The concentration of albumin in CSF, used as a surrogate biomarker of CSF RBC contamination or impaired blood–brain and blood-CSF barriers, was analysed using an Abbot automated analyser (Architect c8000) by spectrophotometric procedures. Other biomarkers of blood contamination such as haemoglobin concentration were analysed by an automated procedure (Advia 2120, Siemens Diagnostics). CSF amino acids were analysed by UHPLC coupled to tandem mass spectrometry detection in a Xevo QT Waters system, as previously reported [23]. Biogenic amines (5-hydroxyindoleacetic (5-HIAA) and homovanillic (HVA) acids) and pterins (biopterin and neopterin) were analysed as biomarkers of serotonin and dopamine deficiencies (and in the case of neopterin, also as a biomarker of neuroinflammatory conditions) by HPLC with electrochemical and fluorescence detection as previously reported [22]. The vitamins thiamine, thiamine-diphosphate (TDP), 5-methyltetrahydrofolate (5-MTHF) and pyridoxal 5´-phosphate (PLP) were analysed by HPLC with fluorescence detection as reported [14, 22, 24]. Typical chromatograms of these procedures are presented in Additional file 3: Figure S2.

Data analysis

The precision of the different techniques was initially calculated using the coefficient of variation (CV = standard deviation/average × 100%) from 20 replicates and was below 10% for all of the metabolites studied, as previously reported [22–24]. Thus, we considered that the effect of blood contamination on CSF samples was not significant when it was lower than 10% when compared with the value obtained in the non-spiked CSF samples. CSF parameters studied here are accredited by the ENAC (ISO 15,189 norm) and certified by AENOR agencies (ISO 9001 norm). CSF amino acids, pterins, and biogenic amines are subjected to external quality control schemes from ERNDIM (data of the results available on request).

Ethical issues

CSF anonymized samples from remnants were collected in our Hospital following our diagnostic protocols, and the study was conducted only once such investigations were concluded. In every case, informed consent was obtained from each patient before performing the lumbar puncture and CSF collection. The Ethical committee of Sant Joan de Déu Hospital approved the study. All samples from the patients were obtained following the 2013 revised Helsinki Declaration of 1964.

Results

The absolute values of the different metabolites studied are depicted in Fig. 1. In Table 1, a list of the metabolites analysed is presented together with the related neurological diseases, the interpretation of a change in the metabolite concentrations and the mean differences in the expected concentration between blood and CSF when CSF was contaminated with 20% of blood under the 2 different experimental conditions designed: condition A: lysed RBC; condition B: removed RBC. Figure 2 is a horizontal bar representation of the

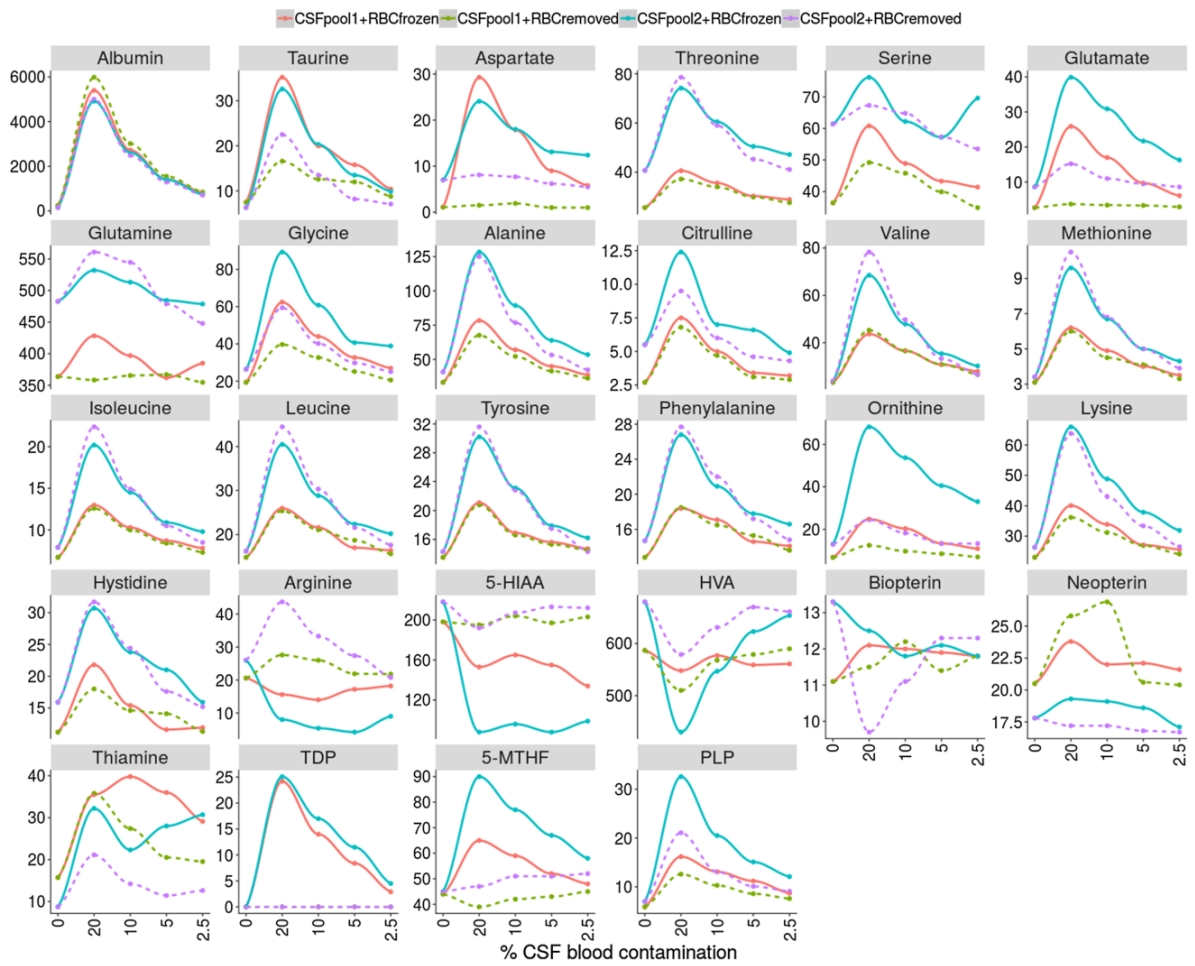

Fig. 1 Graphical representation of the concentrations of the different biomarkers analysed in non-spiked CSF samples, and in CSF samples spiked with 20%, 10%, 5% and 2.5% of whole blood, respectively. Albumin is expressed in g/L, amino acids in µmol/L and biogenic amines, pterins and vitamins in nmol/L

differences stated in Table 1. Aspartate, glutamate, taurine, ornithine, glycine, and citrulline had higher values when RBC were lysed when compared with RBC removed from CSF before freezing. PLP, 5-MTHF, and thiamine also showed this tendency. Arginine, 5-HIAA, and HVA had lower values when RBC were lysed in the CSF samples, while the rest of metabolites studied were consistent between the two different conditions.

In Table 2 and Fig. 1 data from the different spiking conditions (represented as the percentage of variation and absolute values when compared with non-spiked CSF samples, respectively) are shown. The 2.5% spiking condition A (lysed RBC) still had percentages of variation moderately higher than 10% for most metabolites. However, in the spiking condition B (RBC removed from CSF), only some amino acids, thiamine and PLP had variations higher than 10% when compared with the non-spiked CSF sample values. As expected, albumin was still highly elevated under all conditions. TDP, an intracellular form of thiamine, was only increased under the spiking condition A, but undetectable when RBC were removed from CSF by centrifugation. While most metabolites displayed changes in concentrations when RBCs were added to CSF, arginine, 5-HIAA and HVA had decreased values. Pterins and glutamine concentrations did not vary under the different spiking conditions.

Results of the surrogate biomarkers of CSF blood contamination (albumin and haemoglobin) are stated in Additional file 1: Table S1 and Additional file 2: Figure S1.

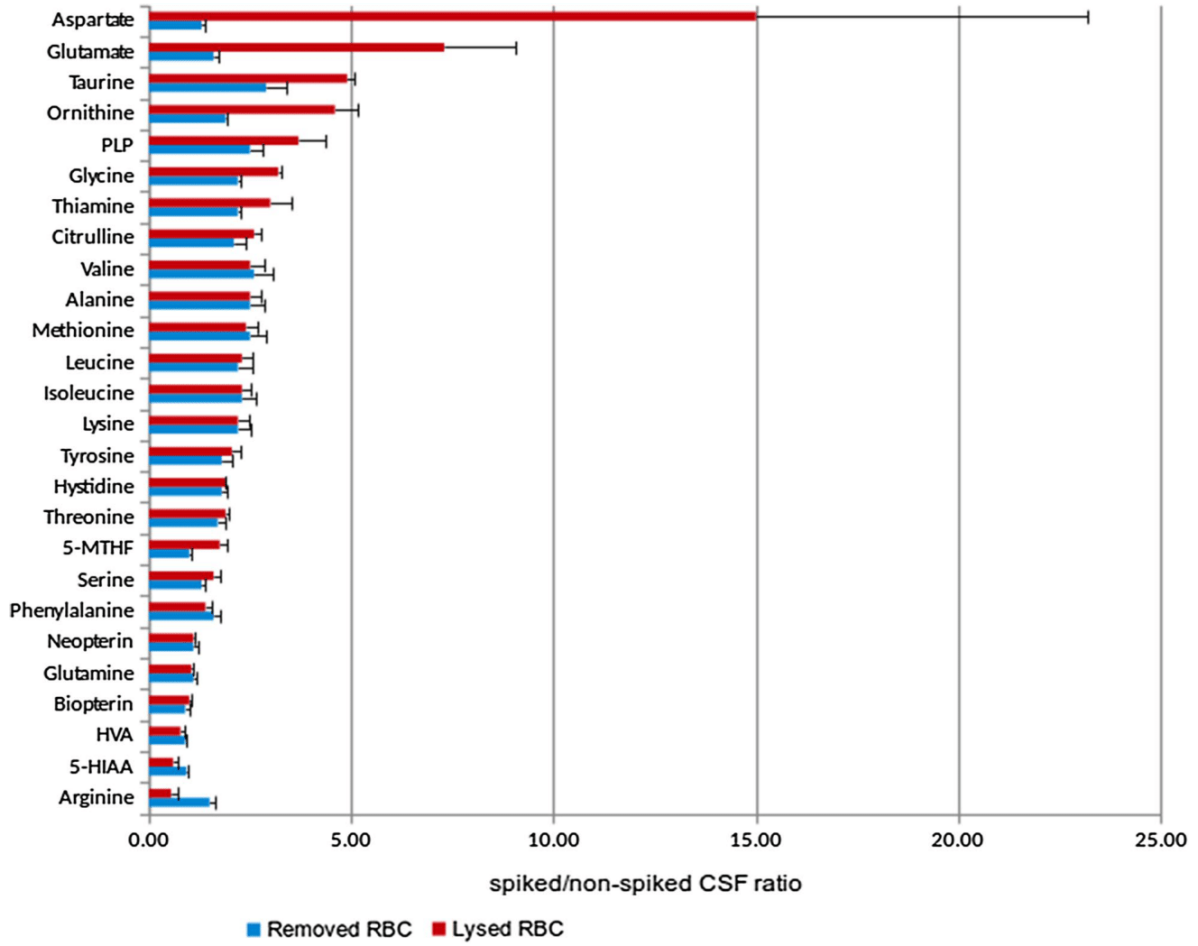

Fig. 2 Horizontal bar representation of the differences between spiked/non-spiked CSF ratio of all metabolites in the two experimental conditions. **a** Red bar: red blood cells (RBC) lysed before CSF freezing. **b** Blue bar: RBC removed from CSF by centrifugation before freezing. CSF samples were spiked with 20% of whole blood

Discussion

CSF metabolomic analysis is a good analytical tool for the study of the neurometabolic conditions stated here [14]. Furthermore, in such diseases, the quantification of these metabolites in blood/urine is not reliable, because they usually display normal or even paradoxical results [25]. This is especially true when the metabolic pathways studied are highly active in the brain, or the genetic blood–brain barrier transport diseases related to vitamins and other metabolites.

CSF RBC contamination is frequent and has been recognized as a substantial confounding factor for proper interpretation of CSF analysis data describing concentrations of amino acids and other molecules [26, 27]. However, literature regarding blood contamination effects on biogenic amines, pterins and vitamins is scarce [28]. The main causes of CSF blood contamination are traumatic lumbar punctures or spontaneous intrathecal bleeding, which can occur in several situations, especially in newborns. Moreover, impaired blood–brain barrier permeability can occur under different conditions, such as in asphyxia and epilepsy [19–21]. Thus, having an estimation of when a misinterpretation of the metabolic profile can occur due to RBC/plasma contamination is important, considering that lumbar puncture is an invasive intervention, that it is difficult to perform, and that the final volume collected is sometimes low in paediatric patients.

Albumin, a protein synthesized in the liver, is a good surrogate biomarker for compromised permeability of

Table 2 Percentage of variation of the different biomarkers measured according to the different volumes of blood spiked into the CSF (expressed as percentage) when compared with the non-spiked CSF samples

Biomarkers (% blood contamination)	A) RBC lysed				B) RBC removed			
	20%	10%	5%	2.5%	20%	10%	5%	2.5%
Albumin	2724	1372	667	323	2877	1379	669	318
Taurine	393	194	112	48	189	91	45	14
Aspartate	1404	842	401	252	42	25	< 10	< 10
Threonine	70	43	21	14	69	38	14	< 10
Serine	45	18	13	< 10	22	15	< 10	< 10
Glutamate	611	394	205	107	57	27	16	< 10
Glutamine	14	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Glycine	229	114	61	43	114	60	21	< 10
Alanine	174	95	46	23	154	72	30	11
Citrulline	151	56	22	< 10	112	42	20	< 10
Valine	138	79	40	23	161	83	36	13
Methionine	141	78	38	20	151	73	39	11
Isoleucine	125	68	34	19	136	69	29	< 10
Leucine	113	62	27	17	123	65	30	< 10
Tyrosine	84	43	20	11	87	41	19	< 10
Phenylalanine	63	38	18	11	66	39	18	< 10
Ornithine	346	256	154	107	86	41	14	< 10
Lysine	113	66	31	16	100	50	22	< 10
Hystidine	93	44	18	< 10	80	42	18	< 10
Arginine	− 47	− 56	− 51	− 39	51	27	< 10	< 10
5-HIAA	− 42	− 37	− 41	− 44	< 10	< 10	< 10	< 10
HVA	− 22	− 11	< 10	< 10	< 10	< 10	< 10	< 10
Biopterin	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Neopterin	12	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Thiamine	198	154	175	169	135	68	37	27
5-MTHF	74	52	33	19	< 10	< 10	< 10	< 10
PLP	270	157	102	60	157	80	45	29
Hemoglobin (g/dL)	3.02	1.55	0.76	0.37				
TDP (nmol/L)	25	14	11	4.5				

Data are expressed as the mean value of the 2 CSF pools from the 2 conditions: A) spiked CSF frozen containing lysed-RBC cells (left columns), and B) spiked CSF with RBC removed by centrifugation prior freezing (right columns). A percentage of variation below 10% (comparing spiked CSF samples to the non-spiked CSF samples) was considered as the cut-off value for a proper interpretation of the results, considering the coefficient of variation for every biomarker measured when procedures were standardized. Haemoglobin and TDP values are expressed in g/dL and nmol/L, respectively since their values in condition B were not detectable. A total of 20 CSF aliquots coming from 50 CSF samples were analysed (Additional file 2: Figure S1)

the blood–brain barrier and also for blood contamination. However, since its concentration in the blood largely exceeds that of the CSF (by approximately 100-fold), its concentration may remain elevated even in the case of low RBC/plasma contamination (in our hands around 1% of blood contamination; data not shown). Haemoglobin measures are an alternative surrogate marker when RBC lysis has occurred, and in our hands, values around 0.35 g/dL of haemoglobin may be a signal for cautious interpretation of the data presented here (Table 2), since it corresponds to a CSF blood contamination approximately from 2.5%, which is the limit where some metabolites may display artefactual results after haemolysis.

With regards to amino acids, several reports have indicated differences between blood and CSF compartments [8], but to our knowledge precise definitions of the limits under which RBC contamination can cause a misinterpretation of the metabolic profiles were not established. While lysed RBCs affected the concentrations of most amino acids at 2.5% of blood contamination, centrifugation of the spiked CSF samples to remove RBC resolved the problem in most cases. In any case, the amino acids that had higher values were aspartate,

glutamate, threonine, ornithine, glycine and citrulline. The explanation for this is that some of these amino acids display higher concentration in RBC when compared with plasma (aspartate, glutamate and threonine) [8]. Amongst the other amino acids, glycine has the highest plasma/CSF ratio [29]. Regarding ornithine, arginase activity is high in RBC, and this would explain our observation that arginine values were lower when RBC lysis occurred while ornithine values increased, as it is the product of the reaction catalysed by arginase [8]. Once again, centrifugation of the samples prevented all of these contamination biases. Some of the results in Table 2 were striking, however. No precise correlations with the expected values were observed for some amino acids considering the different percentages of blood contamination. A possible explanation is that some amino acids are present in CSF at very low concentrations physiologically (close to the quantification limits) and for other amino acids, the matrix effect, common in UPLC-MS/MS technology, could contribute to such differences [22].

Biogenic amines and pterins are synthesized peripherally in some tissues but also in the brain, and no substantial transport has been documented between blood and brain (only OAT3 transporters efflux both 5-HIAA and HVA from CSF to blood [2]). Since their concentrations in blood are similar to those of CSF [9, 10], no significant changes were observed after CSF blood contamination. Interestingly, both 5-HIAA and HVA had lower values when RBC lysis was caused. Autoxidation of these molecules by haemoglobin/free radicals is a potential mechanism explaining this observation [30], and thus, one should be cautious when interpreting data when CSF has not been centrifuged prior freezing, since low 5-HIAA and HVA values are surrogate biomarkers of serotonin and dopamine deficiencies and may be an indication of therapeutic intervention [31]. In any case, centrifugation and RBC removal prior to freezing corrected the results when compared with non-spiked CSF.

Vitamins displayed unpredictable results, except for folate. Folate forms (especially 5-MTHF) are highly concentrated in RBC when compared with plasma and this would explain the positive interference observed when RBC lysis occurred, but not after RBC removal. Only thiamine and PLP had increased values when comparing spiked CSF samples at 2.5% with non-spiked samples under both experimental conditions, although it was less remarkable when RBC removal was performed. These effects were minimized when the CSF blood contamination was 1% (data not shown). Regarding thiamine, active conversion of free thiamine, TMP and TDP occur inside cells (RBCs have a high activity of either thiamine phosphokinase, which phosphorylates thiamine to form TDP, or thiamine phosphatases, which convert TDP to TMP and thiamine) [32]. This would explain the plateau results observed when RBC lysis occurred, results that were minimized when RBCs were removed. In any case, thiamine values are higher in blood than in CSF, and thus, the results should be cautiously interpreted when RBC contamination occurs [12]. TDP, a strictly intracellular thiamine vitamer [32], would be a good surrogate biomarker of RBC lysis in CSF samples since undetectable amounts of TDP were observed when RBCs were removed from CSF. With PLP, the observations were similar, and although less significant, even when RBCs were removed from CSF, PLP displayed higher concentrations in the spiked CSF samples. As with thiamine, a complex intracellular metabolic pathway accounts for the synthesis of the different pyridoxine-related vitamers [13]. Moreover, some of these vitamins can be degraded by nucleophiles and oxygen-derived free-radicals, as CSF has low concentrations of other molecules that can react with these compounds [13]. Thus, results should be analysed cautiously concerning to these two vitamins, since either in thiamine or pyridoxine related disorders, which cause severe neurological phenotypes, diagnostic hallmarks are low CSF thiamine and PLP values [12, 13, 33].

Conclusions

CSF-targeted metabolomic analysis is feasible even when remarkable RBC CSF contamination occurs since CSF centrifugation to remove RBC prior to freezing prevents most of the biases observed. However, data should be cautiously interpreted, especially for some metabolites. CSF albumin, haemoglobin, and TDP can be used as surrogate biomarkers of the potential confounding effect of CSF plasma/RBC contamination.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12987-019-0154-5.

Additional file 1: Table S1. Percentage of blood contamination, albumin and haemoglobin levels from CSF spiked with increasing amounts of blood.

Additional file 2: Figure S1. CSF blood spiking protocol. A total of 20 CSF aliquots were analysed. In the picture, the colours of the 5 CSF sample are presented. Even in the 2.5% spiking condition, the red colour was intense. The median CSF blood contamination observed in our laboratory typically ranged from 0.01 to 0.035 g/dL of a haemoglobine, which is lower than the 0.35 g/dL observed in the 2.5% blood contamination condition.

Additional file 3: Figure S2. Typical chromatograms of the different metabolites analysed in non-spiked CSF samples: (1) Amino acids. (2) Biogenic amines. (3) Pterins. (4) 5-methyltetrahydrofolate. (5) Pyridoxal 5´-phosphate. (6) Thiamine.

Abbreviations

CSF: cerebrospinal fluid; RBC: red blood cells; UHPLC: ultra-high-performance liquid chromatography; 5-HIAA: 5-hydroxyindoleacetic acid; HVA: homovanillic acid; HPLC: high-performance liquid chromatography; TDP:

thiamine-diphosphate; 5-MTHF: 5-methyltetrahydrofolate; PLP: pyridoxal 5´-phosphate; BCAA: branched-chain amino acids.

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Authors' contributions
MB contributed to conception and design, acquisition, analysis and interpretation of data, drafted the initial manuscript and approved the final manuscript as submitted. MC, CS, MCS, LMS, JM, GF, AGC and AO contributed to analysis and acquisition of data, reviewed and revised the manuscript, and approved the final manuscript as submitted. MML and RA contributed to conception and design, analysis and interpretation of data, reviewed and supervised the manuscript, and approved the final manuscript as submitted. Every one of the authors has participated sufficiently in the study, meeting the appropriate authorship criteria, and each has seen, reviewed and approved this version of the manuscript and takes full responsibility for it. We all agree to its submission for publication. Nobody who qualifies for authorship has been omitted from the list of authors. All the authors have complete access to the study data. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Informed consent was obtained from each patient before performing the lumbar puncture and CSF collection or genetic study. The Ethical committee of Sant Joan de Déu Hospital approved the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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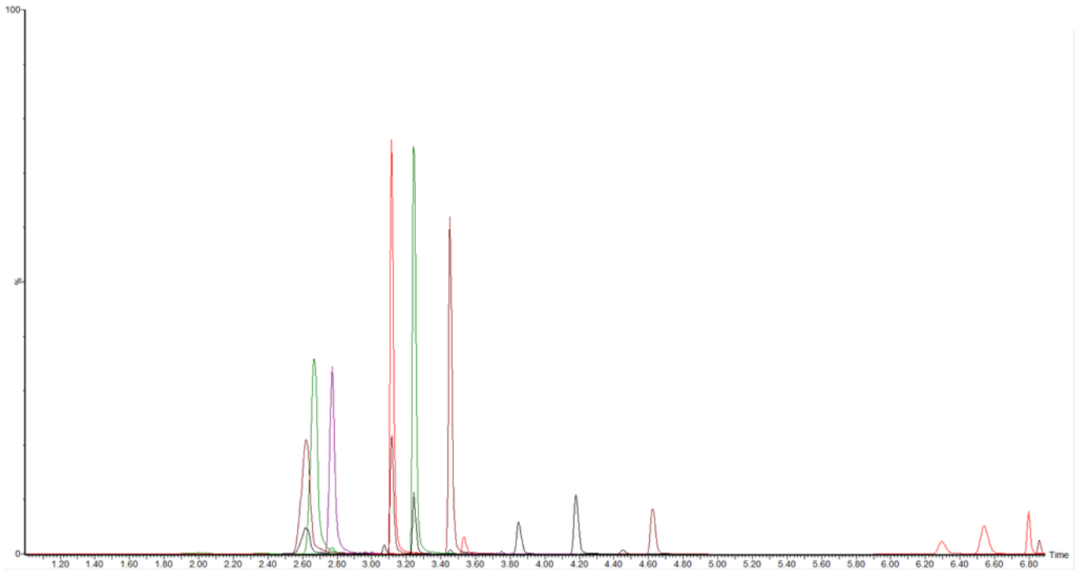
Publisher's Note

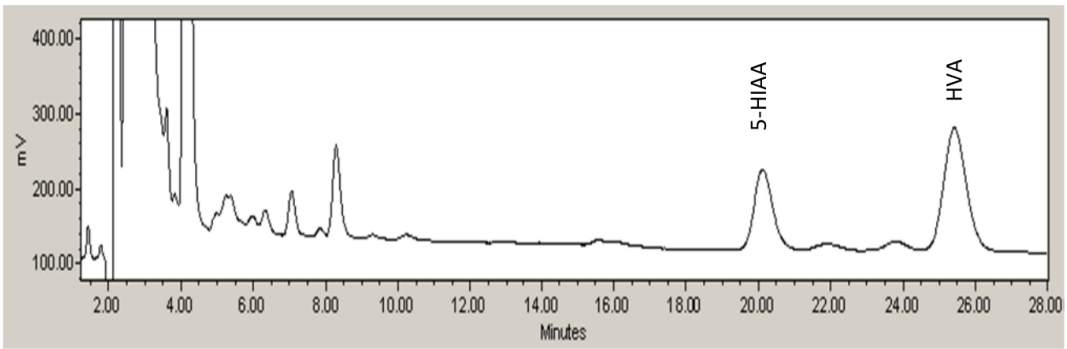
Material suplementari

Supplementary figure 1

1) Amino acid



2) Biogenic amines



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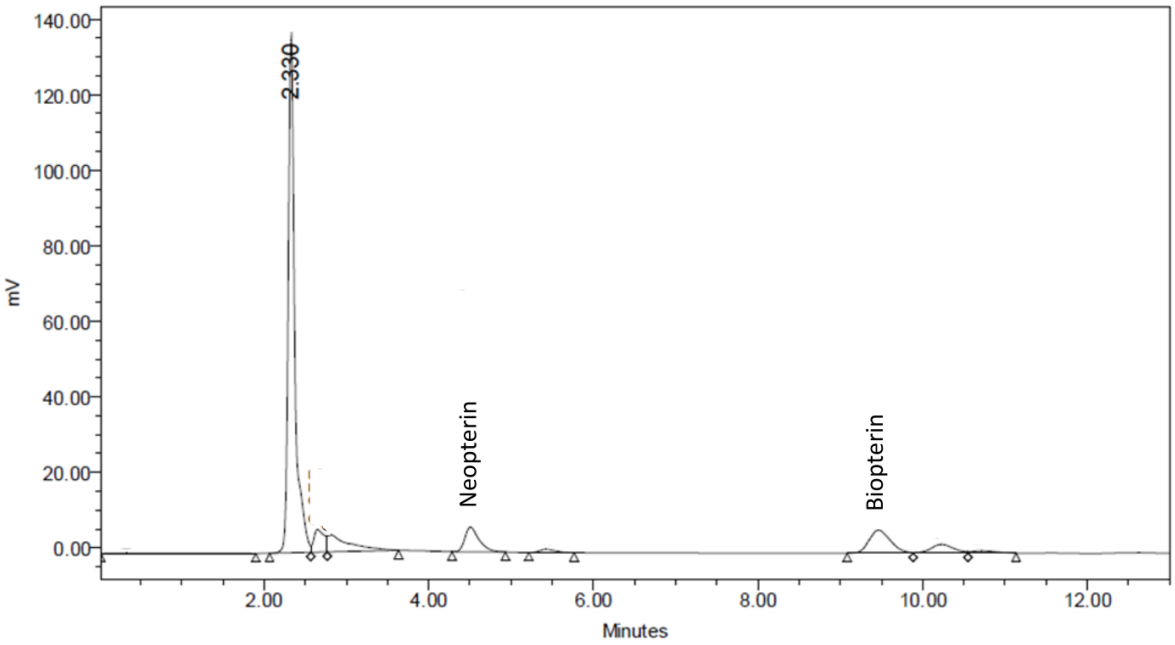

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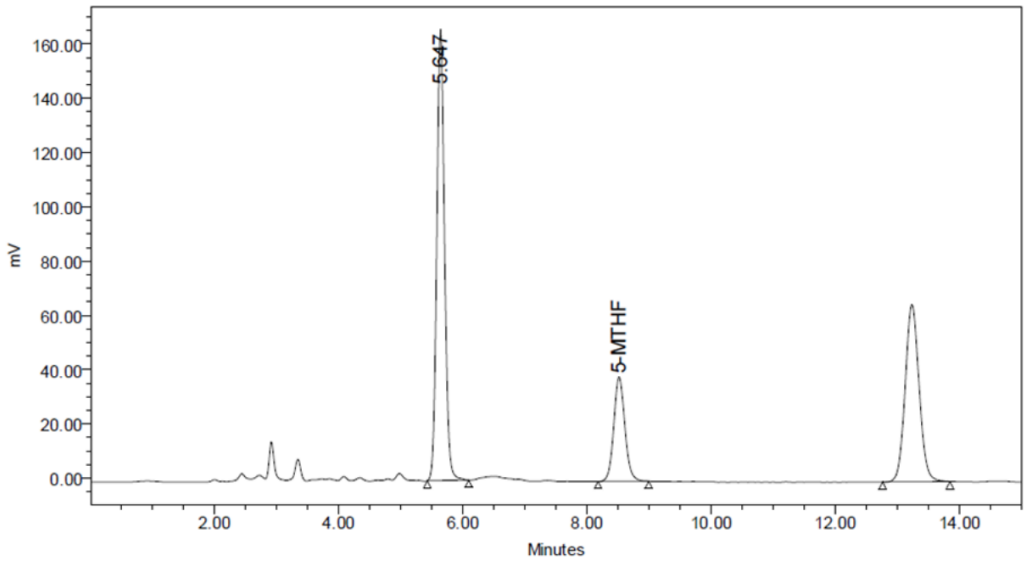

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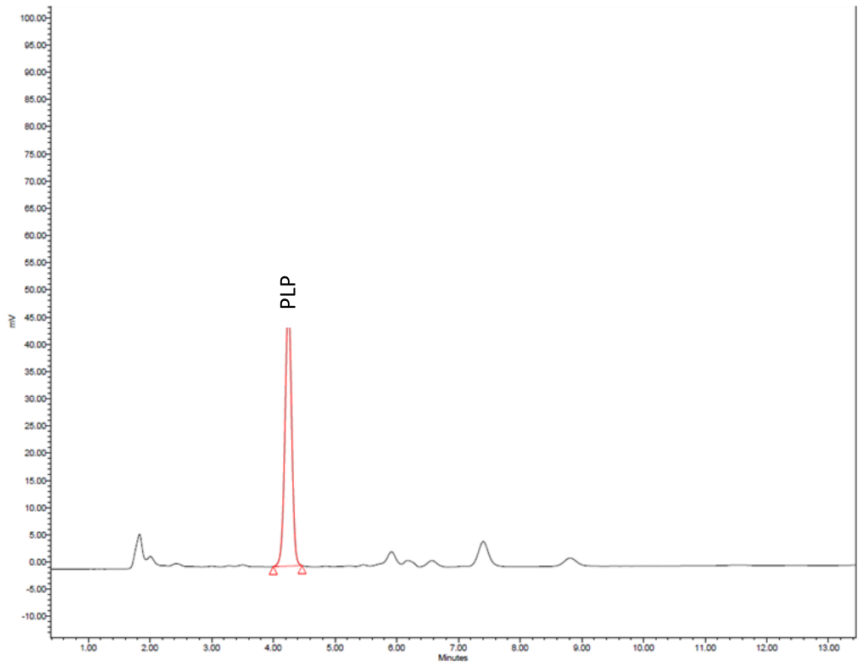
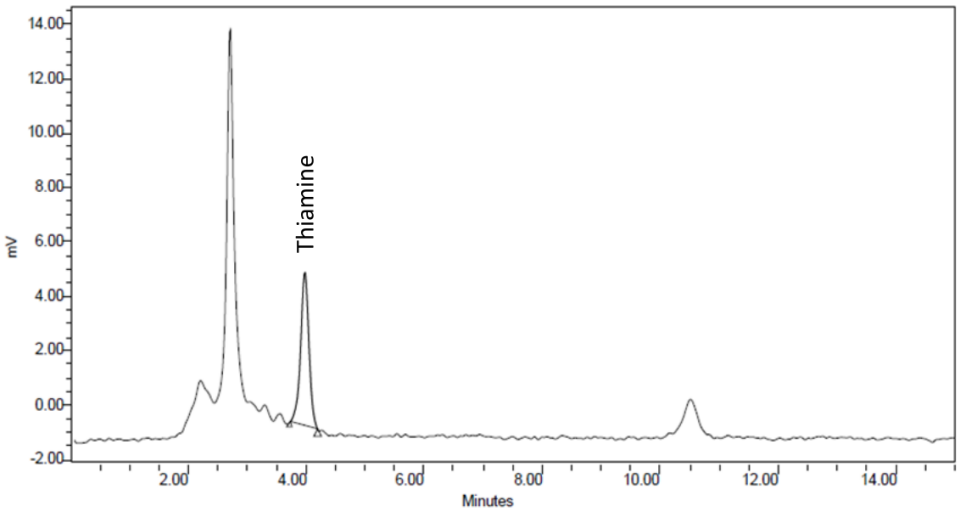


6) Thiamine



OBJECTIU 2: Descripció de protocols analítics basats en cromatografia líquida d'alta resolució (HPLC) per a quantificar amines biògenes i els seus cofactors en líquid cefalorraquidi.

PUBLICACIÓ 2:

Títol: Analysis of human cerebrospinal fluid monoamines and their cofactors by HPLC

Autors: Marta Batllori, Marta Molero-Luis, Aida Ormazabal, Mercedes Casado, Cristina Sierra, Angels García-Cazorla, Manju Kurian, Simon Pope, Simon J Heales & Rafael Artuch.

Referència: *Nat Protoc.* 2017 Nov;12(11):2359-2375.

Resum:

Els procediments per l'anàlisi de monoamines i els seus cofactors s'han anat desenvolupant durant les últimes dècades i s'utilitzen en els laboratoris de referència. La majoria es basen en procediments de cromatografia per HPLC amb diferents sistemes de detecció, principalment electroquímica i de fluorescència, altament sensibles i específics. Tot i el creixent desenvolupament de les tècniques metabolòmiques encara hi ha poca experiència en aquest tipus d'anàlisi.

L'objectiu principal d'aquest treball ha estat realitzar una avaluació exhaustiva de les condicions analítiques per a l'anàlisi d'aquests metabòlits destacant els aspectes crítics dels processos. Aquests protocols descrits, a part de desenvolupar activitats diagnòstiques, permeten també dur a terme activitats d'investigació en l'anàlisi de dopamina i serotonina i metabòlits relacionats.

Síntesi de resultats:

Els procediments descrits en aquest protocol proporcionen mètodes adequats per determinar la concentració de monoamines i els seus cofactors (les pterines i PLP) en mostres de LCR. Els cromatogrames de l'article permeten una identificació ràpida de les condicions genètiques primàries o secundàries que condueixen a alteracions de la dopamina i la serotonina, tal com s'ha descrit anteriorment en altres articles. També es

demostra que es poden determinar els metabòlits actius de la dopamina i la serotonina en cervell.

Addicionalment també es detallen els punts crítics i els problemes que més habitualment poden aparèixer quan es duen a terme aquests procediments analítics i com resoldre'ls.

PROTOCOL

Analysis of human cerebrospinal fluid monoamines and their cofactors by HPLC

Marta Batllori[1,9], Marta Molero-Luis[1,2,9], Aida Ormazabal[1,2], Mercedes Casado[1], Cristina Sierra[1], Angels García-Cazorla[2,3], Manju Kurian[4–7], Simon Pope[8], Simon J Heales[6–8] & Rafael Artuch[1,2]

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The presence of monoamines and their cofactors (the pterins and vitamin B_6 (pyridoxal phosphate (PLP))) 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 dopamine 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 the analysis of 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 PLP requires mixing with trichloroacetic acid to release the protein-bound vitamin, centrifugation, and mixing of the supernatant with phosphate buffer and sodium cyanide for derivatization in alkaline conditions. Monoamines are analyzed by HPLC with coulometric electrochemical detection (ED), pterins are analyzed by HPLC with coupled coulometric electrochemical and fluorescence detection, and PLP is analyzed by HPLC with fluorescence detection. The quantification of all compounds is achieved by external calibration procedures, and internal quality control and standards are analyzed in 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 h.

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 PLP)[1]. Aromatic L-amino acid decarboxylase and tyrosine hydroxylase deficiencies are two relevant genetic diseases (see also **Supplementary Fig. 1** and **Supplementary Table 1**). The quantitative detection of monoamines and their cofactors in the CSF can be used as an indicator for dopamine and serotonin biosynthesis and turnover in the brain, and it is considered to be an important diagnostic marker for a number of the conditions described above. The status of the dopamine and serotonin systems in the CNS 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, enabling a deeper investigation of the monoamine status in a single analysis[2,3]. These metabolites are 3-orthomethyldopa (3-OMD) and methoxyhydroxyphenylglycol (MHPG) for the dopamine pathway and 5-hydroxytryptophan (5HTP) for the serotonin pathway (**Fig. 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 CNS[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 (**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 neurotransmission status in both animal and cellular models, and for the analysis of plasma and urine samples.

Overview of the procedures

The first critical step toward 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

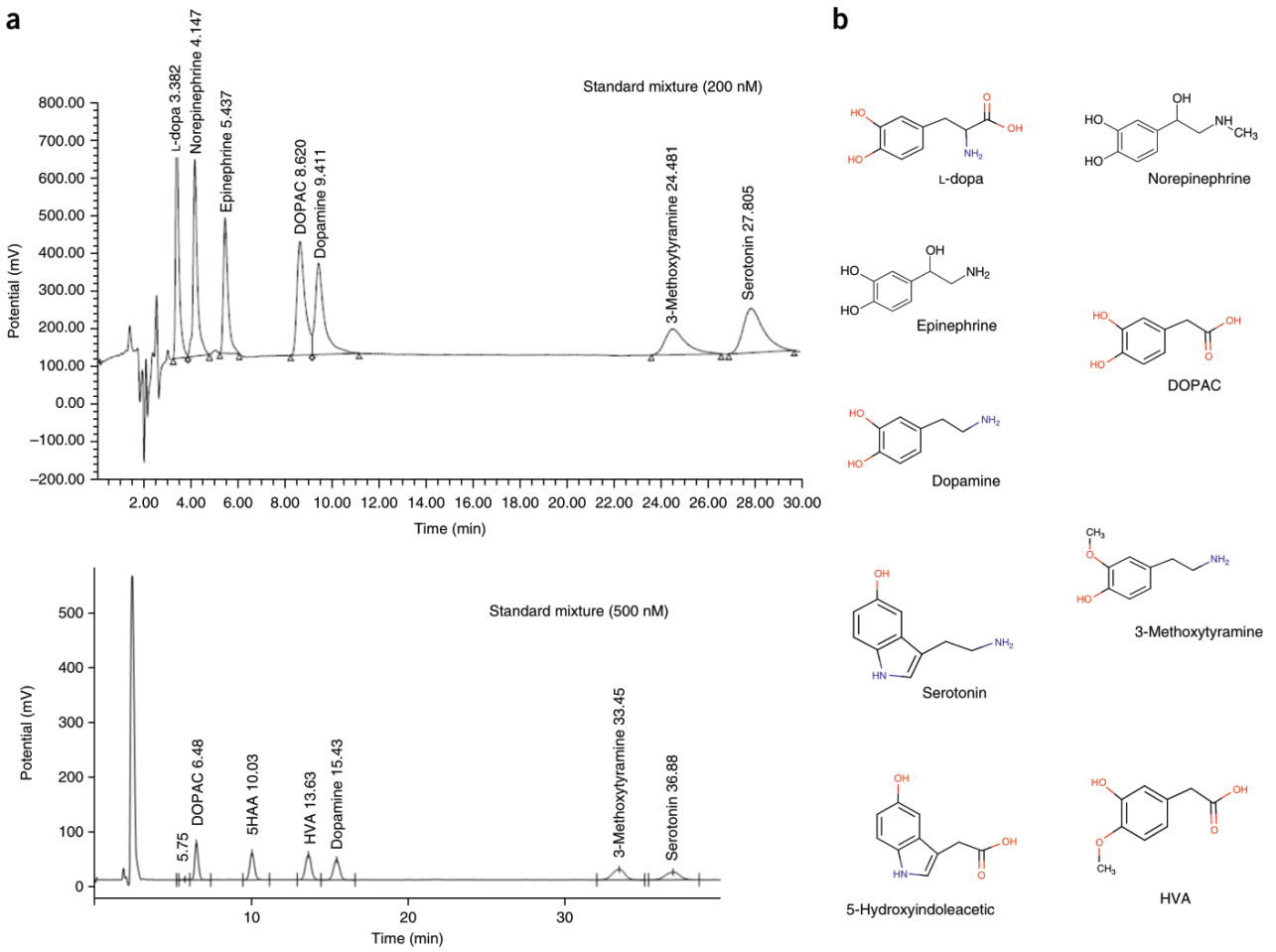

Figure 1 | Chromatograms with different monoamine-related molecules (standard mixture). (**a**) Upper panel (condition 1) shows a chromatogram with seven dopamine and serotonin compounds (L-dopa, norepinephrine, epinephrine, DOPAC, dopamine, 3-methoxytyramine and serotonin). Lower panel (condition 2) shows a chromatogram with six dopamine and serotonin compounds (DOPAC, dopamine, 5HIAA, HVA, 3-methoxytyramine and serotonin). Condition 1. The mobile phase is discussed in the Reagent Setup section. It consists of 75 ml of methanol (7.5%), 13.61 g of sodium acetate trihydrate, 9.60 g of citric acid monohydrate, 0.44 g of EDTA sodium, 0.242 g of octanesulfonic acid and H_2O up to 1 liter. Final pH = 4.00. Injection volume = 30 µl. Voltage: E1 = 50 mV and E2 = 450 mV. Flow rate = 1.3 ml per min. The order of the calibrators is L-dopa, norepinephrine, epinephrine, DOPAC, dopamine, 3-methoxytyramine and serotonin. Condition 2. The mobile phase consists of 320 ml of methanol (16%), 5.44 g of sodium acetate trihydrate (20 mM), 5.25 g of citric acid monohydrate (12.5 mM), 0.074 g of EDTA sodium (0.1 mM), 0.95 g of octanesulfonic acid (2.2 mM) and H_2O up to 2 liters. Final pH = 3.92. Injection volume = 50 µl. Voltage: E1 = 20 mV and E2 = 400 mV. Flow rate = 1.5 ml per min. (**b**) Chemical structures of the molecules in **a**. DOPAC, 3,4-dihydroxyphenylacetic acid.

expert clinician, and strict adherence to the preanalytical 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 regard 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 (pterin analysis) or derivatized fluorescent groups (PLP analysis). In FD, the compound is excited at one wavelength (excitation) and measured at another wavelength (emission) (see **Table 2** for details).

For CSF monoamine quantification (Step 1A of the PROCEDURE), ion-pair HPLC with coulometric ED (HPLC-ED) is the gold standard, because it enables a highly sensitive separation and quantification of these compounds[2,7].

For CSF pterin analysis, HPLC with FD (HPLC-FD) is a 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 (Step 1B of the PROCEDURE) 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 fluorescent compounds (BH_2 and neopterin). For PLP analysis (Step 1C of the PROCEDURE), HPLC-FD offers a single high-sensitivity

TABLE 1 | Overview of the procedures described in this protocol.

	CSF marker	Approach	Option (step)
Biogenic amines	3-OMD	HPLC-ED	1A(i and ii)
	MHPG	HPLC-ED	1A(i and ii)
	5-HTP	HPLC-ED	1A(i and ii)
	5HIAA	HPLC-ED	1A(i and ii)
	HVA	HPLC-ED	1A(i and ii)
Pterins	NP	HPLC-ED-FD	1B(i and ii)
	BH_2	HPLC-ED-FD	1B(i and ii)
	BH_4	HPLC-ED-FD	1B(i and ii)
Pyridoxal phosphate	PLP	HPLC-FD	1C(i and ii)

approach for PLP quantification[8,9]. The results for each technique are reported as absolute values in the nanomole-per-liter range.

Applications of the method

The most common applications of 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[12–14]. Brain microdialysis procedures also allow the determination of the levels 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 FD 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 (**Fig. 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 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 used methods

The methods described here have been routinely used to investigate biogenic amines and pterins in the CSF. Ultra-HPLC–tandem mass spectrometry (UHPLC/MS-MS) methods are increasingly used in bioanalytical investigations, owing 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 toward 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–MS/MS[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[20–22]. However, according to the aforementioned authors, MS/MS detection is less sensitive than FD for pterin quantification. For PLP, a UHPLC–MS/MS approach has been published[23,24] that allows the identification of seven vitamin B_6 vitamers in human CSF samples, although 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].

Taken together, the methodological information presented in this work may be very helpful to both clinical and basic research laboratories.

Limitations

Practical limitations in sample preparation are worthy of consideration. First, sometimes the sample volume is not sufficient to allow 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 to be mainly associated with mass spectrometry procedures. The main limitations of the present protocol are related to the HPLC-ED system. Although HPLC-FD is robust and highly sensitive, HPLC-ED requires an optimum equilibration and preparation of the system, and requires specifically trained operators, so it may be relatively time-consuming. These issues and other ones will be discussed in the present protocol. The establishment of accurate reference values is mandatory for proper data interpretation. CSF samples from 'healthy' pediatric 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 establishment of reference values, as 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 samples as control samples to determine levels of 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 the electrochemical detector is advisable. However, the critical step involves 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, data interpretation should be performed within the context of the clinical information provided by an expert clinician in the field.

TABLE 2 | Summary of the equipment description and setup.

Parameter		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 s	10 s	
	Output	+1 V	−1 V	
	Offset	10%	0%	
Conditioning cell potential			+1,100 mV	
Fluorescence detector conditions	Excitation		360 nm	325 nm
	Emission		440 nm	418 nm
	Gain		1	1
	EUFS		1,000	1,000
Column	Stationary phase	ODS (C-18)	ODS (C-18)	OS (C-8)
	Length and diameter	250 × 5 mm	250 × 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 flow rate, time	Water/methanol 92.5:7.5 (vol/vol) 1 ml/min, 2 h	Water/methanol 50:50 (vol/vol), 1 ml/min, 2 h	Water/acetonitrile 90:10 (vol/vol), 1 ml/min 1 h
Column wash 2	Solvent flow rate, time		Water, 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)

HPLC systems are coupled to ED and FL detectors. EUFS, emission/energy units full scale.

Experimental design

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

Preanalytical 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/her 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

TABLE 3 | Preanalytical protocol for CSF sample collection, management and storage.

Tube	Volume	Biomarkers analyzed
1st tube	5–10 drops (200–400 µl)	Glucose, proteins, cells and lactate
2nd tube	10 drops	Monoamines and PLP/other vitamins
3rd tube[a]	10 drops	Pterins
4th tube	10 drops	Amino acids

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

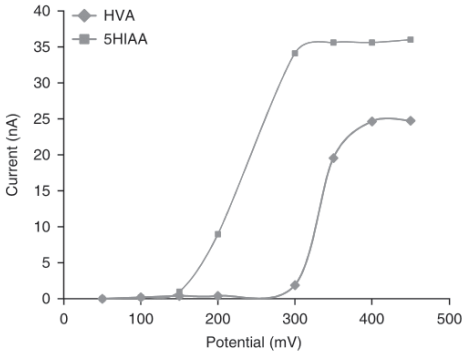

Figure 2 | Hydrodynamic voltammogram for 5HIAA and HVA in CSF. A current/voltage plot is generated after injecting a constant sample volume, which is measured after fixing different potentials. The lowest current that produces the highest analytical response to the electrode should be chosen.

protected from light and stored with stabilizing agents, because the reduced forms are extremely light- and oxygen-sensitive[3]. Because CSF samples have fewer interfering compounds as 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 before HPLC analysis[7]. However, guard column and graphite filters must be regularly replaced to assure a good analytical performance. Sample storage at −70 °C 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 a 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 nonpolar tail. The nonpolar tail interacts strongly with the C18 column, whereas 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 markedly 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 coulometric 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), in which the current is generated, and the auxiliary (E2, or counter) electrode, in which the complementary electrolytic reaction takes place. The potential between the electrodes is held constant, resulting in the complete oxidation of the analyzed compounds of interest. Another electrode, called the reference electrode, is used to keep the potential stable throughout the measurements. The appropriate ED settings are very important to achieving 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 versus applied potential is called the hydrodynamic voltammogram. We advise collecting a hydrodynamic voltammogram plot regularly for each compound, as different variables may influence the results, including factors such as the mobile-phase composition, the type and age of electrodes used and the deterioration of the graphite filter with time[28]. The applied potential at +450 mV is normally

Box 1 | Electrochemical cell cleaning procedure ● TIMING 40 min–2 h

Disconnect the cell from the control module and from the HPLC system. Using a flushing syringe, flush the cell with the solutions indicated below (5–10 ml of each).

1. For general cleaning, flush the cell with deionized water/methanol/deionized water for 10 min at 1 ml/min (pH >5).
2. For lipophilic materials, flush with deionized water/methanol/acetonitrile/THF/acetonitrile/methanol/deionized water at a rate of 1 ml/min for 10 to 15 min each.

! CAUTION NaOH, organic solvents and nitric acid can cause personal injury. Use eye protection and protective clothing, and perform flushing in in a fume hood.

3. For organic materials, flush the system with deionized water/DMSO/deionized water at 1 ml/min for 10 min each.
4. Silica from the column is flushed with deionized water for 10 min at 1 ml/min. Then, using a syringe, the cell is filled with 2 M NaOH (leave it in the cell for 10–30 minutes). Finally, the cell is flushed with deionized water for 10 min at 1 ml/min (until pH is <7).
5. Oxidation/reduction cycle cleaning. Replace the mobile phase with freshly prepared 50 mM phosphate buffer at pH 3, flowing at 1 ml/min. Apply a potential of +1,000 mV to the electrode for 10 min with the phosphate buffer flowing. Do not recycle the phosphate buffer during the treatment. After the treatment, apply a potential of –400 mV for 10 min and then again apply a potential of +1,000 mV for 30 min. 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. Flush the cell with deionized water for 10 min/nitric acid (leave in the cell for 15–30 min)/deionized water (for another 10 min at 1 ml/min) until the pH is >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.

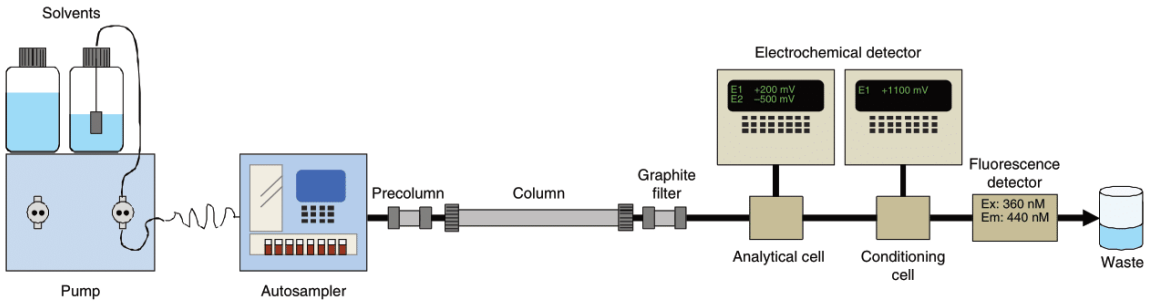

Figure 3 | A representation of a column, the in-line graphite filter and two in-line electrochemical detector cells. The first cell is the analytical cell, which contains electrodes E1 and E2 (the latter is the electrochemical detector). After the sample exits the electrochemical detector cell and is electrochemically detected, it can be oxidized at the conditioning cell for FD of neopterin and BH_2. Em, emission; Ex, excitation.

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, as this leads to a lower background signal. Regarding the composition of electrodes, we used porous graphite electrodes, as 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 the Equipment Setup section 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 pterin[3]. Neopterin, BH_2 and BH_4 are separated using reverse-phase HPLC. BH_4 is measured by ED, where it is oxidized by E1 to quinonoid dihydrobiopterin and reduced back to BH_4 at 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 FD. A postcolumn 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 coelute, they are detected as a single

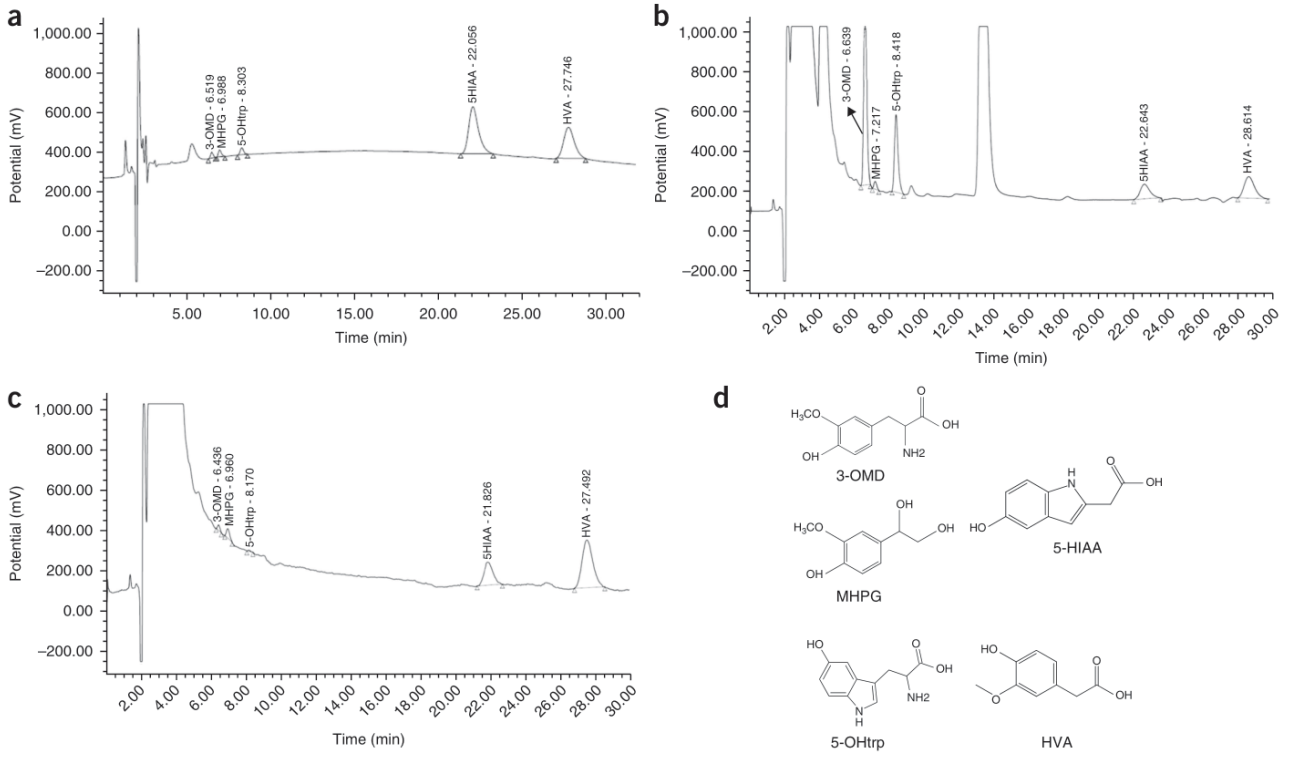

Figure 4 | Chromatograms of monoamines from the standard mixture and CSF samples from pediatric patients. (**a**) A calibration mixture. The elution order is 3-OMD, MHPG, 5-OHtrp, 5HIAA and HVA. (**b**) CSF sample from a 1-month-old patient with increased 3-OMD values. (**c**) CSF sample from a 6-year-old patient. **b** and **c** show a high response in the first 2–4 min of the chromatogram due to the presence of interfering compounds in the CSF sample. (**d**) Chemical structures of the molecules. 5-OHtrp, 5-hydroxy-L-tryptophan.

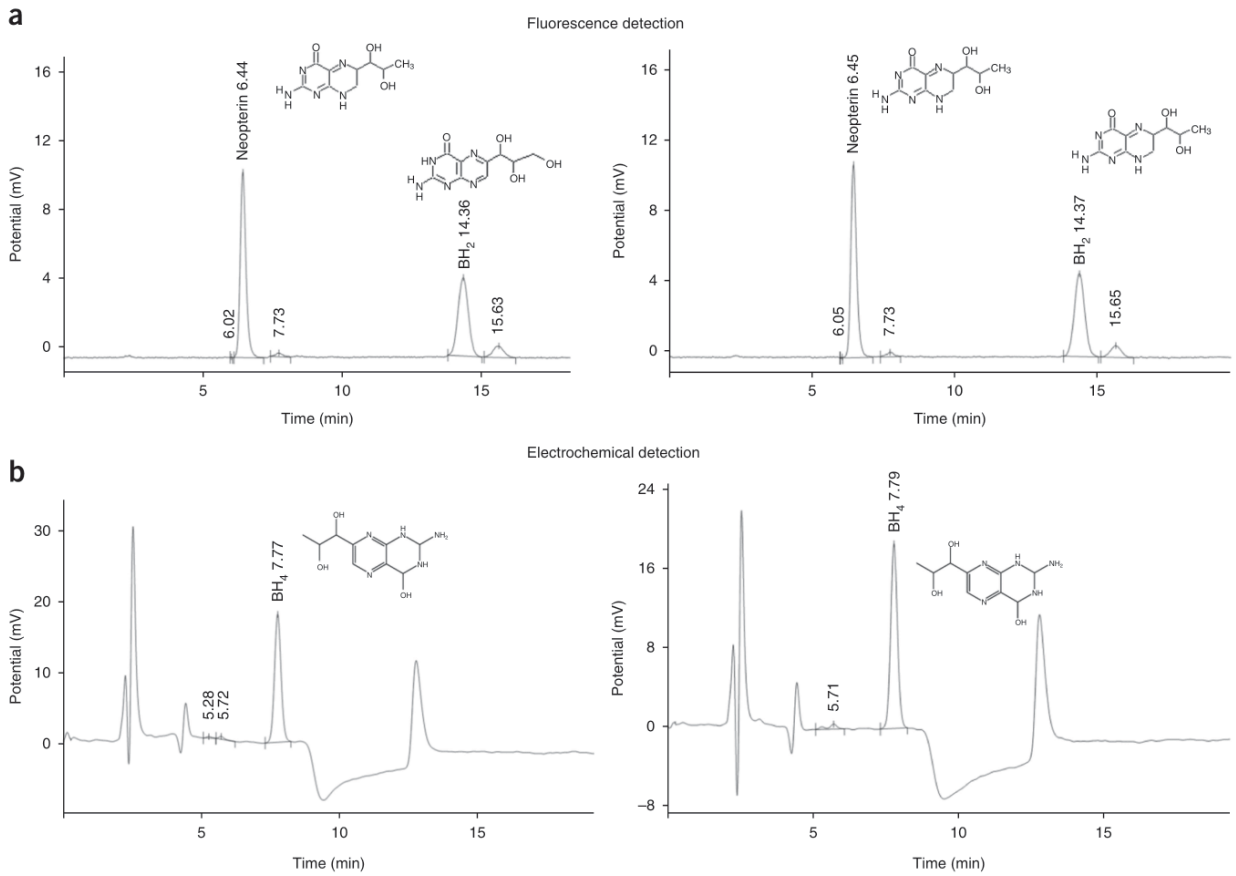

Figure 5 | Chromatograms of pterins from the standard mixture and CSF samples from pediatric patients. Representative chromatograms of BH_4, neopterin and BH_2 in the standard mixture and in a human CSF sample are presented. (**a**) FD: neopterin and BH_2 chromatograms from the standard mixture (left panel) and from a human CSF sample (right panel). The elution order is neopterin and BH_2. (**b**) ED: BH_4 from the standard mixture (left panel) and from a human CSF sample (right panel).

peak by the fluorometer (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 quantifipseccation. CSF PLP quantification is performed by HPLC with FD, 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 **Figures 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, as they are based on human biological matrixes. Since 2014, we have participated in an external quality-control scheme (the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) program) for monoamines and pterins. The ERNDIM control program consists of eight different samples that are analyzed 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 in regard to accuracy, precision, linearity and recovery is graded for each 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: flammable reagents must be stored in an MPA Dresden–approved 90-min EN (European Standards) fire-resistant safety cabinet (Asecos, cat. no. 144470-1), and corrosive or poisonous reagents must be stored in an MPA Dresden–approved chemical cabinet for acids and alkalis (Asecos, cat. no. EN-14727).

▲ CRITICAL Avoid using detergents to clean the glassware for HPLC-ED. This will cause a substantial increase in the background signal. Glassware

PROTOCOL

is rinsed with Milli-Q or ultrapure water and dried with methanol. Use glassware instead of plasticware for the mobile phase and standard solution preparations. Transfer the solvents using glassware.

Human CSF samples CSF (see Reagent Setup for 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 (UK) and Hospital Sant Joan de Déu (Spain). In the UK, the Royal College of Pathologists guidelines (2012) that refer to the Human Tissue Act allow residual anonymized patient material to be used for method performance assessment and quality-control purposes. At Hospital Sant Joan de Déu, the Ethics Committee approved the use of residual and anonymized 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 within the clinical diagnostic workflow, informed consent was always collected as for other medical interventions at 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 CSF-borne pathogens and be trained in the use of correct sample-handling techniques. Some infectious meningoencephalitis cases can mimic genetic diseases and may be unrecognized before the HPLC analysis. Use appropriate personal protective equipment.

▲ 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. Milli-Q water treatment consists of exposure to a UV lamp with two wavelengths to assure the degradation of organic molecules by photo-oxidation. Then, another ultrafiltration system (Quantum) eliminates ionic and organic contaminants to very low trace concentrations. Finally, additional specific filters can be chosen according to the needs of the laboratory (ultrapure water without particle matter, bacteria, lipopolysaccharide, nucleases and proteases).

Monoamine analysis

- Ultrapure water (Type I, 18.2 MΩ·cm; Milli-Q Integral Water Purification System; EMD Millipore, cat. no. ZRXQ003WW)
- Methanol (HPLC grade, ≥99.8%; Merck, cat. no. 1060018)
  ! CAUTION Methanol is toxic and highly flammable, and it should be handled in a 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)
- EDTA dipotassium salt dihydrate (≥98%; Sigma-Aldrich, cat. no. ED2P)
- 1-heptanosulfonic acid sodium salt (Bioxtra; Sigma-Aldrich, cat. no. H8901). This reagent is the ion-pair agent.

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% (thin-layer chromatography (TLC)); Sigma-Aldrich, cat. no. H2255)
- Homovanillic acid (HVA; powder, ≥98%; Sigma-Aldrich, cat. no. H1252)
- Internal quality control, Special Assays Urine (MCA Laboratory, cat. no. SAU-01)
  ▲ CRITICAL Store all standards and controls at −70 °C for up to 1 year.

Pterin analysis

- Ultrapure water (18 MΩ; ELGA PureLab Option-Q DV-25)
- Sodium acetate, AnalaR (VWR, cat. no. 27652.232)
- Citric acid (BDH HiPerSolv, cat. no. 153144M)
- 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 a fume hood.
- Methanol (HPLC-grade) (HiPerSolv for HPLC; VWR, cat. no. 152506X)
  ! CAUTION Methanol is toxic and highly flammable, and it should be handled in a fume hood.
- Sodium hydroxide, pellets (Sigma-Aldrich, cat. no. 221465)

Pterin standards

- D-Neopterin (Sigma-Aldrich, cat. no. N3386)
  ▲ CRITICAL Store D-Neopterin in the dark at −70 °C until the expiry date.
- (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (Sigma-Aldrich, cat. no. T4425) **▲ CRITICAL** Store (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride in the dark at −70 °C until the expiry date.
- 7,8-Dihydro-l-biopterin (Sigma-Aldrich, cat. no. 37272) **▲ CRITICAL** Store 7,8-dihydro-l-biopterin in the dark at −70 °C until the expiry date.
- Internal quality control. This is generated by pooling the clear CSF samples from previous patients with no evidence of pterin disorders. Mix the samples thoroughly, prepare aliquots and immediately freeze them in labeled Eppendorf tubes at −70 °C for up to 1 year.

PLP analysis

- Ultrapure water (Type I, 18.2 MΩ·cm; Milli-Q Integral Water Purification System; EMD Millipore, cat. no. ZRXQ003WW)
- Trichloroacetic acid (TCA; ACS grade, ≥99.5%; Merck, cat. no. 100807)
  ! CAUTION Trichloroacetic acid is corrosive and should be handled in a 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 is a high health hazard (respiratory sensitization, mutagenicity and carcinogenicity); it should be handled in a fume hood.
- Acetonitrile (HPLC grade, ≥99%; Merck, cat. no. 100030)
  ! CAUTION Acetonitrile is toxic and highly flammable and should be handled in a fume hood.
- Orthophosphoric acid (ACS reagent, 85%; Merck, cat. no. 100573)
  ! CAUTION Orthophosphoric acid is corrosive and should be handled in a fume hood
- Semicarbazide hydrochloride (≥95%; Merck, cat. no. 107722)
- Potassium dihydrogen phosphate (KH_2PO_4; ACS grade, ≥99.0%; Sigma-Aldrich, cat. no. P5379)

PLP standards

- PLP plasma/serum calibration standard, lyophilized, 5 × 1 ml (Chromasystems, Teknokroma, cat. no. 36005)
- PLP internal quality control, lyophilized, 5×2 ml (Chromsystems, 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 PLP

- HPLC binary pump (Waters, model no. 1525)
- Refrigerated autosampler injection system (Waters, model no. 717 plus)
  ▲ CRITICAL We advise the use of a refrigerated autosampler, especially if a large number of samples are to be analyzed.
- In-line degasification system, (Waters, model no. AF 186001273)
  ▲ CRITICAL If dissolved gases reach the ED or FD, HPLC pressure oscillations and high-noise background signals will appear (as described in *Coulochem II. Operating Manual*, ESA http://www.vtpup.cz/common/manual/PrF_elanmlab_ESA_Coulochem_manual2_EN.pdf).
- Interface module for equipment communication (Waters, model SAT/IN)
- Integration program (Breeze v3.0; Waters)
- Monoamine column, reverse-phase HPLC column, nucleosil C18 (octadecylsilane (ODS), 5-µm, 250 × 5 mm column; Macherey-Nagel, cat. no. 720041.46).
- Guard ODS column (Teknokroma, cat. no. TR-C-160-1)
- PLP column, HPLC column C8 (octylsilane (OS), 5-µm, 250 × 4.6 mm column; Symmetry Shield, cat. no. WAT 200661)
- Guard C8 column (Teknokroma, cat. no. TR-C-160-3) **▲ CRITICAL** Columns are used only for the indicated applications. The average number of samples analyzed with the same column were 650 for monoamines and 1,450 for PLP.

Pterins

- Pump (Thermo Separation Products, Spectra series model no. P100)
- Refrigerated autosampler (Jasco, model no. AS-2057plus)
- Degasser (Kontron Instruments, model no. DEG 103)

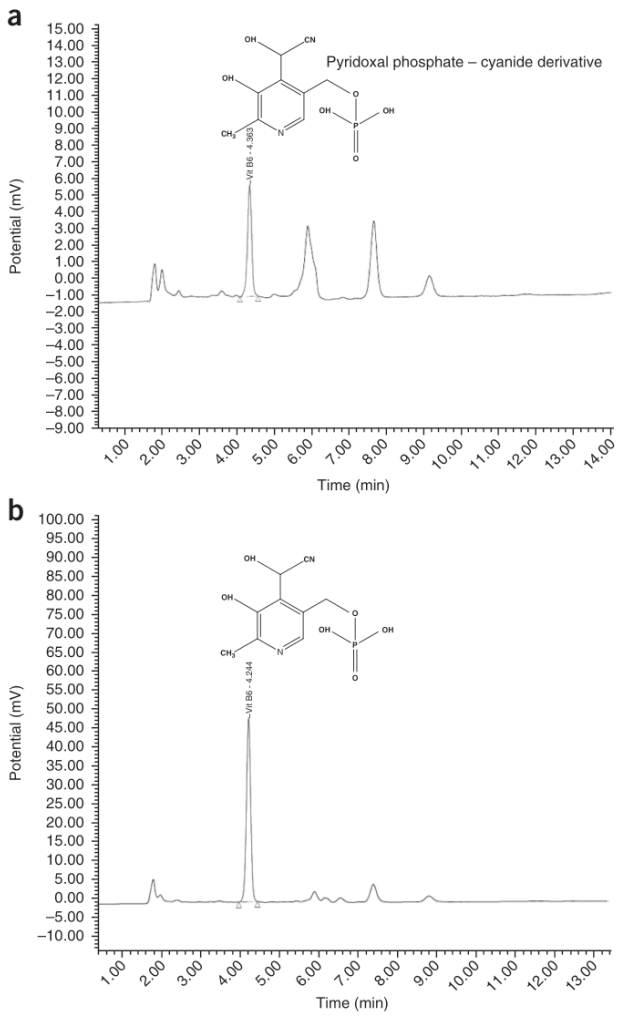

Figure 6 | Chromatograms of PLP from the standard mixture and CSF samples from pediatric patients. (**a**,**b**) Representative chromatograms of the PLP plasma-matrix standard provided by Teknokroma (**a**) and PLP in a human CSF sample (**b**). The chemical structure drawn is the derivatized compound. Vit, vitamin.

- Chromatography column heater (Jones Chromatography, model no. 7970 column block heater)
- PC (DELL, model no. Optiplex 780, with 2.9 GHz and 1.95 GB RAM, running Windows XP)
- Software (Azur data capture system; Datalys Azur v5.0.10.0)
- Reverse-phase HPLC column, C18 (octadecylsilane (ODS), 5-µm, 250 × 4.6 mm with ODS precolumn cartridge; Teknokroma, cat. no. TR-015326) ▲ **CRITICAL** The column is used only for the indicated application. The average number of samples analyzed with the same column was 825.

Common HPLC accessories

- PEEK tubing. 1/16-inch interior diameter, 3 m (Teknokroma, cat. no. 25067) ▲ **CRITICAL** Because of the sensitivity of the chromatographic separation, the interior 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 liter and 5 liter), blue polypropylene screw cap and pouring ring (Duran laboratory, cat. nos. Z305189, Z305197 and Z305200)

Electrochemical detector

- Electrochemical detector (Hucoa-Erlöss, model no. 5200A (Coulochem II))
- Electrochemical detector cell (analytical cell, ESA, model no. 5011; and conditioning cell, ESA, model no. 5021)
- In-line graphite filters (Thermo Fisher Scientific, cat. no. 70-0898). The filters should be placed between the column and the ED cell. ▲ **CRITICAL** This is necessary to remove electroactive impurities before ED. The filters should be regularly replaced.

Fluorescence detector

- Fluorescence detector for pterins. (Intelligent Fluorescence Detector; Jasco, model no. FP-920)
- Fluorescence detector for PLP (Multiwavelength Fluorescence Detector; Waters, model no. 2475)

Accessories for sample and mobile phase preparation

- pH-meter (Crison, model no. GLP21) ▲ **CRITICAL** Calibration of the pH meter by an external accredited company is needed.
- Precision scale (New Classic MF; Mettler Toledo, model no. 104S) ▲ **CRITICAL** Calibration of the precision scale by an external accredited company is needed.
- Freezer set to −70 °C (Nuaire, model no. NU6580E)
- Refrigerated centrifuge (Heraeus, model no. Labofuge 400R)
- Microcentrifuge (for pterin and PLP sample preparation; Nirco, cat. no. 175508).
- Magnetic stirrer with ceramic plate (model no. H20 LBX, 20-liter; Labbox, cat. no. STIH-020-001)
- Stirrer bar (Labbox, cat. no. MAGC-020-005).
- Ductless fume cabinet (Ascent Max; inflow: 187 liters/s or 0.40 ml/s ± 0.025; ESCO, cat. no. ADC-6C1) ! **CAUTION** A ductless fume cabinet is an electrical hazard. Wear rubber-insulated gloves and appropriate shoes.
- Vacuum pump (KNF Laboport and solvent filtration unit; Teknokroma, cat. no. K-953855-1047)
- Automatic pipettes, 10–100, 20–200 and 100–1,000 µl (Nichipet EX; Nichiryo) ▲ **CRITICAL** Pipette calibrations by an external accredited company are required.
- Flasks and bottles, 5, 4, 2 and 1 liters ± 0.4; 500 ml ± 0.250; and 10 ml ± 0.02
- Graduated cylinders, 100 ± 0.5 ml and 1,000 ± 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 to the preparation of the calibration solutions.
- 3-ml Polypropylene round-bottom tube (11 × 55 mm; Daslab; Nirco, cat. no. 175594)
- Filtering membrane filters, nylon, 0.45-µm pore size and 47-mm diameter, package of 50, for mobile-phase filtering (Teknokroma, cat. no. NY 504700)
- 4-mm syringe filter (for 2.5-ml plastic syringes; Ico Plus3; Novico Medica, cat. no. N15649)
- Hydrophilic PVDF membrane (0.22-µm pore size; Millex-GV; Merck Millipore, cat. no. SLGVR04NL)
- 10-kDa centrifugal filters for nonsterile filtration of the samples (Nanosep 10K Omega; Pall Life Sciences, cat. no. OD010c34)
- HPLC vials, 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 for PLP sample preparation (Conterm Poupinel; Selecta, cat. no. 2000200)

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 use this advantage. CSF samples are collected in conventional plastic tubes (10-ml round-bottom polystyrene tubes) 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, and protection from light and blood contamination. Immediately after collection, the tubes (particularly tubes 2–4) must be placed on dry ice and frozen immediately at −70 °C in the dark. If CSF is contaminated with blood, centrifuge the CSF samples (1,500*g* × 10 min at 4 °C) 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.

PROTOCOL

Monoamine mobile phase (acetate–citrate buffer) This buffer contains 0.1 M sodium acetate, 0.1 M citric acid, 1.2 mmol/l EDTA, 1.2 mmol/l 1-heptanosulfonic acid and 75 ml methanol, and it is adjusted to pH 4. Weigh 13.61 ± 0.01 g of sodium acetate into a 1-liter flask, and add water up to 1 liter. 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-heptanosulfonic acid, and add them to a 1-liter glass bottle with a stir bar. To this bottle, add 75 ml of methanol and the acetate–citrate buffer (pH 4) to bring the final volume to 1 liter. 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 Prepare a 401 µM monoamine 3-OMD standard solution by weighing 2.30 ± 0.04 mg of 3-OMD and dissolving it in a 25-ml volumetric flask with water. Prepare 0.5-ml aliquots and store them at −70 °C for up to 1 year.

Monoamine MHPG standard Prepare a 546 µM monoamine MHPG standard solution by weighing 3.10 ± 0.04 mg of MHPG and dissolving it in a 25-ml volumetric flask with water. Prepare 0.5-ml aliquots and store them at −70 °C for up to 1 year.

Monoamine 5-HTP standard Prepare a 270 µM monoamine 5-HTP standard solution by weighing 11.01 ± 0.04 mg of 5-HTP and dissolving it in a 200-ml volumetric flask with water. Prepare 0.5-ml aliquots and store them at −70 °C for up to 1 year.

Monoamine 5HIAA and HVA standard Prepare a monoamine 5HIAA and HVA standard solution (247 µM 5HIAA; 241 µM HVA) by weighing 4.60 ± 0.04 mg of 5HIAA and 2.20 ± 0.04 mg of HVA, and dissolving both in a 50-ml volumetric flask with water. Prepare 0.5-ml aliquots and store them at −70 °C for up to 1 year.

Monoamine calibration working solution Prepare a monoamine calibration working solution in a 100-ml volumetric flask by adding 10 µl each of 3-OMD, MHPG and 5-HTP standards to reach a final concentration of 40.1 nM , 54.6 nM and 27 nM, respectively. Add 200 µl of 5-HIAA and HVA standard to reach a final concentration of 494 nM 5HIAA and 482 nM HVA. 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 of water to the lyophilized commercial vial. Mix gently and incubate the vial for 15 min at room temperature (20–25 °C). It is analyzed for each run to check for data accuracy and to accept or reject the results of the analysis. ▲ **CRITICAL** Store the vial in separate (110-µl) aliquots at −70 °C. The monoamine Internal quality control is stable until the commercial expiry date.

Monoamine neurotransmitter external quality-control scheme (ERNDIM programme) Add 500 µl of water to the lyophilized sample. Mix by vortexing. The sample is ready to be analyzed. This can be stored for up to 1 year at −70 °C. For details of this external quality-control scheme, visit http://www.erndimqa.nl. It is blindly analyzed eight times per year to validate the accuracy of the procedure. The results are available upon request.

Monoamine HPLC system wash solution Monoamine HPLC system wash solution is 500 ml of water with 7.5% (vol/vol) methanol. This solution should be filtered (0.45 µM nylon membrane) and can be stored for up to 1 month at room temperature.

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

Pterin mobile phase Dissolve 27.20 ± 0.01 g of sodium acetate (final concentration 50 mM), 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 liters of ultrapure water in a 2-liter beaker. Bring the volume to 4 liters with water in a volumetric flask. Thoroughly mix and then transfer the buffer to the 5-liter 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 the mixture for 5 min to ensure that the DETAPAC has completely dissolved. Dissolve 15.7 ± 0.1 mg of BH_4 in the mixture and bring the final volume to 100 ml with water. Mix the solution on ice for 30 min with a stir bar and a magnetic stirrer (in the dark), and then prepare 0.5-ml aliquots. ▲ **CRITICAL** The 0.5-ml aliquots should be transferred to Eppendorf tubes and immediately frozen at −70 °C. 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 the solution on ice for 30 min using a magnetic stirrer (in the dark). Then prepare 0.5-ml aliquots. ▲ **CRITICAL** The 0.5-ml aliquots should be transferred to Eppendorf tubes and immediately frozen at −70 °C. The frozen aliquots can be stored for up to 1 year. Because of 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 the solution for 30 min on ice using a magnetic stirrer (in the dark). Then prepare 0.5-ml aliquots. ▲ **CRITICAL** The 0.5-ml aliquots should be transferred to Eppendorf tubes and immediately frozen at −70 °C. The aliquots can be stored for up to 1 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 the mixture for 5 min to ensure DETAPAC dissolution. This solution can be stored for up to 1 year at −70 °C.

Pterin working standard Pour ~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 the contents of the flask by inversion. Fill another 10-ml volumetric flask with ~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 the contents of the flask by inversion. ▲ **CRITICAL** A single working standard solution that contains 50 nM neopterin, 50 nM BH_2 and 50 nM 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 the solution by vortexing. The sample is now ready to be analyzed. It can be stored for up to 1 year at −70 °C. For details regarding this external quality-control scheme, visit http://www.erndimqa.nl. It is blindly analyzed eight times per year to validate the accuracy of the procedure. The results are available upon request.

Pterin HPLC system wash solution Pterin HPLC system wash solution is a 50% (vol/vol) methanol/water solution. This solution can be stored for up to 1 month at room temperature.

Pterin HPLC injector wash solution Pterin HPLC injector wash solution is 10% (vol/vol) methanol in water. This solution can be stored for up to 1 month at room temperature.

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

PLP trichloroacetic acid (10%) Weigh 10 ± 0.01 g of PLP trichloroacetic acid (10%) into a 100-ml flask, and add Milli-Q water to bring the volume to 100 ml. Store the solution between 4 and 8 °C. The solution is stable for 6 months.

PLP dipotassium hydrogen phosphate (3.3 M) Weigh 14.37 ± 0.04 mg of K_2HPO_4 into a 25-ml flask, and add Milli-Q water to bring the volume to 25 ml. Store the solution between 4 and 8 °C. The solution is stable for 1 month.

PLP sodium cyanide (0.04 M) Weigh 64.1 ± 0.04 mg of sodium cyanide into a 25-ml flask, and add Milli-Q water to bring the volume to 25 ml. Store the solution between 4 and 8 °C. 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.

PLP orthophosphoric acid (2.86 M) Pipette 4.08 ml of 85% (vol/vol) orthophosphoric acid into a 25-ml flask, and add Milli-Q water to bring the volume to 25 ml. Store the solution between 4 and 8 °C. The solution is stable for 6 months.

PLP standard Add 1 ml of Milli-Q water to the lyophilized commercial vial. Mix the contents gently and incubate the vial for 10–15 min at room temperature. Swirl the vial to completely dissolve its contents. This solution can be stored for up to 3 months at −20 °C

PLP internal quality control A lyophilized commercial PLP control is available. Add 2 ml of dH_2O to the vial. Gently mix the solution and incubate the vial for 10–15 min at room temperature. Swirl the vial to completely dissolve its contents. This solution can be stored for up to 3 months at −20 °C.

PLP HPLC system wash solution PLP HPLC system wash solution is 10% (vol/vol) acetonitrile in Milli-Q water. This solution can be stored for up to 1 month at room temperature.

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 the Troubleshooting section. **Box 1** provides information on the procedure for cleaning the electrochemical cell.

▲ CRITICAL The samples and freshly prepared mobile phases must be filtered (i.e., free of particulate matter). Check that the columns are stable with regard to the mobile phase and that 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.

PROCEDURE

1| This protocol describes the procedures for characterization of monoamine (option A), pterin (option B) and PLP (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 h for HPLC-ED setup; and 35 min for data acquisition and analysis

(i) *Standard, control and sample preparation (Steps i–vii):* Remove one aliquot of each monoamine standard (3-OMD, MHPG, 5-HTP and 5HIAA/HVA), the internal quality control and the CSF samples (see Reagent Setup) from the −70 °C freezer, 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 aliquot and dilute it at a ratio of 1:100 (vol/vol) in Milli-Q water: prepare the 1:100 dilution by mixing 20 µl of internal quality control with 1,980 µl of Milli-Q water. Prepare the external quality control (see Reagent Setup) according to the ERNIDM schedule, 8 times per year.

(iv) Process the previously prepared CSF samples, calibration solution and internal and external controls by diluting the samples at a ratio of 1:2 (vol/vol) by mixing 125 µl of sample with 125 µl of the chromatographic mobile phase.

▲ CRITICAL STEP For ED, dilution with the mobile phase is required to reduce the sample matrix effects.

(v) Centrifuge the samples at 1,500*g* for 10 min at 4 °C.

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

(vii) Prepare 200-µl aliquots of each sample in labeled chromatography vials and put them into the rack.

(viii) *HPLC-ED setup (Steps viii–xviii):* Before running the standard, controls and CSF samples, the user must connect the HPLC circuit. First, connect the graphite in-line filter before the ED cell.

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

▲ CRITICAL STEP Ensure that the graphite filter is not overtightened, as it can become scratched if overtightened, thereby damaging the ED cell.

? 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.

? TROUBLESHOOTING

(xi) Check for fluid leaks before equilibrating the system.

? TROUBLESHOOTING

(xii) Turn on the computer, and load the Breeze program 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 min using the Breeze program.

? TROUBLESHOOTING

(xv) Purge the injector and the detector with the mobile phase for 10 min using the Breeze program.

PROTOCOL

(xvi) Equilibrate the system at 1.3 ml/min for at least 2 h.
▲ **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 h after the flow rate is restored to 1.3 ml/min the following day.
(xvii) During the equilibration stage, program the electrode parameters (E1 = +50 mV; E2 = +450 mV and R2 = 200 nA) on the ED. Allow equilibration to proceed for at least 1 h until the cell signal is stabilized (i.e., baseline signal is <500 nA).
? TROUBLESHOOTING
(xviii) Program the autosampler in the Breeze program to inject 30 µl of each sample from its position in the rack and set the time for 30 min. Upload the monoamine method stored in the Breeze program. The 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 program. 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 the calibration working solution (see Reagent Setup) and internal quality control (according to the SKML commercial sheet) are within the assigned limits (usually within 2 s.d. 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 must be reinjected, the ED can be turned off.
(xxi) Wash the system with 1 ml/min of 7.5% (vol/vol) methanol (the same percentage as the mobile phase) for 2 h.

(B) Pterin analysis ● TIMING 40 min for standard, control and sample preparation; 3 h for HPLC-ED-FD setup; and 25 min for data acquisition and analysis

(i) *Standard, control and sample preparation (Steps i and ii):* Remove the pterin calibration stock solution, the internal quality control and CSF samples (see Reagent Setup) from the –70 °C freezer. Thaw at room temperature and thoroughly mix individually. Keep the samples 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 the samples for the pterin method. However, some samples do require filtering (high-protein or colored CSFs). Filter such CSF samples through 10-kDa centrifugal filters to remove the major contaminants and impurities.
(ii) Prepare 200-µl aliquots of the samples in amber capped, labeled autosampler vials and position the vials in the rack.
(iii) *HPLC-ED-FD setup (Steps iii–xii):* Before running the standard, controls and CSF samples, the user must connect the HPLC circuit. First, connect the graphite in-line filter before the ED cell.
▲ **CRITICAL STEP** The acquisition software should be operated by the user according to the manufacturer's instructions. Refer to the Equipment Setup section.
▲ **CRITICAL STEP** Ensure that the graphite filter is not overtightened, as it can become scratched if overtightened, thereby damaging the ED cell. The guard cell is placed after the ED and before the fluorescence detector.
? TROUBLESHOOTING
(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.
? TROUBLESHOOTING
(vi) Check for fluid leaks before equilibrating the system.
? TROUBLESHOOTING
(vii) Purge the pump and wash the system with 50% (vol/vol) methanol at a flow rate of 1 ml/min for 2–3 h.
▲ **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 h.
? TROUBLESHOOTING
(viii) Transfer the inlet line to the mobile phase and allow the system to equilibrate at 1.3 ml/min for at least 2 h.
▲ **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 h after the flow rate is restored to 1.3 ml/min the following day.
(ix) After 30 min, turn on the ED and the fluorescence detector. Program 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 +0.5 µA and E2 should be

adjusted so that the current is –0.05 µA; these currents yield the optimal peak area for tetrahydrobiopterin. The voltages applied may vary but usually are as follows: E1=+200 mV; E2 = –500 mV; conditioning cell = +1,100 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.

▲ **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 h.

? TROUBLESHOOTING

(xi) Turn on the computer and load the Azur data capture program to capture and process the data from the electrochemical and fluorescence detectors.

(xii) Program the autosampler to inject 50 µl of the sample, control or standard onto the HPLC and set the run time to 20 min. Upload the pterin method stored in the Azur program. The 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 if the values of the calibration working solution (see Reagent Setup) 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 no CSF sample must be re-injected, turn off the ED and the fluorescence detector.

(xv) Wash the system with ultrapure water at a flow rate of 1 ml/min for at least 1–2 h. Then wash the system with 50% (vol/vol) methanol/water at a flow rate of 1 ml/min for 2 h or overnight.

(C) PLP analysis ● TIMING 40 min for standard, control and sample preparation; 2 h for HPLC-FD setup; and 30 min for data acquisition and analysis

(i) *Standard, control and sample preparation (Steps i–vii):* Remove the PLP calibration stock solution, the internal quality control and CSF samples (see Reagent Setup) from the –70 °C freezer, 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 separate Eppendorf vials, and mix the contents by vortexing. Incubate the samples at 50 °C in the dark for 5 min to completely release the protein-bound vitamin.

(iii) Centrifuge the samples at 12,000*g* (10 min, 4 °C).

(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 to the supernatant in the vial and mix by the contents vortexing (pH 7.4). Incubate the vial at 50 °C in the dark for 25 min.

▲ **CRITICAL STEP** In alkaline conditions, sodium cyanide oxidizes PLP to pyridoxic-5-phosphate, which can be measured by FD.

(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 the major contaminants and impurities.

! **CAUTION** Cyanide/orthophosphoric acid can create toxic hydrogen cyanide, so samples must be prepared and capped in a fume hood.

(vii) Transfer the filtered samples to chromatography 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 must 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 (Waters) instructions.

(ix) Connect the mobile phase to the HPLC system 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 program to capture and process the data from the fluorescence detector.

(xiii) Purge the pump with the mobile phase for 5 min using the Breeze program.

(xiv) Purge the injector and the detector with the mobile phase for 10 min using the Breeze program.

(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 0 (–0.06–0.03 FD signal).

? TROUBLESHOOTING

PROTOCOL

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

(xviii) *Data acquisition and analysis (Steps xviii–xx):* To run the 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 if the values of PLP calibration and internal quality control (according to the Teknokroma commercial sheet) are within the assigned limits (usually within 2 s.d. 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 must be re-injected, the FD can be turned off.

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

? TROUBLESHOOTING

HPLC coulometric ED is a high-sensitivity procedure, as the analyzed compounds are expected to be 100% oxidized. Analytical troubles may be remarkable if ED-cell cleaning and maintenance protocols are not strictly followed. 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 not strictly controlled. Dissolved gases and electroactive compound adsorption to the porous graphite electrode, the presence of electroactive compounds in the mobile phase or the sample, or poor electrode maintenance can lead to low electrochemical signals and unacceptably high background currents with a noisy baseline (as described in the *Coulochem II. Operating Manual* (ESA)). When the ED is flushed with water–methanol–water (as described in **Box 1**), the analytical performance remarkably improves. This cleaning procedure is easy to perform and consists of flushing the cells with water for 30 min (4 ml/min), then with methanol for 30 min (4 ml/min) and finally with water for 30 min (4 ml/min). It is especially useful as a first procedure to be carried out 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 be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
Electrochemical detection			
1A(xiv), 1B(vii)	The baseline is noisy	There is dissolved gas in the pump head	Degas the mobile phase (under helium) and reprime the pump
1A(xvii), 1B(x)		There is dissolved gas in the detector cell	Remove the cell from the system, increase the flow rate for 30 min with water/degassed methanol/water (4 ml/min)
1A(xi), 1B(vi)		The pump head or system leaks	Check the system for leakage
1A(xiv), 1B(vii)		The mobile phase is not properly mixed or degassed	Check the aspect and degas under helium current
1A(x), 1B(v)		Contaminants are eluting from the column	Remove the column from the system and check whether the problem persists
1A and 1B		Electronic problems	Contact maintenance service
1A(xvii), 1B(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 probably present. In that case, replace the mobile phase
1A(x), 1B(v)		Electroactive species are 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

(continued)

TABLE 4 | Troubleshooting table (Continued).

Steps	Problem	Possible reason	Solution
1A(xvii), 1B(x)		Adsorption of impurities onto the electrode that causes decreased current and increased noise	Reverse the potential or clean the cell (**Box 1**)
1A(xi), 1B(vi)		Leaking cell	Check for leaks. Tighten fittings or replace the cell
1A(viii), 1B(iii)	Increased back pressure	Accumulation of particles from the mobile phase or injected samples	Replace the graphite filter. Use freshly prepared and filtered mobile phase and samples
1A(viii and/or x) 1B(iii and/or v)		Accumulation of particles from the column	Replace the graphite filter. Replace the column. Be careful with silica columns with mobile phase pH values >7.5 or <2.5
1A and 1B		Clogged cell	Clean the cell (**Box 1**)
1A(xvii), 1B(x)	Loss of ED cell response	Unstable compounds	Check the stabilities of the analyzed biomarkers (especially 5HIAA and BH_4)
1A(xvii, x)		Decreased ED cell performance	Plot a hydrodynamic voltammogram (sigmoidal curve). Small variations in current may lead to large variations in response. Usually a voltammogram is plotted when the procedure is standardized, when the ED is cleaned or changed or when a significant loss of response is observed
1A and 1B		Adsorption of impurities onto the electrode	Clean the cell (**Box 1**)
Fluorescence detection			
1B(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
1B(x), 1C(xvi)	Failure to ignite at startup, a decrease in sensitivity or noisy baseline	Damaged lamp	Replace the lamp

Columns should stand under mobile phase pH values between 2.5 and 7.5.

● **TIMING**

Option A: monoamine analysis

Steps i–vii, standard, control and sample preparation: 40 min

Steps viii–xviii, HPLC-ED setup: 3 h (1 h to prepare the HPLC system and 2 h 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 for at least 1 h before starting the analysis, in order 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 and ii, standard, control and sample preparation: 40 min

Steps iii–xii, HPLC-ED-FD setup: 3 h (1 h to prepare the HPLC system and 2 h 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 1 h before starting the analysis, in order 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 for chromatographic data analysis)

Option C: PLP analysis

Steps i–vii, standard, control and sample preparation: 40 min
Steps viii–xvii, HPLC-FD setup: 2 h (30 min to prepare the HPLC system and 1.5 h 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)

ANTICIPATED RESULTS

The procedures described in this protocol will provide suitable methods for determining 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[4,29]. Four additional chromatograms depicting the neopterin, BH_4 and BH_2 (**Fig. 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 (**Fig. 6**).

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

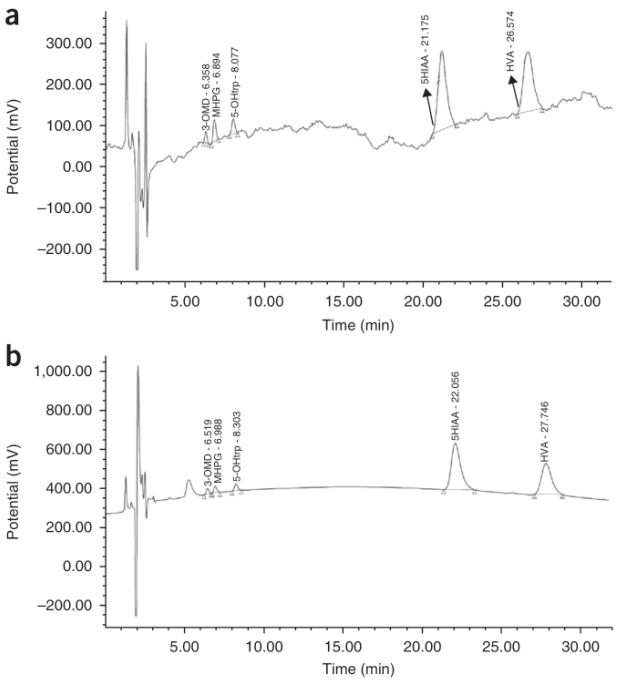

Figure 7 | Experiment that requires troubleshooting to improve the analytical quality. For HPLC-ED, the most common problems usually arise from electrochemical cell contamination or adsorption of electroactive impurities caused by dissolved gases in the system, pump problems, leaks, the mobile phase being not properly mixed or contaminants leaving 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 poor performance is shown in **a**. The result after the ED cell cleaning procedure is shown in **b**.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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OBJECTIU 3: Avaluació de la melatonina com a biomarcador perifèric de l'estat de la serotonina central en pacients amb defectes enzimàtics de les amines biògenes.

PUBLICACIÓ 3:

Títol: Urinary sulphatoxymelatonin as a biomarker of serotonin status in biogenic amine-deficient patients.

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Resum:

Un dels principals problemes de l'estudi dels defectes genètics de la neurotransmissió és l'absència de biomarcadors perifèrics fiables tant pel diagnòstic com per la monitorització del tractament. De fet, els protocols diagnòstics són molt clars en aquest aspecte i remarquen la necessitat d'una anàlisi en LCR. Respecte a la monitorització del tractament no hi ha un consens clar respecte a la freqüència d'aquesta monitorització.

Per a realitzar el seguiment d'aquests pacients, l'única opció que existeix actualment és la realització de puncions lumbars de repetició. Sí que existeix algun marcador perifèric del metabolisme de la dopamina (prolactina), tot i que a la pràctica, presenten gran variabilitat intra i inter individual. Fins a la realització d'aquest estudi, no hi ha hagut cap marcador perifèric que pogués suposar una alternativa al LCR per a l'avaluació del metabolisme de la serotonina en pacients efectes de defectes enzimàtics de les amines biògenes.

L'estreta relació entre la síntesi de melatonina i la serotonina és coneguda de fa temps i, aprofitant que la melatonina ja havia estat utilitzada en algun estudi previ com a metabòlit per a la monitorització d'altres malalties, l'objectiu va ser fer una avaluació d'aquest biomarcador en pacients amb defectes genètics de la neurotransmissió que impliquessin un defecte en la síntesi de serotonina.

Síntesi de resultats:

Aspectes preanalítics

La primera mostra d'orina del matí va mostrar les concentracions més elevades de sulfatoximelatonina (aMT6s), que van caure notablement a la segona mostra del matí (reducció mitjana del 68,7%). La variació intraindividual estudiada en els tres voluntaris va mostrar uns CV del 14,5%, 15,1% i 9,9%, respectivament durant una setmana de seguiment.

Establiment de valors de referència (n = 65):

Es va observar una correlació negativa entre les concentracions de aMT6s en la primera mostra d'orina del matí i l'edat dels pacients. Els valors de referència de la nostra població es van estratificar en tres grups diferents en funció de l'edat.

Pacients amb defectes genètics sense tractament serotoninèrgic

Es va observar una freqüència elevada de deficiència de melatonina especialment en les formes greus: GTPCH autosòmica recessiva (arGTPCH), sepiapterina reductasa (SR) i deficiència de la descarboxilasa d'aminoàcids aromàtics (AADC). En canvi, només 2 casos dels 13 amb defecte de GTPCH autosòmic dominant (adGTPCH), la forma més lleu de defectes, van mostrar una deficiència de melatonina.

Pacients amb defectes genètics i amb tractament serotoninèrgic

Vam estudiar cinc pacients amb deficiència de AADC, un amb deficiència de SR, tres amb deficiència de dihidropterinreductasa (DHPR) i dos amb deficiència de PTPS amb tractament de fàrmacs estimuladors de la síntesi de serotonina o inhibidors de la seva recaptació o degradació. En funció de la malaltia i, probablement, del perfil genètic dels pacients, els resultats van ser diferents: (1) En els pacients amb deficiència de AADC el tractament no normalitzava les concentracions de melatonina, excepte en un cas. No obstant això, sí que es va observar en un altre pacient, que després d'estar 3 mesos en tractament amb IMAOs, les concentracions de aMT6s augmentaven, encara que no arribaven a valors normals. (2) Pel que fa als pacients amb defectes relacionats amb les pterines, alguns van mostrar una disminució de l'excreció aMT6s tot i rebre tractament amb 5HTP. Entre ells, el pacient amb deficiència de SR i el pacient amb deficiència de PTPS van mostrar una excreció de aMT6s notablement reduïda (90,7% i 81,5%, respectivament), mentre que els pacients amb deficiència de DHPR i un dels pacients amb deficiència de PTPS van presentar valors normals aMT6s.

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Urinary sulphatoxymelatonin as a biomarker of serotonin status in biogenic amine-deficient patients

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Melatonin is synthesized from serotonin and it is excreted as sulphatoxymelatonin in urine. We aim to evaluate urinary sulphatoxymelatonin as a biomarker of brain serotonin status in a cohort of patients with mutations in genes related to serotonin biosynthesis. We analized urinary sulphatoxymelatonin from 65 healthy subjects and from 28 patients with genetic defects. A total of 18 patients were studied: 14 with autosomal dominant and recessive guanosine triphosphate cyclohydrolase-I deficiency; 3 with sepiapterin reductase deficiency; and 1 with aromatic L-amino acid decarboxylase deficiency. Further 11 patients were studied after receiving serotoninergic treatment (serotonin precursors, monoamine oxidase inhibitors, selective serotonin re-uptake inhibitors): 5 with aromatic L-amino acid decarboxylase deficiency; 1 with sepiapterin reductase deficiency; 3 with dihydropteridine reductase deficiency; and 2 with 6-pyruvoyltetrahydropterin synthase deficiency. Among the patients without therapy, 6 presented low urinary sulphatoxymelatonin values, while most of the patients with guanosine triphosphate cyclohydrolase-I deficiency showed normal values. 5 of 11 patients under treatment presented low urine sulphatoxymelatonin values. Thus, decreased excretion of sulphatoxymelatonin is frequently observed in cases with severe genetic disorders affecting serotonin biosynthesis. In conclusion, sulphatoxymelatonin can be a good biomarker to estimate serotonin status in the brain, especially for treatment monitoring purposes.

Melatonin (5-methoxy-N-acethyltriptamine) is secreted by the pineal gland and is synthesized from serotonin. Melatonin synthesis is regulated by two specific enzymes: serotonin-N-acetyl transferase (SNAT, EC 2.3.1.5), which is a rate-limiting enzyme, and 5-hydroxyindole-O-methyl transferase (HIOMT EC 2.1.1.4), which transfers a methyl group from S-adenosylmethionine to 2-hydroxyl of N-acetylserotonin (Fig. 1). Melatonin is released from the pineal gland and enters the circulation. Other melatonin sources are the retina, gut, skin, platelets and bone marrow, but their contribution to circulating melatonin is less relevant than that of pineal gland[1]. Melatonin is metabolized in the liver to 6-hydroxymelatonin by cytochrome CYP1A2 (EC 1.14.14.1), and it is excreted in urine as sulphatoxymelatonin (aMT6s) and, to a lower extent, as glucuronide conjugate[1]. Urine aMT6s excretion

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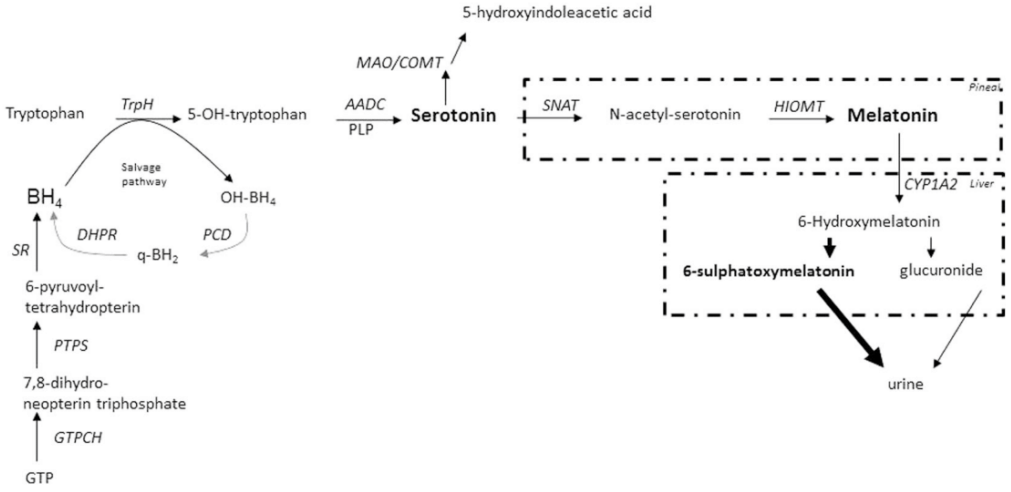

Figure 1. Melatonin pathway. Melatonin synthesis occurs in pineal gland (and other tissues) and its metabolism produces 6-sulphatoxymelatonin, the main urine melatonin metabolite. Serotonin and melatonin are marked in bold type. Synthesis and salvage pathways (grey arrows) of the tetrahydrobiopterin (BH_4) are also represented. Enzymes are in cursive. AADC: aromatic L-amino acid decarboxylase; BH_4: tetrahydrobiopterin; COMT: catechol O-methyltransferase; CYP1A2: cytochrome P450 isoform CYP1A2; DHPR: dihydropteridine reductase; GTP: guanosine triphosphate; GTPCH: GTP cyclohydrolase I; HIOMT: 5-hydroxyindole-O-methyltransferase; MAO: monoamine oxidase; OH-BH_4: hydroxy-tetrahydrobiopterin; PCD: pterin-4a-carbinolamine dehydratase; PTPS: 6-pyruvoyl-tetrahydropterin synthase; q-BH_2: quinonoid-dihydrobiopterin; SNAT: serotonin N-acetyltransferase; SR: sepiapterin reductase; TrpH: tryptophan hydroxylase; PLP: pyridoxal phosphate.

closely correlates to the plasma melatonin profile[1,2] and is a good indicator of melatonin secretion from the pineal gland[3]. Thus, it has been suggested that the measurement of urinary aMT6s may be a good biomarker of serotonin status in the brain[4]. Yano *et al.* reported that blood melatonin and urine aMT6s levels may serve as biomarkers reflecting brain serotonin synthesis in subjects with phenylketonuria (PKU)[4].

There are several genetic alterations affecting brain serotonin and dopamine biosynthesis (Fig. 1): aromatic L-amino acid decarboxylase (AADC) deficiency (OMIM#608643), pyridoxal phosphate (PLP) deficiency (pyridoxamine 5-phosphate oxidase deficiency, OMIM#610090), antiquitin deficiency (OMIM#266100) and tetrahydrobiopterin (BH_4) disorders including the following deficiencies: dominant and recessive form of guanosine triphosphate cyclohydrolase-I (GTPCH-1, OMIM#128230 and OMIM#233910 respectively), 6-pyruvoyltetrahydropterin synthase (PTPS, OMIM#261640), sepiapterin reductase (SR, OMIM#612716), dihydropteridine reductase (DHPR, OMIM#261630) and primapterinuria (OMIM#264070). Other disorders such as tyrosine hydroxylase deficiency and the recently reported transportopathies[5,6] impair dopamine biosynthesis in particular[7]. The clinical presentation of these disorders include symptoms related to autonomic dysfunction, which manifest as sweating, temperature dysregulation, hypersalivation, nasal congestion and psychiatric signs[8] such as bad behaviour and autistic features. Movement disorders are caused mainly by dopamine dysregulation, which includes gait disturbances, dystonia, dyskinesia, Parkinsonism, tremor, oculogyric crises, palpebral ptosis and axial hypotonia[7,9]. However, the symptoms that are thought to be related to serotonin deficiency are more difficult to clinically assess.

To estimate serotonin deficiency in these conditions, the analysis of cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5HIAA) is the most appropriate tool[10]. Furthermore, most of the patients harbouring these disorders received different treatment approaches to restore normal serotonin (and dopamine) levels in the brain. To evaluate the biochemical response to treatment, patients often undergo a lumbar puncture for the monitoring of brain serotonin status by 5HIAA quantification.

To evaluate whether urine aMT6s is a reliable biomarker for serotonin status in the brain, we first established normal values for aMT6s in urine in a healthy population. In a second step, we analysed urine samples from patients with pathogenic mutations in genes involved in the serotonin biosynthetic pathway.

Methods

Subjects. The reference values (RVs) for urinary aMT6s were established in 65 healthy control subjects (36 females; age range 2–61 years; mean = 10.0) with similar age to our patient population. Lighting environment during the night when the morning urine was recovered was controlled (in darkness). The duration of sleep on that night was between 8–11 hours. Exclusion criteria were the presence of any pharmacological conditions affecting serotonin status, evidence of drug or alcohol abuse and a history of shift work or travel across two or more time zones in the preceding month. After establishing the RVs, urine aMT6s was studied in 28 patients with genetic defects affecting serotonin biosynthesis (age range 2–55 years; mean = 13.0). Details of these patients are described in Tables 1 and 2. Out of these 28 cases, patients 1–18 were patients without treatment (naive patients) or patients on L-dopa/carbidopa therapy (Table 1), and the rest of cases (18bis-28) were under serotoninergic

Patients	Date of birth (age at analyses time)	Disease (#OMIM) *Gene* Mutations	aMT6s µmol/mol creatinine (ref. values)	% aMT6s reduction	CSF 5HIAA nmol/L (reduction, %)	Treatment	Dose (units)	Duration
1	1960 (56 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	4.1 (6.3–37.9)	−34.9	NP	L-dopa/carbidopa	300 mg/d	15y
2	1963 (53 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	8.4 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	400 mg/d	5y
3	1966 (50 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	18.2 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	375 mg/d	25y
4	1996 (20 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	12.7 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	75 mg/d	15y
5	2002 (14 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	19.8 (11.9–66.2)	No reduction	236 (no reduction) At 1 y of age (diagnose time)	L-dopa/carbidopa	130 mg/d	12y
6	1992 (24 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	38 (6.3–37.9)	No reduction	NP	No. Naive patient		
7	1998 (18 years)	dGTPCH (#233910) *GCH1* c.235_240delCTGAGC (p.L79_S80del)	21.2 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	62.5 mg/d	10 y
						Trihexypenidyl	6 mg/d	6y
8	1963 (52 years)	dGTPCH (#233910) *GCH1* c.235_240delCTGAGC (p.L79_S80del)	6.5 (6.3–37.9)	No reduction	NP	No. Naive patient		
9	2006 (10 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	13.4 (11.9–66.2)	No reduction	NP	No. Naive patient		
10	1974 (42 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	12.1 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	200 mg/d	7 y
11	1950 (66 years)	dGTPCH (#233910) *GCH1* c.265C>T (p.Q89X)	18.1 (6.3–37.9)	No reduction	NP	L-dopa/carbidopa	NA	NA
12	1971 (45 years)	dGTPCH (#233910) GCH1 c.68C>T (p.P23L)	19.8 (6.3–37.9)	No reduction	NP	No. Naive patient		
13	1967 (49 years)	dGTPCH (#233910) GCH1 c.265C>T (p.Q89X)	1.9 (6.3–37.9)	−69.8	NP	No. Naive patient		
14	2006 (10 years)	rGTPCH (#128230) GCH1 c.68C>T/c.265C>T(p.P23L/p.Q89X)	34.2 (11.9–66.2)	No reduction	NP	L-dopa/carbidopa	120 mg/d	4y
15	1997 (19 years)	SR (#612716) *SPR* c.304G>T/c.448A>G(p.G102C/p.R150G)	2.5 (6.3–37.9)	–60.3	25 (−60.3) At 11 y of age (diagnose time)	L-dopa/carbidopa	180 mg/d	8 y
16	1992 (24 years)	SR (#612716) *SPR* c.304G>T/c.448A>G(p.G102C/p.R150G)	4.8 (6.3–37.9)	−23.8	NP	L-dopa/carbidopa	300 mg/d	7 y
17	1990 (26 years)	SR (#612716) *SPR* c.304G>T/c.448A>G(p.G102C/p.R150G)	0.8 (6.3–37.9)	−87.3	NP	No. Naive patient		
18*	2014 (1 years)	AADC (#608643) *DDC* c.1041+1G>C/c.323G>A(IVS11 ds G-C +1 /p.S108N)	1.1 (19.4–79.1)	−94.3	1 (−99.4) At 1 y of age (diagnose time)	No. Naive patient		

Table 1. Biochemical, genetic and medical information of 18 patients (naive and under L-dopa/carbidopa treatment) (patients 1–18). *Patient 18 was studied in baseline conditions. The percentage of aMT6s reduction represents the decrease in aMT6s levels compared with the lower limit of the reference values in each age group. mo: months; ref. values: reference values; w- weeks; y: years; NA: not available; NP: not performed.

treatment (Table 2) including serotonin precursors (5-hydroxytryptophan, (5HTP)), monoamine oxidase inhibitors (MAOIs), selective serotonin re-uptake inhibitors (SSRI), cofactors that enhance serotonin/melatonin biosynthesis (BH_4, PLP and folinic acid) and melatonin treatment. Other treatments that were applied are also stated in Tables 1 and 2. Patient 18 was studied twice, at diagnosis (stated as patient 18 in Table 1) and after 3 months on MAOIs therapy (stated as patient 18bis in Table 2).

Patients	Year of birth (age at analyses time)	Disease (#OMIM) *Gene* Mutations	aMT6s µmol/mol creatinine (ref. values)	% aMT6s reduction	CSF 5HIAA nmol/L (reduction, %)	Treatment	Dose (units)	Duration
18 bis*	2014 (2 years)	AADC (#608643) *DDC* c.1041+1G>C/c.323G>A(IVS11 ds G-C +1 /p.S108N)	9.2 (19.4–79.1)	−52.6	**No further analyses on treatment**	**Selegiline**	0.5–0.5–0 mg	3 mo
19	2006 (10 years)	AADC (#608643) *DDC* c.1040G>A/c.1040G>A(p.R347Q/p.R347Q)	3.5 (11.9–66.2)	−70.6	10 (−94.1) At 1.5 y of age(diagnose time)	PLP	100 mg/d	3 mo
						Selegiline	10mg/d	2 w
						Ropinirol	5 mg /d	2 w
20	2009 (6 years)	AADC (#608643) *DDC* c.1040G>A/c.1040G>A(p.R347Q/p.R347Q)	7.4 (19.4–79.1)	−61.8	63 (−62.9) At 6 mo of age (diagnose time)	PLP	100 mg /d	2 w
						Selegiline	10 mg /d	2 w
21	2013 (3 years)	AADC (#608643) *DDC* c.799T>C /c.799T>C(p.W267R/p.W267T)	20.7 (19.4–79.1)	No reduction	27 (−84.1) At 1 y of age(diagnose time)	**Selegiline**	10 mg/d	Chronic treatment
						Ropinirol	2,25 mg/d	
22	2008 (8 years)	AADC (#608643) *DDC* c.367G>A/c.734C>T(p.G123R)/p.T245I)	285 (11.9–66.2)	Increase	25 (−85.3) At 11 mo of age (diagnose time)	Folinic acid	15 mg/d	6 y
						PLP	100–100–100 mg	6 y
						Bromocriptine	7.5–7.5–7.5 mg	7 y
						Selegiline	10.5–10.5–10.5 mg	7 y
						Fluoxetine	6 mg/d	4 y
						Melatonin	3 mg (12 drops) at night	5 y
23	2004 (11 years)	SR (#612716) *SPR* c.751A>T/c.751A>T (p.K251X/p.K251X)	1.1 (11.9–66.2)	−90.7	9 (−94.7) At 23 mo of age (diagnose time)	L-dopa/carbidopa	120 mg/d	10 y
						5HTP	60 mg/d	10 y
						Folinic acid	20 mg/d	10 y
24	2011 (5 years)	DHPR (#261630) *QDPR* c.661C>T/c.609dupA (p.R221Ter/P204TfsTer7)	900 (19.4–79.1)	Increase	158 (no reduction) At 12 mo of age (analysis on treatment)	L-dopa/carbidopa	160 mg/d	Chronic treatment
						5HTP	50 mg/d	
						Folinic acid	20 mg/d	
						BH_4	300 mg/d	
						Clonazepam	1,5 mg/d	
						Zonisamide	100 mg/d	
						Prednisone	8 mg q.o.d	
						Fluoxetine	5 mg/d	
25	2011 (5 years)	DHPR (#261630) *QDPR* c.609dupA/c.466G>A (p.P204TfsTer7)/p.A156T)	19.5 (19.4–79.1)	No reduction	9 (−94.7) At 2 y of age (diagnose time)	L-dopa/carbidopa	100 mg/d	Chronic treatment
						5HTP	50 mg/d	
26	2011 (5 years)	DHPR (#261630) *QDPR* c.384_387del/c.665C>T (p.Gly129Alafs*/p.Pro222Leu)	29.5 (19.4–79.1)	No reduction	133 (no reduction) At 5 years of age (analysis on treatment)	L-dopa/carbidopa	13.5 mg/d	Chronic treatment
						5HTP	8.5 mg/d	
27	2005 (11 years)	PTPS (#261640) *PTS* c.98A>G / c.297C>A(p.Asp33Gly/p.Tyr99Ter)	19.6 (11.9–66.2)	No reduction	65 (−61.8) At 1 year of age (on treatment) 169 (no reduction)At 11 years of age(on treatment)	L-dopa/carbidopa	33 mg/6 h	Chronic treatment
						5HTP	36 mg/6h	
						BH_4	150 mg/d	
						Calcium folinat	15 mg/d	
						Levothyroxine	50 µg/d	
28	2005 (11 years)	PTPS (#261640) *PTS* c.260C>T/c.260C>T(p.P87L/p.P87L)	2.2 (11.9–66.2)	−81.5	194 (no reduction) At 8 years of age(analysis on treatment)	L-dopa/carbidopa	400 mg/d	Chronic treatment
						5HTP	100 mg/d	
						BH_4	100 mg/d	

Table 2. Biochemical, genetic and medical information of 11 patients on treatment related to serotoninergic pathway (patients 18bis–28). *Patient 18bis was studied after serotoninergic treatment. Serotoninergic drugs are highlighted in bold. The percentages of aMT6s and cerebrospinal fluid (CSF) 5HIAA reduction were calculated from the lower limit of the reference values for each age group. aMT6s: urine 6-sulphatoxymelatonin expressed as µmol 6-sulphatoxymelatonin/mol creatinine. CSF 5HIAA values are expressed as nmol/L. q.o.d.: every other day. 5HTP: 5-hydroxytryptophan; BZP: benzodiazepines; Dopamine ag: dopamine agonist; MAOIs: monoamine oxidase inhibitors; mo: months; PLP: pyridoxal phosphate; ref. values: reference values; w- weeks; y: years.

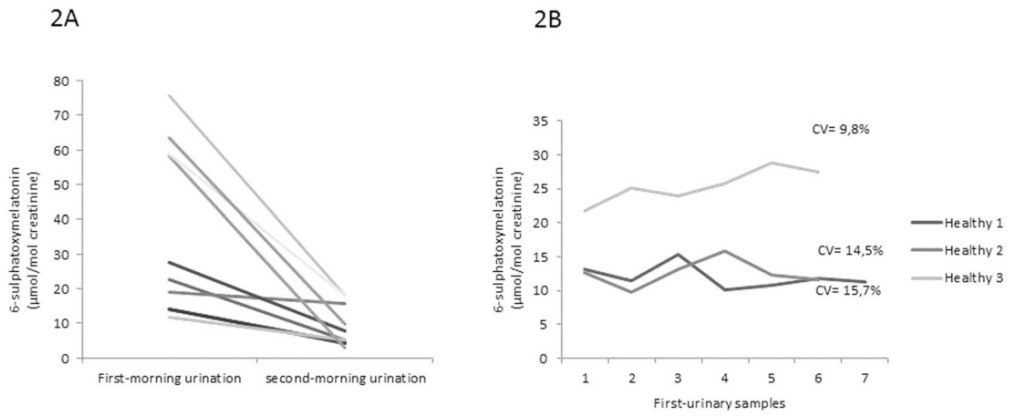

Figure 2. Assay variables. (**A**) Urine 6-sulphatoxymelatonin (aMT6s) values in the first and second morning urine samples in 10 healthy subjects. (**B**) Intra-individual variation of urinary aMT6s excretion in three healthy volunteers during 7 consecutive days.

Ethical issues. All samples from the patients were obtained in accordance with the 2013 revised Helsinki Declaration of 1964. For biochemical and genetic investigations, informed consent was collected from patients or their guardians. The Ethical Committee of Sant Joan de Déu Hospital approved the study.

Samples. For both the controls and the patients, the first morning urine samples were collected, centrifuged and stored at −20 °C until analysis. From the 28 patients, a total of 29 urine samples were collected, because, in one case with AADC deficiency, we analysed one urine sample at baseline conditions and again after therapy (patient 18). A total of 18 urine samples were taken from patients not treated with serotoninergic therapy (Table 1, patients 1–18). A further 11 urine samples were collected from subjects on different combinations of serotoninergic drugs (Table 2, patients 18bis-28).

In Tables 1 and 2, we also stated CSF 5HIAA values when available (13 out of 28 patients). Most of the CSF analyses were done during the treatment monitoring process (Table 2).

Urinary aMT6s analysis validation. *Assay variables.*

- To study urinary melatonin excretion changes related to the time of sampling, we analysed aMT6s values in the first and second morning urine samples in 10 healthy subjects (age range: 4–33 years) (Fig. 2A).
- To study the intra-individual variations of urinary aMT6s excretion, urinary aMT6s was analysed in three healthy volunteers during 7 consecutive days.
- To evaluate assay imprecision, we calculated the within-run and between-run coefficients of variation (CV = standard deviation/mean aMT6s values*100) in 10 replicates.
- After these metrological studies, RVs were established in the first morning urine samples, as reported by other groups[4,11,12].

Urinary aMT6s was analysed by duplicate using a competitive ELISA kit (IBL; ref RE54021) and the optical density measured with a photometer (ATOM S.A. Barcelona, Spain) at 405 nm and 630 nm. Coefficients of variation of the duplicates were calculated. Creatinine concentration was determined by an automated spectrophotometric assay in the Architect c8000 analyser (Abbott). Results are expressed as µmol aMT6s/mol creatinine.

Statistical analysis. The Kolmogorov–Smirnow test was used to assess the distribution of the data. Because the data did not follow a Gaussian distribution, different non-parametric tests were applied. The Spearman simple correlation test was used to determine the correlations between urinary aMT6s and patient age. The Kruskal–Wallis and Mann–Whitney U tests were used to compare urinary aMT6s excretion among the different age groups and also between GTPCH naive patients and those under L-dopa/carbidopa treatment. Statistical calculations were performed using SPSS 23.0 software.

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Assay variables. The first morning urine sample showed the highest aMT6s concentrations in the 10 healthy cases, which remarkably dropped down in the second morning sample (Fig. 2A). The mean reduction from the first to the second urine samples was 68.7%.

The intra-individual variation studied in the three volunteers showed a CV value of 14.5%, 15.1% and 9.9%, respectively (Fig. 2B).

Group	Median (µmol aMT6s /mol creatinine)	Min-Max
1 (2–6 years) n = 14	41.5	19.4–79.1
2 (7–14 years) n = 29	25.3	11.9–66.2
3 (> 15 years) n = 22	18.6	6.3–37.9

Table 3. Urine 6-sulpatoxymelatonin (aMT6s) values of control population. Reference values were established in three different groups and are expressed as median, minimum and maximum values. The Mann-Whitney U test showed statistical differences between groups 1 and 2 (U = 28.8; $p < 0.001$), between groups 1, 2 and 3 (U = 9.0; $p < 0.001$) and between groups 2 and 3 (U = 34.0; $p < 0.001$). Units = µmol aMT6s /mol creatinine.

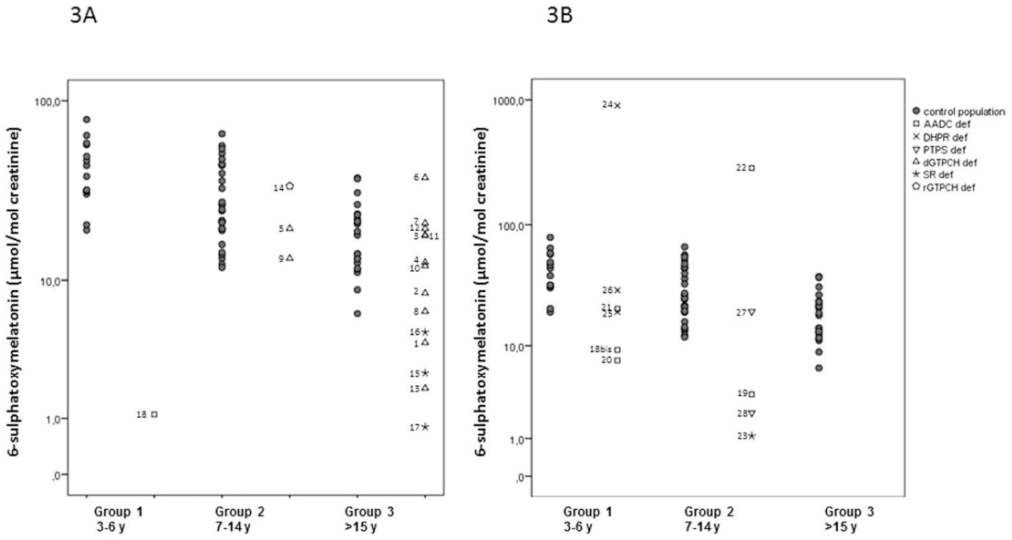

Figure 3. Representation of urine 6-sulphatoxymelatonin (aMT6s) values from the reference population and patients with serotonin defects without serotoninergic treatment (**A**) and with serotoninergic treatment (**B**). Each disease is represented by different geometric shapes: square-AADC deficiency; cross-DHPR deficiency; inverted triangle-PTPS deficiency; triangle-adGTPCH deficiency; star-SR deficiency, and hexagon-arGTPCH deficiency. Each number matches with the patient's number as shown in Tables 1 and 2. AADC: aromatic L-amino acid decarboxylase; DHPR: dihydropteridine reductase; GTPCH: guanosine triphosphate cyclohydrolase I; PTPS: 6-pyruvoyl-tetrahydropterin synthase; SR: sepiapterin reductase.

Regarding the imprecision assay studies, within-run CV was 17.0% for aMT6s = 11.5 ng/mL and between-run CV was 10.6% for aMT6s = 114.6 ng/mL.

Urinary aMT6s was analysed by duplicate. In the 80% studied samples, the variation of the duplicates was <10%, in the 10% studied samples was 10–15% and in the other 10% of studied samples the variation was >15%.

Establishment of reference values. In the healthy population (n = 65), a negative correlation was observed between urine aMT6s in the first morning urine sample and the age ($r = -0.591$; $p < 0.001$, Spearman test). RVs for our population were stratified according to age in three different groups as statistical differences were found (U = 28.8, $p < 0.001$ in groups 1–2; U = 9.0, $p < 0.001$ in groups 1–3; U = 34.0, $p < 0.001$ in groups 2–3; Table 3; Fig. 3).

Patients not treated with serotoninergic treatment (Table 1). Naive patients (patients 6, 8, 9, 12, 13, 17 and 18) and cases under L-dopa/carbidopa therapy (patients 1–5, 7, 10, 11, 14–16) were included (n = 18). Out of 18 cases, we studied 13 patients with autosomal dominant GTPCH (adGTPCH) deficiency (patients 1–13); 1 patient with autosomal recessive GTPCH (arGTPCH) deficiency (patient 14); 3 patients with SR deficiency (patients 15–17); and 1 patient with AADC deficiency (patient 18). We compared the results with those from our RVs (Table 1 and Fig. 3A). Regarding patients with adGTPCH deficiency (n = 13), only 2 presented decreased urine aMT6s values (patients 1 and 13; 34.9% and 69.8% of aMT6s reduction, respectively). No statistical differences were observed between adGTPCH patients and the control group. Patient with arGTPCH form (patient 14) had normal aMT6s values, whereas patients with SR and AADC deficiencies had very low urine aMT6s values compared to their age reference group.

No differences were observed in urinary aMT6s values when compared GTPCH naive patients (median = 13.4) and those under L-dopa/carbidopa therapy (median = 18.1; U = 20.5; $p = 0.797$).

Patients under serotoninergic treatment (Table 2). We studied five patients with AADC deficiency (patients 18bis–22), one with SR deficiency (patient 23), three with DHPR deficiency (patients 24–26) and two with PTPS deficiency (patients 27–28). Details of the different treatments and results are stated in Table 2 and Fig. 3B. Three AADC deficiency cases (patients 18bis–20) presented low urine aMT6s values, while patient 21 showed normal aMT6 values. Patient 18bis was studied twice, at diagnosis (stated as patient 18) and after 3 months on MAOIs therapy (stated as patient 18bis). In this condition, aMT6s values increased from 1.1 to 9.2 µmol/mol creatinine, although they remained below the RVs (Table 1 and Table 2). Patient 22 was on melatonin therapy and therefore had a higher aMT6s concentration than RVs.

Regarding patients with pterin-related defects, some showed decreased aMT6s excretion despite receiving 5HTP treatment. Among them, the SR-deficient case (patient 23) and PTPS-deficient case (patient 28) showed remarkably reduced urinary aMT6s excretion (90.7% and 81.5%, respectively), while the DHPR-deficient cases (patients 24–26) and one PTPS-deficient case (patient 27) presented normal aMT6s values.

Discussion

This is the first report that assessed urinary aMT6s concentrations in genetic conditions that cause a severe effect on brain serotonin synthesis. Regarding assay variables, we corroborated that the first morning urine was the most suitable sample because the majority of melatonin is metabolized and excreted into urine as aMT6s[4]. Thus, any urine leak during the night would noticeably decrease aMT6s concentrations[12]. This fact would explain the unexpected low aMT6 value observed in two of our patients under serotoninergic treatment, who probably had troubles in urine sample collection (patient 18, who was 2 years old, and patient 23, because he had urinary continence problems).

Our analytical studies have shown that intra-individual variation only ranged from 9.9% to 15.1% (Fig. 2B). Furthermore, the metrological variations obtained were also reasonable for a proper interpretation of the results. Only 10% of patients presented CV of the duplicates higher than 15%, which was probably due to cross-reaction phenomena related to immune-based laboratory methods[11]. Thus, such samples were not considered in the study and should be reanalysed.

Regarding RVs, we established three different age groups (Table 3). Our results can be explained by the fact that melatonin synthesis reaches the highest rate at the age of 3–6 years, and later it decreases progressively by 80% until adult age[1]. These RVs are similar to others previously reported[13], which supports the reproducibility of the ELISA method for aMT6s quantification in different populations.

Considering patients without serotoninergic treatment we studied 13 patients with adGTPCH deficiency, and 11 of them presented normal values of aMT6s. The adGTPCH deficiency is the mildest disease among those studied here, because the patients have a mild to moderate reduction in dopamine and serotonin biomarkers in CSF and were even normal in some cases. In fact, in patient 5 (the index case of one family[14]), CSF 5HIAA concentrations were normal at the time of diagnosis, which suggests minimal or no alteration in brain serotonin status. Moreover, most of our adGTPCH-deficient patients harboured the same mild mutation (p.Q89X) at the *GCH1* gene as patient 5, and this fact would explain that adult cases from this cohort present a very mild (or even symptom-free) phenotype, as previously reported.

arGTPCH deficiency usually show PKU and has an early onset with a more severe clinical course than the adGTPCH deficiency[15]. Urine aMT6s levels were also normal in one case (patient 14) with arGTPCH deficiency, who showed normal phenylalanine levels and a phenotype resembling the dominant form of GTPCH deficiency, which suggested high GTPCH residual activity.

SR deficiency is inherited in an autosomal recessive manner. Patients present with a diurnally fluctuating motor disorder, and in most cases, it is associated with cognitive delay and severe neurologic dysfunction. The three patients reported here are siblings and they showed an important reduction of aMT6s levels (60.3%, 23.8% and 87.3%). In the index case (patient 15), the reduction of CSF 5HIAA at the time of diagnosis was also remarkable (Table 1). These three patients presented a mild phenotype with a late-onset presentation[16]. Moreover, they were under treatment with only L-dopa/carbidopa, as 5HTP was trialled some years ago, but the treatment was discontinued due to side effects (vomiting and diarrhoea). They presented a novel mutation in the *SPR* gene that affects splicing, which was reported as a mild change[16]. In SR deficiency, the dopamine and serotonin pathways are usually severely affected[17], and the low levels of aMT6s could be a reflection of the impaired brain serotonin status.

Patient 18, with a severe form of AADC deficiency (at age of 1 year, she showed hypotonia, oculogyric crises and dystonia), presented, as expected, an extremely low value of urinary aMT6s, which was related to the concomitant dramatic reduction of the CSF 5HIAA values.

It has been reported that L-dopa therapy may be toxic to serotoninergic neurons in cell cultures by oxidative mechanisms producing highly reactive quinone species that reduce serotoninergic neurons[18]. These findings also have been observed "*in vivo*" by similar oxidative mechanisms producing a significant decrease in serotonin and 5HIAA metabolite[19], as well as affecting the behaviour and cognitive functions in animal models[19]. However, no differences were observed when compared urinary aMT6s values between naive GTPCH patients and those under L-dopa/carbidopa treatment. It is interesting that carbidopa treatment (an inhibitor of peripheral AADC activity) does not seem to affect urine aMT6s excretion, emphasizing that the contribution of peripheral melatonin is less relevant than that of pineal gland[1].

Regarding patients under serotoninergic treatment, three AADC-deficient patients showed low aMT6s concentrations despite serotoninergic treatment. In patient 18, urinary aMT6s excretion increased after 3 months of MAOIs therapy, which suggests that this therapy improves serotonin and melatonin status, although the aMT6s value was still below the normal values. Two patients (patients 19–20) with a severe phenotype, extremely low CSF 5HIAA levels at diagnosis, and who were under MAOIs and PLP therapy, showed reduced aMT6s urinary

excretion, while patient 21 with a moderate phenotype, who was also on therapy with MAOIs and PLP, showed normal urinary aMT6s values. An explanation for these data is that AADC is the most severe condition affecting brain serotonin status[20] with a very reduced capacity of serotonin and melatonin biosynthesis. Further investigations are required to determine whether long-term MAOIs treatment can normalize aMT6s excretion in AADC patients. Patient 22 with a mild phenotype, who was on MAOIs, SSRI and melatonin therapy, showed higher aMT6s values than the RVs.

Regarding patients with BH_4-related defects, patient 23 with SR deficiency was diagnosed at 23 months of age and presented the typical phenotype with psychomotor retardation, hypotonia, ataxia and extrapyramidal signs[16]. CSF neurotransmitter studies showed a classical SR deficiency pattern with severely decreased 5HIAA values in CSF. We found very low urinary aMT6s values despite the patient being under 5HTP therapy, which should have increased serotonin and melatonin biosynthesis (Fig. 1). His parents reported night urinary incontinency problems, a fact that could explain the unexpectedly low aMT6s values of the patient.

All DHPR-deficient patients had normal urine aMT6s values. Although these patients were under different treatment regimens, all of them received 5HTP, the serotonin precursor. Patient 24 was also on long-lasting treatment with SSRI, folinic acid and BH_4. At 12 months of age (when she was already under this treatment), she presented normal CSF 5HIAA values. At 5 years of age, she showed high urine aMT6s values, which suggested that the combination of all these drugs may have led to this result. In fact, it has been suggested that some SSRI drugs may increase plasma melatonin values[21]. Patients 25 and 26 undertook only 5HTP therapy, and both showed normal aMT6s excretion, which supports the hypothesis that this serotonin precursor is effective in increasing urinary aMT6s excretion. However, the reduced size of our series strongly advises the development of clinical trials to confirm or rule out this hypothesis.

PTPS-deficient patient 27 was diagnosed by a newborn screening program, and at 1 year of age (when she was already on treatment), she showed mildly reduced CSF 5HIAA values. Currently, this patient is receiving 5HTP, BH_4, and calcium folinate as serotoninergic drugs. The last CSF analysis reported normal CSF neurotransmitter concentrations, which suggest adequate therapy; the urinary aMT6s values were also normal. Unexpectedly, patient 28 had very low aMT6s values despite being under 5HTP and BH_4 treatment, and had underwent a previous CSF 5HIAA analysis that showed normal results. In this case, the doctor and the family confirmed that the urine was correctly sampled; one explanation we have, may be related to individual variations on CYP1A2 activity. CYP1A2 is one of the major isoforms of cytochrome P450 in the liver and metabolizes a number of clinical drugs and several important endogenous compounds, such as melatonin[22]. There is a large inter-individual variability in the expression and activity of CYP1A2, which is caused mainly by genetic (several polymorphisms) and environmental factors[23]. Other explanation woud be a possible cerebral folate deficiency, since HIOMT needs S-adenosylmethionine (SAM) as a cofactor for melatonin biosynthesis. However, CSF 5-methyltetrahydrofolate values were normal in this patient.

A limitation of this study is that aMT6s determination strictly depends on a proper sample collection, which may be difficult in severely handicapped infants. Moreover, in some cases, the inter-individual variations of CYP1A2 activity[22,23] could contribute to unexpected variations in urinary aMT6s excretion. Another important aspect of melatonin fluctuation is that it is greatly influenced by dim light, whereas very bright light can block melatonin production[11]. Finally, the simultaneous measurement of both CSF 5HIAA and urinary aMT6s in larger series of patients should be done to stablish a correlation between these two variables.

In conclusion, we studied 28 patients with different genetic serotoninergic metabolism defects and found that decreased excretion of aMT6s is frequently observed in all patients with more severe disorders. No consistent alterations were documented in the adGTPCH deficiency, which is the mildest disease studied here. Sulphatoxymelatonin can be a non-invasive good biomarker to estimate serotonin status in the brain, especially for treatment monitoring purposes.

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Author Contributions

M.B. and M.M.L. contributed to conception and design, acquisition, analysis and interpretation of data, drafted the initial manuscript, and approved the final manuscript as submitted. A.O. contributed to analysis and interpretation of data, reviewed and revised the manuscript, and approved the final manuscript as submitted. L.A., J.D.L.H., J.A.F.R., L.G.G.S., S.I.M., R.D., J.C., T.O., B.Z., R.P., A.G.C., F.S. and E.L.L. contributed to acquisition of data, reviewed and revised the manuscript, and approved the final manuscript as submitted. R.A. contributed to conception and design, analysis and interpretation of data, reviewed and supervised the manuscript, and approved the final manuscript as submitted. Every one of the authors has participated sufficiently in the study, meeting the appropriate authorship criteria, and each has seen, reviewed and approved this version of the manuscript and takes full responsibility for it. We all agree to its submission for publication. Nobody who qualifies for authorship has been omitted from the list of authors. All the authors have complete access to the study data. Considering that the work has been performed with the collaboration of different Hospitals of Spain, and also from Greece and Germany, we believe that we are 17 authors who have contributed and participated in this multidisciplinary work.

Additional Information

Competing Interests: Yes there is potential Competing Interest. MedDay Pharmaceuticals gave economical support for the purchase of melatonin ELISA kits.

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OBJECTIU 4: Avaluació retrospectiva del fenotip clínic i perfils bioquímics d'amines biògenes en líquid cefalorraquidi de pacients diagnosticats genèticament d'una malaltia mitocondrial.

PUBLICACIÓ 4:

Títol: Cerebrospinal fluid monoamines, pterins, and folate in patients with mitochondrial diseases: systematic review and hospital experience.

Autors: Marta Batllori, Marta Molero-Luis, Aida Ormazabal, Raquel Montero, Cristina Sierra, Antonia Ribes, Julio Montoya, Eduardo Ruiz-Pesini, Mar O'Callaghan, Leticia Pias, Andrés Nascimento, Francesc Palau, Judith Armstrong, Delia Yubero, Juan D. Ortigoza-Escobar, Angels García-Cazorla, Rafael Artuch.

Referència: *J Inherit Metab Dis*. 2018 Nov;41(6):1147-1158.

Resum:

Les malalties mitocondrials són un grup de trastorns neurometabòlics amb diferents mecanismes fisiopatològics que acaben conduint principalment a un dèficit energètic. Tenint en compte que la neurotransmissió és un procés biològic amb elevada demanda energètica i que no és infreqüent observar fenotips clínics relacionats amb trastorns en la neurotransmissió, principalment dopaminèrgica, podria ser que aquest procés es veiés afectat en pacients amb malalties mitocondrials. Per altra banda, les malalties mitocondrials encara presenten actualment, importants dificultats diagnòstiques motiu pel qual pensem que la realització d'una anàlisi metabolòmica en LCR podria aportar informació en l'estudi d'aquests pacients tant des d'un punt de vista de diagnòstic com de tractament.

Per tant, i tenint en compte la manca de treballs científics en pacients amb malalties mitocondrials vam creure oportú avaluar, tant des del punt de vista bioquímic com fenotípic, una cohort llarga de pacients amb diagnòstic genètic de malaltia mitocondrial.

Síntesi de resultats:

Alteracions en els valors d'HVA i 5-HIAA

En vuit de 29 pacients, la concentració d'HVA en LCR va ser alta; en 5 pacients, va ser baixa, i en els 16 pacients restants, els valors de HVA van ser normals. Els vuit casos amb valors elevats d'HVA tenien síndromes associades a mutacions en el mtDNA (cinc delecions del mtDNA, dues mutacions puntuals) i un presentava mutacions bial·lèliques al gen *POLG*, que comportaven la depleció del mtDNA.

En tres pacients amb síndromes de deleció/depleció del mtDNA, tant el 5-HIAA com l'HVA van estar simultàniament elevats.

En sis dels 29 pacients, el 5HIAA estava disminuït i, en tres d'ells, concomitantment amb l'HVA. En els vint pacients restants, el 5-HIAA va ser normal.

Clínicament, cinc dels 29 casos van presentar predominantment signes dopaminèrgics. Aquests cinc casos presentaven mutacions en gens nuclears. No hi va haver una associació significativa entre les alteracions en les concentracions d'HVA i la presència d'un quadre clínic predominantment de disfunció dopaminèrgica. Des del punt de vista radiològic no va haver-hi una associació entre l'afectació dels ganglis basals i el fenotip clínic, ni tampoc amb les concentracions de l'HVA. Alguns pacients amb alteracions en els nivells dels neurotransmissors van presentar una ressonància cerebral normal.

El pacient 11, que presentava mutacions en SLC19A3, tenia valors baixos d'HVA en LCR i troballes anormals de 18F-FDOPA (fluorodihidroxifenilalanina) en el PET (bilateral, asimètric (esquerra> dreta) i una depleció irregular i greu dopaminèrgica a l'estriat) i, després de rebre tractament amb L-dopa, presenta una millora parcial dels signes clínics de distonia-parkinsonisme. L'administració de L-dopa es va indicar a una dosi de 4 mg / kg / dia (2 g / dia). Durant el període d'administració, van aparèixer moviments involuntaris (tics del cap) i, per tant, la dosi de L-dopa es va augmentar molt lentament. Va rebre L-dopa durant deu mesos.

Deficiència cerebral de folat amb valors elevats d'HVA en LCR

Els pacients amb valors elevats de lactat en LCR no van mostrar una associació significativa ni amb l'HVA ni amb un fenotip clínic concret. No es van detectar fenotips clínics específics atribuïbles a la deficiència de serotonina i no es van poder demostrar associacions entre l'estat de 5HIAA i el fenotip clínic o radiològic. Només dos pacients van presentar una depressió relacionada a una deficiència serotoninèrgica. Respecte a

les pterines, no va haver-hi cap associació entre els valors de la neopterina i biopterina i les concentracions de les monoamines. Quatre dels 29 estudiats van presentar valors de neopterina superiors a 61 nmol/L, que és el cut-off prèviament establert per events inflamatoris.

Hi va haver una associació estadísticament significativa entre concentracions elevades d'HVA i baixes de folat (X^2-square=10.916, *p=0.001*) i elevades de proteïnes en LCR (X^2-square=10.429, *p=0.001*). Set dels vuit pacients amb valors elevats d'HVA en LCR van mostrar dèficit de folat cerebral i nivells elevats de proteïnes en LCR. Cinc d'ells van presentar delecions en el mtDNA associades a Síndrome de Kearn-Sayre; un, una mutació puntual en el gen *ATPase6* del mtDNA i un altre una mutació del gen*POLG*. Tres pacients d'aquests van presentar valors de 5-HIAA francament elevats. Quatre dels 21 pacients estudiats van mostrar una lleugera deficiència de folat cerebral sense associació a valors elevats d'HVA.

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ORIGINAL ARTICLE

Cerebrospinal fluid monoamines, pterins, and folate in patients with mitochondrial diseases: systematic review and hospital experience

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Abstract

Mitochondrial diseases are a group of genetic disorders leading to the dysfunction of mitochondrial energy metabolism pathways. We aimed to assess the clinical phenotype and the biochemical cerebrospinal fluid (CSF) biogenic amine profiles of patients with different diagnoses of genetic mitochondrial diseases. We recruited 29 patients with genetically confirmed mitochondrial diseases harboring mutations in either nuclear or mitochondrial DNA (mtDNA) genes. Signs and symptoms of impaired neurotransmission and neuroradiological data were recorded. CSF monoamines, pterins, and 5-methyltetrahydrofolate (5MTHF) concentrations were analyzed using high-performance liquid chromatography with electrochemical and fluorescence detection procedures. The mtDNA mutations were studied by Sanger sequencing, Southern blot, and real-time PCR, and nuclear DNA was assessed either by Sanger or next-generation sequencing. Five out of 29 cases showed predominant dopaminergic signs not attributable to basal ganglia involvement, harboring mutations in different nuclear genes. A chi-square test showed a statistically significant association between high homovanillic acid (HVA) values and low CSF 5-MTHF values (chi-square = 10.916; $p = 0.001$). Seven out of the eight patients with high CSF HVA values showed cerebral folate deficiency. Five of them harbored mtDNA deletions associated with Kearns-Sayre syndrome (KSS), one had a mitochondrial point mutation at the mtDNA *ATPase6* gene, and one had a *POLG* mutation. In conclusion, dopamine deficiency clinical signs were present in some patients with mitochondrial diseases with different genetic backgrounds. High CSF HVA values, together with a severe cerebral folate deficiency, were observed in KSS patients and in other mtDNA mutation syndromes.

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Introduction

The monoamines dopamine and serotonin are neurotransmitters displaying key roles in the brain. Dopaminergic and serotoninergic neurons are widespread and inter-connected in the brain, which explains their important role in many physiological functions such as control of movement; neuroendocrine secretion; cognitive and emotional mechanisms; and the autonomous control of breathing, temperature, sleep, and mood (Carlsson 2001; Kurian et al. 2011). Primary genetic diseases affecting their metabolism have been related to a growing group of neurological syndromes whose symptoms and signs often overlap with other neurological conditions.

Figure 1 depicts the metabolic pathways for dopamine and serotonin metabolism and known genetic conditions leading to dopamine and serotonin deficiency (Rodan et al. 2015; Ng et al. 2015; Kurian et al. 2011). The presentation of

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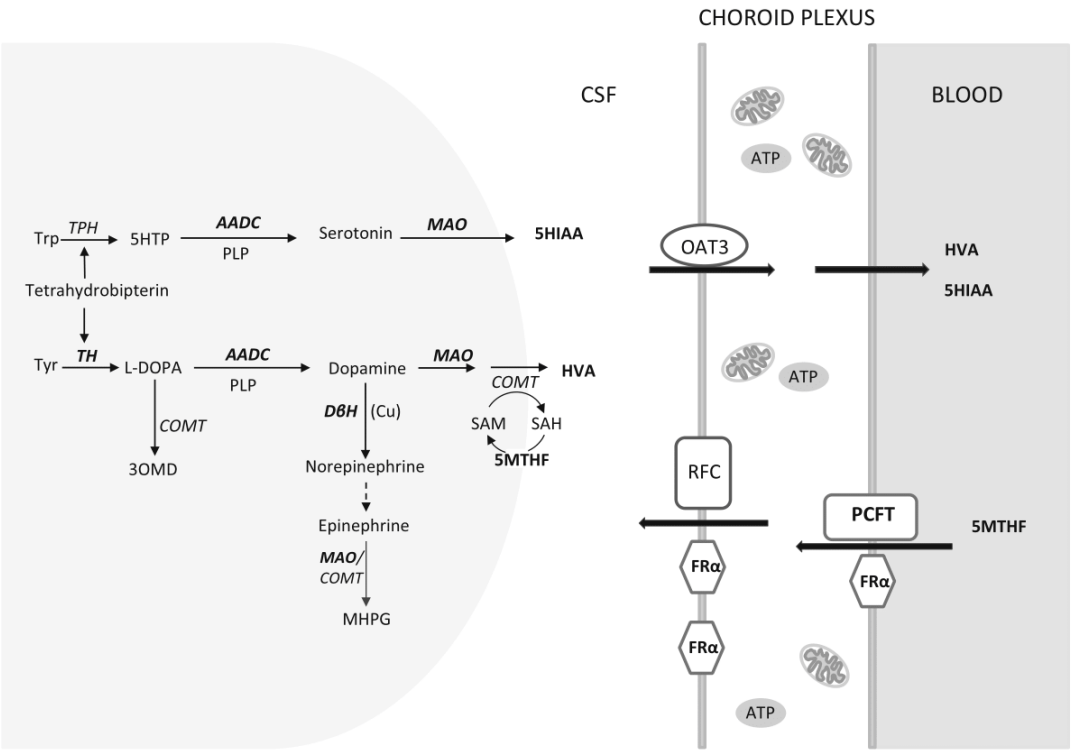


Fig. 1 Schematic representation of dopamine and serotonin metabolism-related pathways together with the choroid plexus folate and monoamines transport systems. AADC: aromatic L-amino acid decarboxylase; ATP: adenosine triphosphate; COMT: catechol O-methyltransferase; CSF: cerebrospinal fluid; Cu: copper; DβH: dopamine beta hydroxylase; FRα: folate receptor alpha; 5HIAA: 5-hydroxyindoleacetic acid; 5HTP: 5-hydroxytryptophan; 5-MTHF: 5-methyltetrahydrofolate; HVA: homovanillic acid; L-DOPA: 3,4-dihydroxyphenylalanine; MAO: monoamine oxidase; MHPG: 3-methoxy-4-hydroxyphenylglycol; OAT3: organic anionic transporter type 3; 3OMD: 3-O-methyldopa; PCFT: proton-coupled folate transporter; PLP: pyridoxal phosphate; RFC: reduced folate carrier; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; TH: tyrosine 3-hydroxylase; TPH: tryprophan-5-hydroxylase; Trp: tryptophan; Tyr: tyrosine. Genetic defects are highlighted in bold and italics

the primary defects ranges from cognitive and motor delay (often with diurnal variation), epilepsy, and autonomic dysfunction to neuropsychiatric signs such as anxiety or autistic spectrum disorder (Pearl et al. 2005; Kurian et al. 2011). Many of these clinical features overlap with other genetic neurological conditions (Molero-Luis et al. 2013a, b), emphasizing the importance of deep clinical and biochemical phenotyping.

The analysis of CSF monoamine biomarkers includes measuring the levels of 3-orthomethyldopa, 3-methoxy 4-hydroxyphenylglycol and homovanillic acid (HVA), and 5-hydroxytryptophan and 5-hydroxyindoleacetic acid (5HIAA) for the assessment of the dopamine and serotonin pathways, respectively (Hyland et al. 1993; Ormazabal et al. 2005). Especially relevant are the end degradation products, HVA and 5HIAA, which may provide insight into disturbances of these monoamines in biosynthesis, catabolism, transport, and removal from the brain (Marecos et al. 2014). Measurement of neopterin and biopterin provides further insights into tetrahydrobiopterin status, an essential cofactor for dopamine and serotonin biosynthesis (Kurian et al. 2011). Moreover, CSF neopterin concentrations above 61 nmol/L act as a biomarker for inflammatory/immune events (Molero-Luis et al. 2013a, b). There are a large number of neurological disorders caused by both environmental and genetic conditions that also exhibit abnormal monoamine profiles (Van Der Heyden et al. 2003; Serrano et al. 2010; García-Cazorla et al. 2007; De Grandis et al. 2010; Molero-Luis et al. 2013a, b; Horvath et al. 2016; Kuster et al. 2018).

5-methyltetrahydrofolate (5-MTHF) is an essential cofactor for methylation reactions in the brain and other organs via S-adenosylmethionine. It participates, for example, in the catabolism of dopamine to its final metabolite HVA. Cerebral folate deficiency (defined as low CSF 5-MTHF values with normal blood folate status) has been described as associated with different neurogenetic and environmental conditions (Pérez-Dueñas et al. 2011) and specifically to some mitochondrial disorders (Garcia-Cazorla et al. 2008a).

Mitochondrial diseases are a large and heterogeneous group of genetic disorders (as a result of mutations in either mitochondrial [mtDNA] or nuclear genes) leading to a dysfunction of the mitochondrial oxidative phosphorylation

process and other energy metabolism–related pathways. It has been suggested that energy-metabolism defects can affect the neurotransmitter system (Garcia-Cazorla et al. 2008a, b) because synapses are enriched in high potential-dependent mitochondria (Ly and Verstreken 2006). It is known that the synapse engages in high-energy consumption, and mitochondria provide up to 90% of the ATP needed to maintain the synaptic membrane potential (Ly and Verstreken 2006). This also seems to fuel numerous steps of the vesicle cycle such as scission, uncoating, refilling with neurotransmitters, and vesicular transport to the synaptic terminal (Murthy and De Camilli 2003). Thus, it seems plausible that an altered neurotransmitter status can be observed in this group of disorders. Some patients with mitochondrial diseases have presented with clinical features of parkinsonism and other movement disorders (Garcia-Cazorla et al. 2008a, b; Ghaoui and Sue 2018; Kuster et al. 2018; Tzoulis et al. 2016). More consistent is the association between cerebral folate deficiency and mitochondrial disorders, with the choroid plexus probably being the target organ for this association (Tanji et al. 2000).

Thus, in this work, we aimed to review the most up-to-date knowledge about the association between mitochondrial diseases and CSF monoamine and 5-MTHF status, and retrospectively assess the clinical phenotype and the CSF status of these biomarkers in patients with different genetic diagnoses of mitochondrial energy-metabolism diseases.

Materials and methods

Subjects We recruited, over the past 12 years, 29 patients with genetically confirmed mitochondrial diseases harboring mutations in either nuclear or mtDNA genes. Clinical, biochemical, radiological, and molecular data of these 29 patients are presented in Table 1.

Samples Blood, urine, CSF samples, and when indicated, skin/muscle biopsies (for diagnosis of mtDNA deletion or depletion syndromes) were collected for diagnostic purposes. CSF samples were obtained by a protocolized lumbar puncture as previously described (Ormazabal et al. 2005). DNA from blood and muscle samples was isolated using standard procedures. Samples were taken in accordance with the 2013 revised Helsinki Declaration of 1964. The parents of the patients signed informed consent and the ethical committee of Sant Joan de Déu Hospital approved the study.

Clinical exploration Four expert neuropediatricians reviewed the patient's clinical records and established age at onset, gene-related phenotype, and signs and symptoms of impaired neurotransmission: (1) dopamine deficiency: parkinsonism-dystonia, dyskinesia, axial hypotonia/limb hypertonia, oculogyric crisis, ptosis, hypersalivation; and (2) serotonin deficiency: insomnia, depression, temperature instability, abnormal gastrointestinal motility. Each neuropediatrican reviewed a subset of cases. Details of the clinical phenotypes are given in Table 1.

Neuroradiological exploration Magnetic resonance imaging (MRI)/magnetic resonance spectroscopy (MRS) studies were done: basal ganglia disturbances (most of them presenting as Leigh syndrome) or other features (white matter and corpus callosum lesions and ischemic strokes) were assessed. Details of the radiological phenotype are provided in Table 1.

Biochemical analysis CSF monoamines (HVA and 5HIAA), pterins (neopterin and biopterin), and 5-methyltetrahydrofolate (5-MTHF) concentrations were analyzed using high-performance liquid chromatography with electrochemical and fluorescence detection procedures as previously reported (Ormazabal et al. 2005; Ormazabal et al. 2006). CSF lactate and total protein values were analyzed by automated spectrophotometric analysis.

Genetic analysis Because the patients were studied over the past 12 years, different approaches were applied for genetic diagnosis. The different mtDNA mutations were studied by specific procedures, including Sanger sequencing, Southern blot, and real-time PCR, as previously reported (O'Callaghan et al. 2015; Montiel-Sosa et al. 2013). Nuclear DNA was assessed either by Sanger sequencing, depending on the suspected causative gene, or, over the past 4 years, by next-generation sequencing (NGS) using customized (Yubero et al. 2016) or commercial panels (TruSight One Sequencing Panel, Illumina) in MiSeq or NextSeq500 sequencers (Illumina). Progenitor studies were performed to evaluate the inheritance model and to establish the molecular diagnosis.

Statistical analysis and data assessment Reference values for monoamines are strongly related to age. We categorized the clinical, radiological, and biochemical data as normal or impaired (see Table 1). Then, statistical associations among the variables were studied with the chi-square test for the whole group of patients. Quantitative data are displayed in Table 1 and Figs. 2 and 3.

Systematic review The literature published from 1980 to March 2018 were systematically searched in PubMed. To avoid any risk of bias, general search terms were chosen (mitochondria/mitochondrial, oxidative phosphorylation, monoamines, homovanillic, 5-hydroyindoleacetic, pterins, folate, 5-methyltetrahydrofolate). Inclusion criteria were studies in humans, monoamines and/or folate analyzed in the CSF, and patients with mitochondrial diseases.

Table 1 Main clinical and biochemical and genetic diagnosis from the 29 cases included in the study are stated. Patients were ordered according to high, low, and normal cerebrospinal fluid HVA concentrations compared with our reference ranges. The whisker indicates patients previously reported

Patient	Age (years)/sex	Disease (OMIM) *gene (OMIM)*	CSF HVA nmol/L	CSF HIAA nmol/L	CSF 5MTHF nmol/L	CSF neopterin/biopterin nmol/L	Dopaminergic symptoms (yes or not)	Clinical and radiological features
1	9 years/F	Kearns-Sayre syndrome (#530000) mtDNA deletion	1297 ↑	387 ↑	18 ↓	87↑/28	No	Hypoglycemia, failure to thrive, nephrocalcinosis, ptosis, ophthalmoplegia. MRI (9y) normal. MRS normal.
2	4 years/F	Mitochondrial DNA depletion syndrome 4A (Alpers type) (#203700) *POLG* (*174763)	1134 ↑	405 ↑	3 ↓	31/27	Yes	Parkinsonism, hypotonia/pyramidal signs, gait impairment, epileptic status, psychomotor regression. MRI (4y)—T2W calcarine sulcus hyperintensities
3*	7 years/M	Kearns-Sayre syndrome (#530000) mtDNA deletion	844 ↑	474 ↑	8 ↓	55/32	No	Cognitive regression, abnormality of retinal pigmentation, renal insufficiency, hearing loss, MRI (10y) Caudate ischemic lesions, leukoencephalopathy MRS lactate peak
4	3 years/M	Leigh syndrome (#256000) *MTTV* (*590105)	802 ↑	255	63	24 / 12	No	Psychomotor regression, status epilepticus, axonal neuropathy, dystonia. MRI (2Y) t2W putamen and thalamus hyperintensities—Leigh syndrome
5	3 years/F	Leigh syndrome (#256000) - *MTATP6* (*516060)	802 ↑	379	25 ↓	44 / NA	No	Hearing loss, gait impairment, exercise intolerance, dysarthria, dysphagia, failure to thrive. MRI (3y) T2W caudate and putamen hyperintensities - Leigh syndrome
6*	12years/M	Kearns-Sayre syndrome (#530000) mtDNA deletion	670 ↑	266	4 ↓	31/13	No data	No data
7*	22years/M	Kearns-Sayre syndrome (#530000) mtDNA deletion	487 ↑	109	1 ↓	37/9 ↓	No	Cognitive regression, tremor, ataxia, dysarthria, atrioventrincular block, hearing loss, ptosis, abnormality of retinal pigmentation. MRI (10y) T2W brainstem, caudate and putaminal hyperintensities. CT (10y) basal ganglia calcification.
8*	34 years/F	Kearns-Sayre syndrome (#530000) mtDNA deletion	442 ↑	134	24 ↓	26/20	No	Failure to thrive, hearing loss, abnormality of retinal pigmentation, ophthalmoplegia, ataxia, myoclonus axonal neuropathy. MRI (34y) T2W caudate and thalamic hyperintensities, ventriculomegaly, cerebellar atrophy. MRS lactate peak.—Leigh syndrome CT (37y) caudate and thalamic calcification.
9	1 year/F	Multiple mitochondrial dysfunctions syndrome 1 (#605711) *NFU1* (*608100)	293 ↓	219	37 ↓	21 / 14	Yes	Psychomotor regression, hypotonia, nystagmus, dystonia, hypotonia. MRI (2y) Diffuse leukoencephalopathy
10	8 years/M	Leigh syndrome (#256000)/NARP SYNDROME MTATP6 (*516060)	195 ↓	155	51	33/9 ↓	No	Cognitive impairment, ataxia, gait disturbance, dysarthria, tics, axonal neuropathy. MRI (8y)—T2W caudate, putamen hyperintensity MRS normal. Leigh syndrome.
11*	16 years/M	Thiamine metabolism dysfunction syndrome (#607483) *SLC19A3* (*606152)	155 ↓	63 ↓	31 ↓	8 ↓/13	Yes	Agitation, coma, status dystonicus, akinetic-rigid syndrome, tremor, nystagmus, ptosis, dysarthria, vertigo, facial dyskinesias, pyramidal signs, rabdomyolisis, seizures.

Table 1 (continued)

Patient	Age (years)/sex	Disease (OMIM) *gene (OMIM)*	CSF HVA nmol/L	CSF HIAA nmol/L	CSF 5MTHF nmol/L	CSF neopterin/biopterin nmol/L	Dopaminergic symptoms (yes or not)	Clinical and radiological features
								MRI (26y) – T2W caudate, putamen and medial thalamus hyperintensities—Leigh syndrome
12	11 years/M	MERRF syndrome (#545000) *MTTK* (*590060)	123 ↓	54 ↓	112	17 / 8 ↓	No	Intellectual disability, myoclonic seizures, ophthalmoplegia, hearing loss. MRI (11y) normal
13	13 years/M	CARDIOMYOPATHY WITH SKELETAL MYOPATHY *MTTL1* (*590050)	84 ↓	48 ↓	85	26/8 ↓	No	Strabismus, ptosis, hypertrophic cardiomyopathy, exercise intolerance, seizures, depression. MRI (13y): Normal
14	0.2 years/F	Fatal infantile lactic acidosis *KARS* (*601421)	895	256	NA	NA/NA	Yes	Failure to thrive, irritability, vomiting, diarrhea, oculogyric crisis, tremor, arterial hypertension, anemia, hypoglycemia. MRI (0.2y)—T2W left frontal WM hyperintensity. Focal leukoencephalopathy.
15*	0.3 years/F	Combined oxidative phosphorylation deficiency 1 (#609060) *GFM1* (*606639)	880	344	69	7 ↓/10 ↓	Yes	Irritability, failure to thrive, dystonia, cognitive impairment, hypotonia, pyramidal signs, hearing loss. MRI (5y)—thin corpus callosum, white matter volume loss
16	1 year/M	Mitochondrial DNA depletion syndrome 4A (Alpers type)(#203,700) *POLG* (*174763)	608	320	82	64↑/18	No	Failure to thrive, global developmental delay, status epilepticus, myoclonus. No neuroimaging data.
17	1 year/F	Mitochondrial DNA depletion syndrome 5 (encephalomyopathic with methylmalonic aciduria) (#612073) *SUCLA2* (*603921)	560	184	91	10 /27	No	Dystonia, hypotonia, hearing loss, cognitive impairment. MRI (1.5y): T2W caudate hyperintensity—MRS normal—Leigh syndrome
18	0.5 years/M	Mitochondrial complex I deficiency (#252010)/Cockayne syndrome, type A (#216400) *NDUFAF2*(*609653) /*ERCC8* (*649412)	553	194 ↓	200	126↑ / 17	No	Encephalopathy, seizures, psychomotor regression, microcephaly, hearing loss, congenital cataract, failure to thrive. MRS (0.5y): delayed myelination
19	0.2 years/F	Leigh syndrome (#256000) *MTATP6* (*516060)	541	394	101	78↑ / 26	No	Hypotonia/pyramidal signs, tachypnea, nystagmus, hearing loss, hearing loss, seizures. MRI (0.2y) T2W globus pallidus, putamen, thalamus hyperintensities—Leigh syndrome
20*	1 year/M	Thiamine metabolism dysfunction syndrome (@607483) *SLC19A3* (*606152)	522	254	74	13 / 23	No	Irritability, hypotonia, status dystonicus, tremor, opistotonus, dysphagia, nystagmus, strabismus, ataxia, weight loss, hepatomegaly, jaundice. MRI (1.2y) T2W caudate, putamen and medial thalamus hyperintensities—Leigh syndrome
21	0.9 years/F	Epileptic encephalopathy 5q14.3 deletion (*612881)	465	310	76	20 / 16	Yes	Global developmental delay, hypotonia/pyramidal signs, seizures. MRI (0.9y): dysplastic corpus callosum. MRS normal.

Table 1 (continued)

Patient	Age (years)/sex	Disease (OMIM) *gene (OMIM)*	CSF HVA nmol/L	CSF HIAA nmol/L	CSF 5MTHF nmol/L	CSF neopterin/biopterin nmol/L	Dopaminergic symptoms (yes or not)	Clinical and radiological features
22*	7 years/M	Pyruvate dehydrogenase E1-alpha deficiency (#312170) *PDHA1* (*300502)	437	257	72	8 ↓/20	No	Encephalopathy, axonal neuropathy, ataxia, dysarthria. MRI (9y): T2W globus pallidus, dentate nuclei, patchy corticosubcortical hyperintensities—Leigh syndrome
23	1 year/M	Pearson marrow-pancreas syndrome (#557000) mtDNA deletion	429	170	124	7 ↓/13	No	Cognitive impairment, hearing loss, pancreatic insufficiency, hypoglycemia, failure to thrive. MRI (6y) normal. MRS normal.
24	13 years/F	Melas syndrome (#540000) *MTTL1* (*590050)	368	111	81	13/13	No	Vomiting, diarrhea, dysarthria, seizures, right hemiparesis, ptosis, hearing loss, depression, cognitive impairment, anemia. MRI (15Y)—multiple ischemic strokes. MRS lactate peak
25	7 years/F	Pyruvate dehydrogenase E1-alpha deficiency (#312170) *PDHA1* (*300502)	364	136 ↓	33 ↓	25/25	No	Encephalopathy, gait disturbance, dystonia, dysarthria, photophobia. MRI (7y) T2W caudate, putamen, globus pallidus and cerebral peduncle hyperintensities. MRS normal. Leigh syndrome
26	13 years/F	Leigh syndrome(#256000) /NARP SYNDROME MTATP6 (*516060)	240	168 ↓	39	17 / 11 ↓	No	Global developmental delay, hypotonia, vomiting, axonal neuropathy, ataxia, abnormality of retinal pigmentation. MRI (16y)—T2W caudate, putamen, cerebellar cortex hyperintensities. MRS normal—Leigh syndrome
27*	10years/M	Pyruvate dehydrogenase E1-alpha deficiency (#312170) *PDHA1* (*300502)	236	118	55	9 ↓ / 13	No	Hypotonia, vomiting, ptosis, nystagmus, upgaze palsy, dystonia, ataxia. MRI (12y)—T2W globus pallidus hyperintensity. Left globus pallidus cyst. Leigh syndrome
28	13 years/F	Mitochondrial myopathy *MTTA* (*590000)	208	50	30 ↓	9 ↓/18	No	Cognitive impairment, Wolf-Parkinson-White syndrome, pes cavus, exercise intolerance. MRI (13y): normal
29	11 years/M	Pyruvate dehydrogenase E1-alpha deficiency (#312170) *PDHA1* (*300502)	187	52	65	8 ↓/8 ↓	No	Cognitive impairment, dystonia, right hemiparesis. MRI (14y) TW2 left globus pallidus hyperintensity, normal MRS—Leigh syndrome

*Patient 3 was the first published case in our series with cerebral folate deficiency (Pineda et al. 2006); patients 3–6 have been published by Tondo et al. (biochemical aspects of KSS) and by Serrano et al., (neuroradiological features in KSS); cases 11 and 20 were published by Ortigoza-Escobar et al. (2016), but neither monoamines nor folate results were reported; case 15 was individually reported, but no details about dopamine or monoamine disturbances were done (Brito et al. 2015); and cases 22 and 27 were previously published, but neither monoamine nor folate studies were reported in that work (Asencio et al. 2016). *MRI* magnetic resonance imaging, *MRS* magnetic resonance spectroscopy, *T2W* T2-weighted

Additional clinical studies reporting dopamine deficiency detected by SPECT were also included. Exclusion criteria were studies in animal/cellular models and patients with neurodegenerative conditions, such as Parkinson's disease or Alzheimer syndrome. A total of 28 articles were found, half of them dealing with monoamine analysis and the others with cerebral folate deficiency in mitochondrial disorders. Regarding our series (Table 1), CSF monoamines and folate status have been previously reported for patients 3–6 (Pineda et al. 2006; Serrano et al. 2010; Tondo et al. 2011). Cases 11, 15 20, 22, and 27 have been previously published (Brito et al. 2015; Ortigoza-Escobar et al. 2016; Asencio et al. 2016)

but neither CSF monoamines nor folate results were reported in those works.

Results

HVA and 5-HIAA disturbances Biochemical results of the entire cohort of patients are given in Fig. 2 and Table 1. In eight of 29 patients (patients 1 to 8 in Table 1), CSF HVA was high; in 5, it was low (Patients 9 to 13), and in the remaining 16 patients (Patients 14 to 29), HVA values were normal. The eight cases with high HVA values had mtDNA mutation syndromes (five mtDNA deletions, two point mutations) and one was diagnosed with biallelic mutations in *POLG*, leading to mtDNA depletion. In three patients with mtDNA deletion/depletion syndromes (Table 1), 5-HIAA was high concomitantly with high CSF HVA values. In six of the 29 patients, 5HIAA was decreased in three of them concomitantly with HVA. In the remaining 20 patients, 5-HIAA was normal.

Clinically, five out of 29 cases showed predominant dopaminergic signs not attributable to basal ganglia involvement (Table 1). These five cases harbored mutations in nuclear genes with different biological functions. A chi-square test did not show significant associations between impaired HVA values and the presence of a clinical picture with predominant dopaminergic dysfunction signs. In MRI studies, no association could be demonstrated between basal ganglia involvement with either the clinical phenotype or CSF HVA status. A normal brain MRI was observed in some patients with impaired neurotransmitters (patients 1, 12, 13).

Patient 11, harboring SLC19A3 mutations, had low CSF HVA values and abnormal 18F-FDOPA (fluorodihydroxyphenylalanine) PET findings (bilateral, asymmetric (left>right) and irregular severe dopaminergic depletion in the striatum), and after receiving treatment with L-dopa showed partial improvement of clinical signs of dystonia-parkinsonism. L-dopa administration was indicated at a dose of 4 mg/kg/day (2 g/day). During the administration period, involuntary movements (head tics) appeared and thus the dose of L-dopa was increased very slowly. He received L-dopa for 10 months.

Patients with high CSF lactate values did not show an association with either HVA or a clinical or radiological phenotype. No specific clinical phenotypes attributable to serotonin deficiency were detected, and no associations could be demonstrated between 5-HIAA status and the clinical or radiological phenotype. Only two patients (cases 13 and 24) presented with depression as serotonin-deficiency-related clinical sign. Regarding pterins, no consistent associations could be found between the neopterin and biopterin concentrations with the monoamine values. In four out of 29 cases, the neopterin concentration was higher than 61 nmol/L (the established cut-off value for identification of inflammatory events).

Cerebral folate deficiency in patients with high CSF HVA values

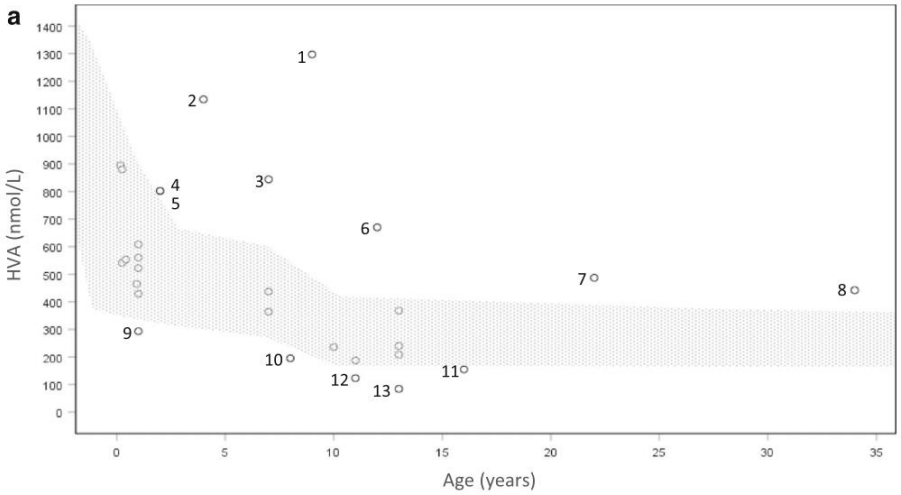
A chi-square test showed there was a statistically significant association between high HVA values and low CSF folate values (chi-square = 10.916; $p = 0.001$) and high CSF protein concentration (chi-square = 10.429; $p = 0.001$). Seven of the eight patients with high CSF HVA values showed cerebral folate deficiency and high CSF protein levels (Fig. 3). Five of them harbored mtDNA deletions associated with KSS, one a mitochondrial point mutation in the mtDNA *ATPase6* gene and one *POLG* mutations (Table 1). Values of 5-HIAA were remarkably increased in three of them. Four of the remaining 21 patients showed mild cerebral folate deficiency with no association with high HVA values.

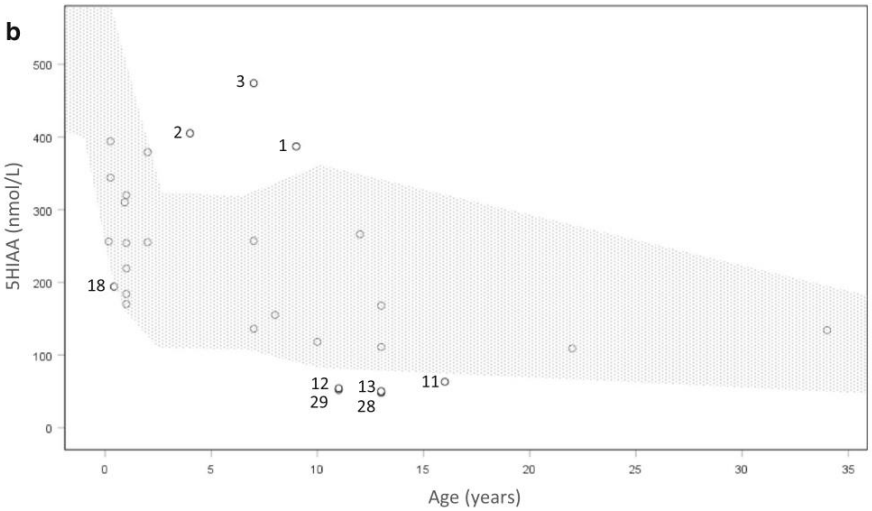
Discussion

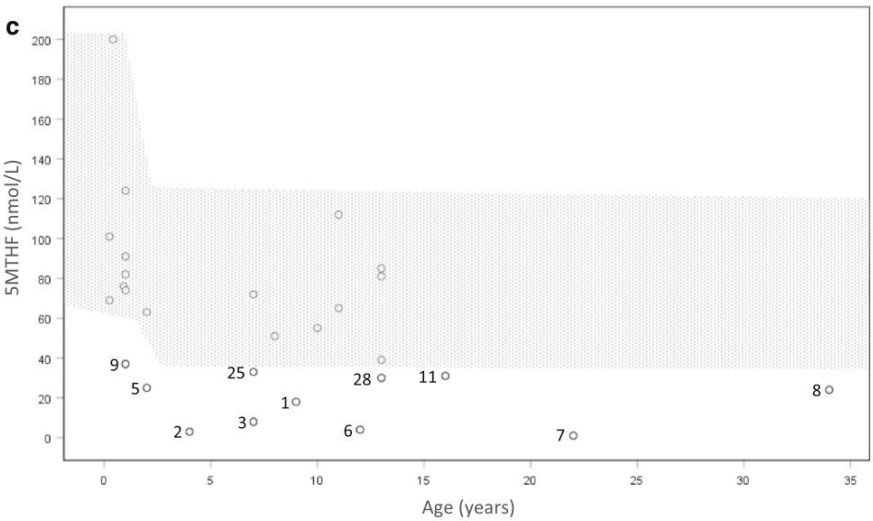
In this work, we assessed clinical, radiological, and CSF biochemical data in a series of patients with mitochondrial diseases to provide further insight into monoamine (dopamine and serotonin) disturbances in these patients. The relationship between mitochondrial dysfunction and Parkinson's disease has been extensively investigated. Energy failure, impaired mitochondrial turnover and quality control, oxidative damage from excessive mitochondrial reactive oxygen species (ROS), impaired mitochondrial calcium buffering and endoplasmic reticulum-mitochondria contacts, loss of mitochondrial mass, and impaired mitochondrial dynamics have all been shown to be involved in the pathophysiology of Parkinson's (Haddad and Nakamura 2015).

The signs and symptoms of secondary neurotransmitter deficiencies in mitochondrial diseases are markedly heterogeneous (Kuster et al. 2018). From a clinical point of view, few reports about monoamine disturbances in mitochondrial diseases patients have been published. It has, however, been reported that some mitochondrial disease patients present with parkinsonism features (Garcia-Cazorla et al. 2008a, b; Moran et al. 2011; Kuster et al. 2018). Tzoulis et al. (2016) reported a dopamine transporter imaging study (DAT scintigraphy: 185 MBq I-123 ioflupane-SPECT) in mitochondrial diseases patients, and concluded that nigrostriatal denervation occurred exclusively in the subset of disorders due to defective mtDNA replication and maintenance. In patients with POLG disease, striatal denervation became detectable by DAT scanning after the age of 25, and the severity of the nigrostriatal defect showed a strong correlation with increasing age. Additional studies have confirmed the association between *POLG* mutations and dopamine deficiency (Invernizzi et al. 2008; Tzoulis et al. 2013; Miguel et al. 2014).

In our series, the five patients who showed prominent dopaminergic-deficiency clinical features without basal


a
HVA (nmol/L)
Age (years)
1
2
3
4
5
6
7
8
9
10
11
12
13


b
5HIAA (nmol/L)
Age (years)
1
2
3
11
12
13
18
28
29


c
5MTHF (nmol/L)
Age (years)
1
2
3
5
6
7
8
9
11
25
28

◀ **Fig. 2** Biochemical data in CSF: **a** HVA, **b** 5HIAA, and **c** 5-MTHF concentrations of the entire cohort of mitochondrial diseases patients are displayed. Numbers of the circles correspond to the patients' codes in Table 1. The shadowed area represents the reference intervals according to age

ganglia lesions harbored mutations in different genes (*KARS*, *GFM1*, *NFU1*, *POLG*, and a 5q14.3 deletion that includes the *COX7C* gene) (Table 1). The genetic and pathophysiological heterogeneity of this subgroup is remarkable. Kuster et al. also reported dopamine-deficiency clinical features in cases of *POLG* and *NFU1* mutations, but in general, the descriptions in the literature are anecdotal. In fact, it is difficult to differentiate the clinical signs of dopamine deficiency from those derived from the other pathophysiological mechanisms involved in mitochondrial diseases. The lack of association observed in our study between the biomarkers of dopamine deficiency (CSF HVA values) and either the clinical or radiological phenotype reinforces the idea that searching for new biomarkers is mandatory to elucidate the pathophysiological basis of parkinsonism and other dopamine deficiency-related features in mitochondrial diseases. The same lack of association was found with pterin values, since most of the observed low pterin values were not very low, with probably no impact on monoamine biosynthesis. Interestingly, four out of the 29 displayed a high CSF neopterin concentration, indicating, as previously reported (Hasselmann et al. 2010), the presence of inflammatory events in isolated cases with mitochondrial diseases.

The only statistical association we found was that of high CSF HVA values with both very low 5-MTHF and high CSF protein concentrations, corresponding to patients with mtDNA mutations (mainly deletions causing KSS and 2 further cases harboring mutations in the mtDNA *ATPase* and *POLG* genes). A few authors have previously reported this characteristic biochemical profile (Allen et al. 1983; Dougados et al. 1983; Pineda et al. 2006; Ramaekers and Blau 2004; Ramaekers et al. 2007; Hasselmann et al. 2010; Serrano et al. 2010; Tondo et al. 2011; Grapp et al. 2013) associated with mtDNA mutations and in some cases with POLG deficiency (Kuster et al. 2018). Because this profile has not been observed in other mitochondrial or non-mitochondrial diseases (Pérez-Dueñas et al. 2011), a combination of these biomarkers may be useful in the study of patients harboring mtDNA mutations. However, the mechanism explaining this association has not been fully elucidated nor has the clinical consequences regarding high CSF HVA values. The most consistent hypothesis is that the choroid

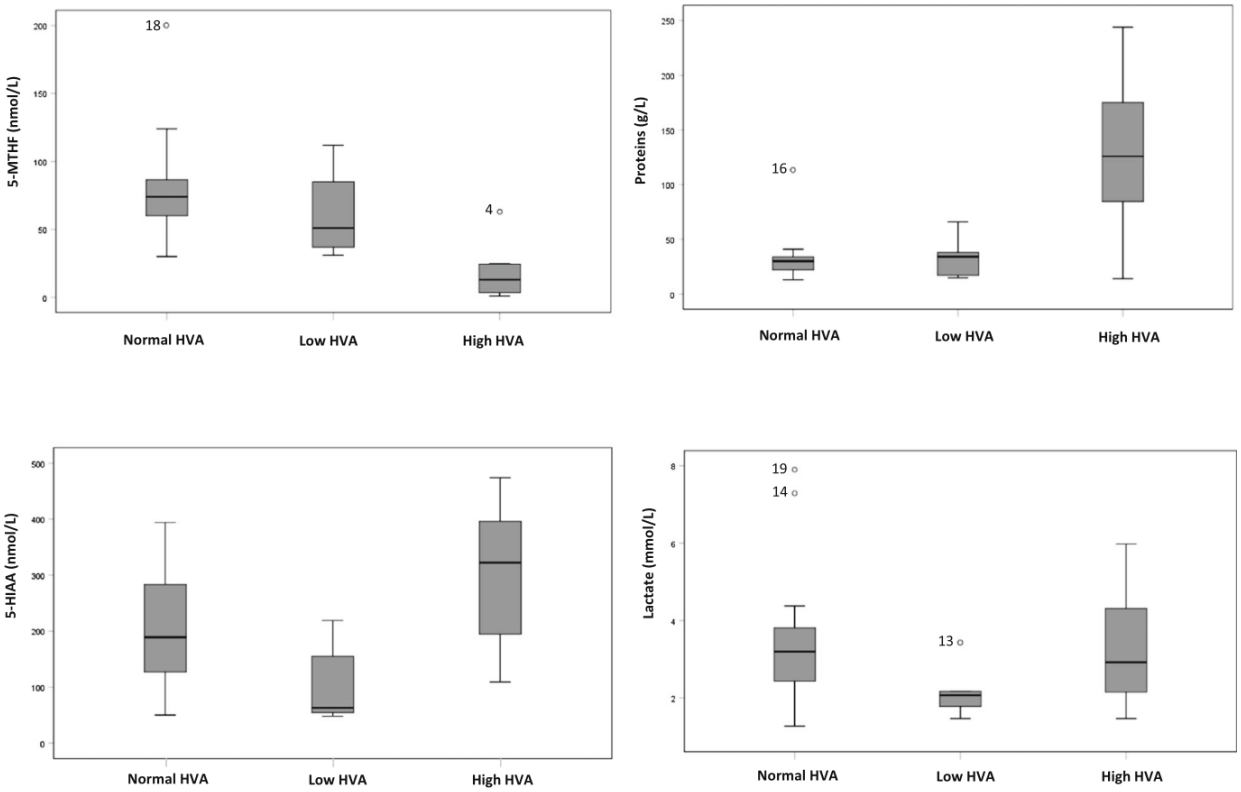


Fig. 3 Box plot representation of 5-MTHF, proteins, 5HIAA, and lactate levels, depending on whether patients have normal, low, or high CSF HVA concentrations. The length of the boxes indicates the interquartile space (p25–p75); the horizontal line into the box represents the median (p50), and the circles indicate outlier values

plexus may be a target organ in some mtDNA mutation syndromes.

In Fig. 1, choroid plexus folate (folate receptor alpha; FRα) and monoamine transporters (organic anionic transporter type 3; OAT3) are displayed. To control the homeostasis of CSF and ensure proper brain nutrition and protection, it has been described as a very complex transport system between the apical and the basolateral sides of the choroid plexus epithelial cells (Spector 2010; Spector & Johanson 2010). The choroid plexus controls the quantity and the composition of the CSF by secreting molecules into the CSF and excreting others from the CSF into the blood (Orešković et al. 2017). Two additional features are worth mentioning: (1) the choroid plexus is enriched in mitochondria because the active transporter systems that consume ATP are responsible for folate influx into the CSF and monoamine efflux from the CSF to the blood (Alebouyeh et al. 2003; Mori et al. 2003), and (2) it has been suggested that the accumulation of mutated mitochondria in the choroid plexus of KSS patients leads to failure of absorption and transport, and/or secretion of proteins, folate, and monoamines (Tanji et al. 2000). Thus, the lack of ATP due to mutated mitochondria cannot provide the energy needed to transport molecules against the gradient into the CSF, although this lack of energy does not fully explain cerebral folate deficiency in KSS in particular or in mitochondrial diseases in general (Garcia-Cazorla et al. 2008a, b). On the other hand, the accumulation of these mutated mitochondria in the choroid plexus probably contributes to an anatomical destruction that prevents the correct transport of molecules through the choroid plexus. It has been reported that in KSS patients, there is a correlation between 5-MTHF concentrations and levels of FRα expression (Grapp et al. 2013). Other mechanisms such as increased generation of reactive oxygen species and/or loss of CSF antioxidants may be additional factors to consider with regard to the development of a central 5-MTHF deficiency in mitochondrial disorders (Aylett et al. 2013).

The high HVA values could be explained by a putative OAT3 deficiency in the choroid plexus (Spector 2010). Further evidence that a functional deficiency of OAT3 leading to decreased export of HVA from the CSF may be involved comes from the consideration of the folate deficiency. 5-methyltetrahydrofolate provides the methyl group (as S-adenosylmethionine) for COMT, which is required for the catabolism of dopamine to HVA. In folate deficiency, the activity of COMT is likely compromised with the expected outcome being decreased CSF HVA. In KSS, the increased HVA therefore points to a mechanism involving the trapping and accumulation of HVA within the CSF even in the presence of decreased production due to a lack of COMT activity.

High CSF HVA values can also reflect impaired dopaminergic status, as occurs in genetic DAT1 deficiency (Kurian et al. 2011). The loss of function of the DAT1 transporter in the synapses would lead to massive dopamine degradation into HVA in the synaptic cleft environment, leading to its increased values and a dopamine-deficient status with severe clinical consequences. Dopaminergic neurotransmission is highly dependent on energy and, thus, could be impaired in mitochondrial disorders.

Thus, an excessive formation of HVA, via MAO and COMT, could result in excessive consumption of the folate pool via S-adenosylmethionine due to a potential failure of vesicular loading of dopamine in energy deficiency states. Moy et al. (2007) provided direct evidence that mitochondrial impairment and metabolic stress can cause striatal dopamine efflux via the DAT and suggested that disruptions in dopamine homeostasis resulting from energy impairment may contribute to the pathogenesis of neurodegenerative diseases. However, the lack of a specific dopaminergic phenotype in KSS patients would partially rule out these hypotheses. Although it is an unspecific biomarker, CSF lactate values were not associated with the clinical, radiological, or biochemical disturbances in this study.

In conclusion, clinical signs of dopamine deficiency were present in some mitochondrial diseases' patients with different genetic backgrounds. High CSF HVA values, together with a severe cerebral folate deficiency, were observed in KSS patients and in other mtDNA mutation syndromes. No clinical signs of dopaminergic deficiency were present in either KSS patients or in most mitochondrial diseases patients. Although there is little data currently in the literature, impaired dopaminergic neurotransmission may be an additional pathophysiological mechanism in some cases of POLG deficiency.

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Compliance with ethical standards

Conflict of interest M. Batllori, M. Molero-Luis, A. Ormazabal, R. Montero, C. Sierra, A. Ribes, J. Montoya, E. Ruiz-Pesini, M. O'Callaghan, L. Pias, A. Nascimento, F. Palau, J. Armstrong, D. Yubero, J. D. Ortigoza-Escobar, A. García-Cazorla, and R. Artuch declare that they have no conflict of interest.

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DISCUSSIÓ

Els defectes genètics de la neurotransmissió han estat estudiats per diferents grups durant les últimes dècades. La principal conclusió de tots aquests grups investigadors és que el diagnòstic encara recau en la realització de proves bioquímiques especialitzades en LCR, tot i que l'arribada de les tècniques diagnòstiques per NGS està canviant el paradigma diagnòstic molt ràpidament. La utilització del LCR ha demostrat ser la mostra més adequada pel diagnòstic de moltes d'aquestes patologies pel fet que s'ha vist que l'anàlisi dels biomarcadors d'aquestes patologies en perifèria (per exemple sang o orina) no són representatives del SNC, ja que presenten concentracions dins els límits de la normalitat. A més a més, les tècniques bioquímiques aplicades que s'utilitzen per fer les anàlisis es podrien considerar proves "clàssiques" perquè utilitzen HPLC amb detecció electroquímica i de fluorescència, metodologia molt emprada i amb un llarg recorregut en els laboratoris especialitzats en diagnòstic de malalties que afecten la neurotransmissió.

Aquesta primera reflexió ens deixa en un atzucac, però que alhora ens ofereix diferents camins. Un dels reptes que és necessari resoldre és la validació bioquímica de biomarcadors en fluids biològics perifèrics per al diagnòstic i sobretot pel monitoratge terapèutic d'aquestes patologies cròniques. Actualment, la literatura al respecte és escassa, fet que fa pensar que les investigacions futures aniran en aquesta direcció. Aquests tipus d'investigacions seran especialment rentables amb l'aplicació de les tècniques de metabolòmica que, tot i que s'estan utilitzant de forma extensiva en altres malalties hereditàries, l'experiència en el camp de la neurotransmissió és encara molt escassa. No obstant això, tenim clar que en un període breu de temps aquests sistemes metabolòmics substituiran els procediments més tradicionals. Aquest fet no desmereix la importància dels resultats obtinguts en aquesta tesi, ja que, mentre tot això no sigui possible, s'han de seguir tenint clares les condicions preanalítiques i analítiques perquè, tal com hem exposat i demostrat, poden afectar i molt la interpretació dels resultats.

Per tant, en aquesta tesi, hem abordat els reptes de la investigació en biomarcadors de la neurotransmissió d'una manera holística començant per una investigació exhaustiva dels factors preanalítics i analítics relacionats amb aquests tipus d'anàlisi i, com a metàfora dels temps moderns, la recerca de nous biomarcadors que ens permeti fer

un control i monitoratge dels tractaments dels dèficits genètics rars del metabolisme de la serotonina.

Per últim, per completar aquest procés d'investigació translacional, vam decidir investigar els resultats dels neurotransmissors en pacients que pateixen unes malalties extremadament greus que presenten encara molts interrogants diagnòstics i fisiopatològics com són les malalties mitocondrials.

1. Estudi preanalític per avaluar la influència de la contaminació hemàtica en les mostres de líquid cefalorraquidi a l'hora de realitzar les proves bàsiques d'un perfil metabòlic.

L'anàlisi metabolòmic dirigit del LCR ha demostrat ser una bona eina analítica per a l'estudi de vies neurometabòliques específiques i malalties relacionades i per explorar el transport de diversos metabòlits des de la sang al SNC. Diverses condicions neurogenètiques són causades per trastorns específics en aquests processos. A més, en aquestes malalties, la quantificació d'aquests metabòlits en sang / orina no és fiable, ja que generalment mostren resultats normals o fins i tot paradoxals (Wassenberg T et al., 2010). Això és especialment cert quan les vies metabòliques estudiades són altament actives en el cervell o en les malalties genètiques de transport de la BHE relacionades amb les vitamines i altres metabòlits.

La contaminació del LCR amb sang és freqüent i ha estat reconeguda com un factor de confusió substancial a l'hora de realitzar una adequada interpretació dels resultats quantitatius quan s'analitzen aminoàcids i altres metabòlits en LCR (Aasebø E et al., 2014 i Krishnamurthy V et al., 2019). No obstant això, la literatura sobre els efectes de la contaminació sanguínia en l'anàlisi d'amines biògenes, pterines i vitamines és escassa (Verbeek M.M. et al., 2008). Les principals causes de contaminació sanguínia del LCR són puncions lumbars traumàtiques o sagnat intratecal espontani, fets que es poden donar en diverses situacions, especialment en els nadons. A més, l'alteració de la permeabilitat de la BHE pot ocórrer en diferents condicions, com en l'asfíxia i l'epilèpsia (Tan R et al., 2017; Krueger M et al., 2019 i Klebe D et al., 2020). En aquest context i considerant que la punció lumbar és una intervenció invasiva, que és difícil de realitzar i que el volum final recol·lectat és sovint baix en pacients pediàtrics, és important tenir una estimació de fins a quin punt la contaminació hemàtica del LCR ens pot conduir a una mala interpretació del perfil metabòlic.

L'albúmina, una proteïna sintetitzada en el fetge, és un bon biomarcador secundari per determinar el nivell d'afectació de la permeabilitat de la BHE i també per avaluar el grau de contaminació sanguínia. No obstant això, atès que la seva concentració en sang supera àmpliament la del LCR (aproximadament 100 vegades), la seva concentració pot romandre elevada fins i tot en casos de contaminació mínima per glòbuls vermells / plasma (en el nostre estudi ja s'aprecia en dilucions 1:100-1:1000;

dades no mostrades). La determinació de la concentració d'hemoglobina és un marcador substitutiu alternatiu quan s'ha produït la lisi dels glòbuls vermells i, en el nostre estudi, concentracions que van des de 0,17 a 0,35 g/L d'hemoglobina poden ser un senyal d'alerta a l'hora de fer una interpretació prudent dels resultats (consultar taula 2 del primer article).

Pel que fa als aminoàcids, diversos articles han indicat diferències entre la sang i el LCR (Duran M., 2008), però fins on nosaltres sabem, no s'han establert els límits precisos en què la contaminació per eritròcits pot provocar una mala interpretació dels perfils metabòlics. Mentre que els eritròcits lisats van afectar les concentracions de la majoria d'aminoàcids a la dilució d'1:40, la centrifugació de les mostres de LCR amb la posterior separació de la sang contaminant va resoldre el problema en la majoria dels casos. En qualsevol de les dues situacions, els aminoàcids que tenien valors més alts eren l'aspartat, el glutamat, la treonina, l'ornitina, la glicina i la citrulina. L'explicació d'això és que alguns d'aquests aminoàcids presenten una concentració més gran en els eritròcits en comparació amb el plasma (aspartat, glutamat i treonina) (Duran M., 2008).

Entre els altres aminoàcids, la glicina és la que té la proporció més gran plasma/LCR (Swanson MA et al., 2015). Pel que fa a l'ornitina, l'activitat de l'arginasa és alta en eritròcits i això explicaria la nostra observació que els valors d'arginina eren menors quan es produïa la lisi dels eritròcits mentre que els valors d'ornitina augmentaven, ja que, és el producte de la reacció catalitzada per l'arginasa (Duran M., 2008). Una vegada més, la centrifugació de les mostres va evitar tots aquests biaixos provocats per la contaminació. Per altra banda, en alguns aminoàcids no es va observar la correlació esperable tenint en compte els diferents factors de dilució. Una possible explicació és que alguns aminoàcids estan presents en LCR a concentracions molt baixes fisiològicament (propers als límits de quantificació) i per a altres aminoàcids, l'efecte matriu, habitual en la tecnologia UPLC-MS / MS, podria contribuir a aquestes diferències (Batllori M et al., 2017)

Les amines biògenes i les pterines se sintetitzen perifèricament en alguns teixits, però també al cervell i no s'ha descrit cap transportador substancial entre la sang i el cervell (només els transportadors OAT3 eliminen el 5-HIAA i el HVA del LCR cap a la sang (Spector R., 2010)). Atès que les seves concentracions en sang són similars a les del LCR

(Coppus AW et al., 2007 i Mori S et al., 2003), no es van observar canvis significatius després de la contaminació del LCR amb sang. Curiosament, el 5-HIAA i el HVA van tenir valors més baixos quan es va produir la lisi de glòbuls vermells. L'autooxidació d'aquestes molècules per hemoglobina/radicals lliures és un mecanisme potencial que explica aquesta observació (Kato Y et al., 2016), i per tant, s'ha de tenir cura a l'hora d'interpretar les dades quan el LCR no s'ha centrifugat abans de la congelació, ja que, els valors baixos de 5-HIAA i HVA són potencials biomarcadors de les deficiències de serotonina i dopamina i pot ser una indicació d'intervenció terapèutica (Ng J et al., 2015). En qualsevol cas, la centrifugació i l'eliminació de glòbuls vermells abans de la congelació va corregir els resultats en comparació amb el LCR sense contaminar.

Les vitamines van mostrar resultats inesperats, excepte el folat. Les diferents formes de folat (especialment 5-MTHF) estan altament concentrades en els glòbuls vermells en comparació amb el plasma i això explicaria la interferència positiva observada quan es va produir la lisi dels glòbuls vermells, però no després de l'eliminació dels glòbuls vermells. Només la tiamina i el PLP van augmentar els valors quan es van comparar les mostres de LCR contaminades en ambdues condicions experimentals a la dilució 1:40, amb mostres no contaminades, encara que va ser menys notable quan es van eliminar els glòbuls vermells per centrifugació. Aquests efectes es van minimitzar quan la dilució va arribar a 1:100 (dades no mostrades). Pel que fa a la tiamina, la conversió activa de tiamina lliure, TMP i TDP passa dins de les cèl·lules (els glòbuls vermells tenen una elevada activitat de tiamina fosfocinasa, que fosforila la tiamina per formar TDP, o la tiamina fosfatasa, que converteix TDP a TMP i tiamina) (Collie JTB et al., 2017). Això explicaria els resultats observats, valors pràcticament iguals en totes les dilucions, quan es va produir la lisi de glòbuls vermells. Aquests resultats es van minimitzar quan es van eliminar els glòbuls vermells per centrifugació. En qualsevol cas, els valors de tiamina són més alts en sang que en LCR i, per tant, els resultats s'han d'interpretar amb cautela quan es produeix contaminació per glòbuls vermells (Ortigoza-Escobar JD et al., 2016).

La TDP, una isoforma de la tiamina estrictament intracel·lular (Collie JTB et al., 2017), seria un bon biomarcador substitutiu de la lisi dels eritròcits en mostres de LCR, ja que es van observar quantitats indetectables de TDP quan es van eliminar els eritròcits. Amb el PLP, les observacions eren similars i, encara que menys significatives, fins i tot

quan es van eliminar els glòbuls vermells del LCR, el PLP va mostrar concentracions més elevades en les mostres de LCR contaminades. Com amb la tiamina, una via metabòlica intracel·lular complexa explica la síntesi de les diferents isoformes relacionades amb la piridoxina (Footitt EJ et al., 2011). A més, algunes d'aquestes vitamines poden ser degradades per nucleòfils i radicals lliures derivats d'oxigen, ja que, el LCR té concentracions baixes d'altres molècules que poden reaccionar amb aquests compostos (Footitt EJ et al., 2011). Així, els resultats han de ser analitzats amb precaució respecte a aquestes dues vitamines, ja que, en trastorns relacionats amb la tiamina o la piridoxina que causen fenotips neurològics greus, els biomarcadors diagnòstics són nivells baixos de tiamina i PLP en LCR (Ortigoza-Escobar JD et al., 2016; Footitt EJ et al., 2011; Zeng WQ et al., 2005).

2. Descriure diversos protocols analítics basats en cromatografia líquida d'alta resolució (HPLC) per a la quantificació d'amines biògenes i els seus cofactors en líquid cefalorraquidi.

Tal tal comcom hem desenvolupat en aquesta tesi, el primer pas crític per a l'anàlisi de les amines biògenes i els seus metabòlits i cofactors és el procediment de la recollida de les mostres de LCR, així com la seva correcta centrifugació i emmagatzematge un cop ha arribat al laboratori. Les condicions de recollida de la mostra són claus per a la correcta interpretació dels cromatogrames que es descriuen en aquest protocol.

Per a l'anàlisi dels neurotransmissors i els seus cofactors s'han utilitzat HPLC amb diferents mètodes de separació cromatogràfica, així com diferents tipus de detecció en funció del tipus de molècula que estem estudiant. Així, per a la quantificació de monoamines en LCR s'ha descrit un protocol utilitzant un HPLC de parell iònic amb detecció electroquímica (HPLC-ED), el gold standard. Per a l'anàlisi de pterines s'ha descrit un procediment emprant un HPLC amb detector electroquímic i de fluorescència (HPLC-ED-FD) en sèrie que permet quantificar la neopterina total, la BH4 i el seu precursor BH2. I, finalment, per a l'anàlisi del PLP s'ha descrit un protocol per HPLC amb detector de fluorescència (HPLC-FD).

Aquestes tècniques s'han utilitzat freqüentment en els laboratoris de diagnòstic de malalties genètiques primàries que provoquen deficiències en la neurotransmissió i altres malalties greus d'inici precoç que poden estar relacionades amb alteracions en la via de la dopamina i la serotonina cerebrals. La informació metodològica presentada en aquest treball pot ser de gran ajuda tant per aquests tipus de laboratoris com també per laboratoris de recerca bàsica.

Limitacions: un dels factors que cal tenir en compte són les limitacions pràctiques en la preparació de mostres. En primer lloc, de vegades, el volum de la mostra no és suficient per permetre totes les investigacions necessàries. A més, la contaminació del LCR amb sang després d'una punció lumbar traumàtica pot provocar l'autoxidació d'alguns compostos (tal com hem vist en el primer article d'aquesta tesi). Pel que fa als efectes de matriu, en LCR són menys importants que en sang o d'altres teixits, i s'ha descrit que s'associen principalment a procediments d'espectrometria de masses.

Les principals limitacions d'aquest protocol estan relacionades amb el sistema HPLC-ED. Tot i que HPLC-FD és robust i altament sensible, l'HPLC-ED requereix una

preparació i un procés d'equilibratge de l'equip òptims. Aquest procediment cal que el realitzin persones específicament formades, de manera que pot requerir temps. Aquestes qüestions i altres es tracten en el present protocol. L'establiment de valors de referència propis de cada laboratori és necessari i obligatori per a una interpretació adequada de les dades. Per establir-los es necessiten mostres de LCR procedents de controls pediàtrics "sans", però són difícils d'obtenir fora de l'ambient hospitalari. Com que hi ha una forta correlació entre els nivells de monoamines, pterines i PLP i l'edat, es necessita un tamany de mostres de LCR elevat per a l'establiment de valors de referència, ja que s'han de definir diversos intervals en funció de l'edat. Vam aprofitar les puncions lumbars recollides als nostres hospitals per a diagnòstics de meningitis vírica i bacteriana. Un cop descartades aquestes malalties, vam utilitzar els excedents de les mostres com a mostres control per determinar nivells d'amines biogèniques, pterines i PLP.

Nivell d'expertesa

Aquests procediments analítics han de ser realitzats per professionals competents. El procediment més complicat és el que implica la detecció electroquímica i es recomana una formació especial per a aquells que gestionen el detector electroquímic. No obstant això, el pas crític consisteix en la interpretació de dades perquè es requereix un alt grau d'experiència per identificar les condicions genètiques esmentades a la taula 1 de la introducció d'aquesta tesi i per interpretar els nombrosos trastorns secundaris de la neurotransmissió, de les pterines i del PLP. Per tant, la interpretació de les dades s'ha de realitzar dins del context de la informació clínica proporcionada per un clínic expert en la matèria.

Disseny experimental

L'optimització de les determinacions d'amines biògenes, pterines i PLP es divideix en dues etapes: (1) factors preanalítics (protocol de recollida i emmagatzematge de mostres) i (2) factors analítics (optimització dels paràmetres de HPLC-ED i FD, i interpretació de dades).

1. Factors preanalítics. El LCR és la mostra biològica ideal per diagnosticar la majoria de trastorns d'amines biògenes. Es requereix una recollida protocol·litzada de punció lumbar per a una anàlisi fiable de les monoamines, pterines i PLP. Com que hi ha un gradient rostrocaudal és important comparar els valors d'un pacient amb

els seus propis valors de referència, que s'estableixen utilitzant la mateixa fracció de LCR (Ormazabal A et al., 2005; Hyland K et al., 1993).

Com que la lisi de glòbuls vermells provoca l'oxidació d'alguns metabòlits d'amines, les mostres contaminades amb sang han de ser centrifugades immediatament i el sobrenedant transparent de LCR s'ha de transferir a un nou tub. Per mesurar la neopterina, BH2 i BH4, la mostra de LCR ha d'estar protegida de la llum i emmagatzemada amb agents estabilitzadors, perquè les formes reduïdes són extremadament sensibles a la llum i l'oxigen (Blau N. & Beat T, 2008). Com que les mostres de LCR tenen menys compostos interferents en comparació amb altres matrius biològiques, com sang, plasma, extractes de teixits o orina, no es requereixen procediments extensius de purificació o extracció, i només es necessiten passos de dilució i filtració abans de l'anàlisi HPLC (Hyland K et al., 1993). No obstant això, la columna de protecció i els filtres de grafit s'han de substituir periòdicament per garantir un bon rendiment analític. Les mostres s'han d'emmagatzemar sempre a -70 °C protegides de la llum (consultar taula 3 de l'article).

2. Factors analítics.
    a. Respecte a la quantificació de monoamines. L'HPLC-ED és el procediment més utilitzat per a la quantificació de monoamines. Aquest enfocament es basa en la separació per cromatografia de parells iònics, amb una fase mòbil que consisteix en un tampó citrat-acetat a un pH àcid que es complementa amb un agent de parells iònics (àcid heptanosulfònic o equivalent) (Ormazabal A et al., 2005; Hyland K et al., 1993). L'agent de parells iònics té un grup iònic al cap i una cua no polar. La cua no polar interactua fortament amb la columna C18, mentre que el grup iònic del cap es projecta cap a la fase mòbil. Aquesta modificació de columna genera una atracció entre l'agent de parells d'ions i els compostos ionitzats, cosa que permet la retenció i l'elució cromatogràfica. Les condicions crítiques de separació que poden canviar notablement el temps d'elució d'un compost inclouen el valor del pH de la fase mòbil i l'agent de parells iònics (Snyder LR et al., 2010). La detecció de monoamines es realitza mitjançant un detector electroquímic que s'utilitza per determinar el corrent generat entre dos

elèctrodes col·locats a la cel·la de detecció. Aquests elèctrodes són l'elèctrode de treball (E1), en què es genera el corrent, i l'elèctrode auxiliar (E2, o comptador), en què té lloc la reacció electrolítica complementària. El potencial entre els elèctrodes es manté constant, donant lloc a l'oxidació completa dels compostos d'interès analitzats. Un altre elèctrode, anomenat elèctrode de referència, s'utilitza per mantenir el potencial estable durant les mesures. Els paràmetres adequats són molt importants per aconseguir una oxidació completa de l'anàlit. El potencial adequat es pot determinar mesurant el corrent oxidatiu de l'anàlit (a una concentració constant) en un interval de potencials d'elèctrodes de treball. La representació gràfica del corrent generat enfront del potencial aplicat s'anomena voltamograma hidrodinàmic. Aconsellem recollir regularment un diagrama de voltamograma hidrodinàmic per a cada compost, ja que diferents variables poden influir en els resultats, inclosos factors com la composició de fase mòbil, el tipus i l'edat dels elèctrodes utilitzats i el deteriorament del filtre de grafit amb el temps (Snyder LR et al., 2010). El potencial aplicat a +450 mV és normalment suficient per a una oxidació electroquímica adequada de les monoamines. Aquesta oxidació dóna lloc a un corrent que el detector pot detectar i és directament proporcional a la concentració de la monoamina. A la figura 2 de l'article es mostra un exemple de voltamograma hidrodinàmic per a 5HIAA i HVA. L'ideal seria escollir el corrent més baix que produeix la resposta analítica més alta a l'elèctrode, ja que això condueix a un senyal de fons inferior. Pel que fa a la composició dels elèctrodes, hem utilitzat elèctrodes porosos de grafit, ja que tenen una gran superfície. Això permet que l'eluent flueixi a través dels elèctrodes i, per tant, maximitzi l'àrea de contacte amb compostos electroactius, augmentant la sensibilitat de l'anàlisi. S'han de prendre precaucions especials per a l'estabilització, manteniment i neteja de les cèl·lules electroquímiques, tal com es descriuen a l'article.

b. Respecte a la quantificació de pterines. L'HPLC amb detectors electroquímics i de fluorescència en línia és el mètode escollit per mesurar la neopterina, la BH_2 i la BH_4 en LCR en el mateix cromatograma. La

neopterina, la BH_2 i BH_4 se separen mitjançant HPLC de fase inversa. La BH_4 es mesura per detecció electroquímica, on és oxidada a l'E1 a quinonoide dihidrobiopterina i es redueix de nou a BH_4 a l'E2. A continuació, el detector utilitza el corrent generat per aquesta reacció de reducció per determinar la concentració de BH_4. La neopterina total (dihidroneopterina i neopterina) i BH_2 es poden mesurar en la mateixa injecció mitjançant un detector de fluorescència. Una oxidació postcolumna de la dihidroneopterina i la BH_2, mitjançant una cèl·lula condicionadora que utilitza un potencial òptim, oxida la dihidroneopterina a neopterina i la BH_2 a biopterina. Com que la dihidroneopterina i la neopterina coelueixen, són detectades com un únic pic pel fluoròmetre (excitació = 360 nm; emissió = 440 nm), que proporciona una mesura total de neopterina. La BH_2 es detecta i es mesura en el mateix cromatograma. A la figura 3 de l'article es mostra una representació esquemàtica de les cèl·lules electroquímiques.

c. Respecte a la quantificació de PLP. La quantificació en LCR del PLP es realitza mitjançant HPLC amb FD, tal com s'ha descrit anteriorment (Bates CJ et al., 1999; Ormazabal A et al., 2008). La fase mòbil consisteix en un tampó fosfat. El PLP és derivatitzat amb cianur de sodi en condicions alcalines. Els detalls analítics d'aquests procediments es resumeixen a la taula 2 de l'article i els cromatogrames típics dels diferents procediments es mostren a les figures 4-6.

Resolució de problemes

La detecció electroquímica coulomètrica per HPLC és un procediment d'alta sensibilitat, ja que s'espera que els compostos analitzats s'oxidin al 100%. Els problemes analítics poden ser notables si no es compleixen estrictament els protocols de neteja i manteniment de cèl·lules. En els procediments descrits en aquest protocol, els problemes de rendiment més freqüents solen ser causats per la detecció electroquímica i no pels procediments de separació de HPLC. Aquests problemes solen produir-se quan l'agent de parells iònics i els valors de pH no es controlen estrictament. Els gasos dissolts i l'adsorció de compostos electroactius a l'elèctrode de grafit porós, la presència de compostos electroactius a la fase mòbil

o a la mostra o un manteniment deficient dels elèctrodes poden provocar senyals electroquímics baixos i corrents de fons inacceptablement elevats amb una línia de base sorollosa (tal com es descriu a Coulochem II. Manual d'operacions (ESA)).

Quan el detector electroquímic es renta amb aigua-metanol-aigua (tal com es descriu al quadre 1 de l'article), el rendiment analític millora notablement. Aquest procediment de neteja és fàcil de realitzar i consisteix a rentar les cèl·lules amb aigua durant 30 min (4 mL/min), després amb metanol durant 30 min (4 mL/min) i finalment amb aigua durant 30 min (4 mL/min). És especialment útil com a primer procediment que s'ha de dur a terme quan els paràmetres analítics del DE no són bons. Si la cel·la del DE no funciona correctament, a la Taula 4 de l'article hi ha informació completa sobre la resolució de problemes i informació addicional de resolució de problemes per a la resta de procediments.

3. Avaluar la melatonina com a biomarcador perifèric de l'estat de la serotonina central en pacients amb defectes enzimàtics de les amines biògenes.

Aquest és el primer article que va avaluar les concentracions urinàries de sulfatoximelatonina (aMT6) en pacients amb malalties genètiques que causen un efecte sever en la síntesi de serotonina cerebral. Pel que fa a les variables d'assaig, corroborem que la primera orina del matí va ser la mostra més adequada, ja que la major part de melatonina es metabolitza i s'excreta en l'orina com a aMT6s (Yano S et al., 2013). Per tant, qualsevol pèrdua d'orina durant la nit disminuiria notablement les concentracions de aMT6s (Gholipour T et al., 2015). Aquest fet explicaria l'inesperat valor baix de aMT6 observat en dos dels nostres pacients amb tractament serotoninèrgic, que probablement van tenir problemes en la recol·lecció de mostres d'orina (pacient 18, que tenia dos anys i pacient 23, perquè tenia problemes de continència urinària).

Els nostres estudis analítics han demostrat que la variació intraindividual està en el rang de 9,9% a 15,1% (Fig. 2B de l'article). A més, les variacions metrològiques obtingudes també van ser raonables permetent una correcta interpretació dels resultats. Només el 10% dels pacients presentaven CV dels duplicats superiors al 15%, probablement deguts a fenòmens de reacció creuada ja coneguts i inherents a algunes tècniques de laboratori basades en mètodes immunològics (Benloucif S et al., 2008). Per tant, aquestes mostres no es van considerar en l'estudi i haurien de ser reanalitzades.

Pel que fa als valors de referència, hem establert tres grups d'edat diferents (consultar la taula 3 de l'article). Els nostres resultats es poden explicar pel fet que la síntesi de melatonina assoleix la taxa més alta a l'edat de 3-6 anys, i després disminueix progressivament, fins a un 80%, a l'edat adulta (Claustrat B & Leston J, 2015). Aquests valors de referència són similars a altres grups (Rosen R et al., 2009), mostrant l'elevada reproductibilitat del mètode ELISA per quantificar aMT6s en diferents poblacions.

Fixant-nos en els pacients sense tractament serotoninèrgic, es van estudiar 13 pacients amb dèficit d'adGTPCH i 11 d'ells van presentar valors normals d'aMT6. La deficiència d'adGTPCH és la malaltia més lleu entre les estudiades aquí, perquè els pacients presenten una reducció lleu-moderada en els biomarcadors de dopamina i serotonina

en LCR i fins i tot, en alguns casos, tenien valors normals. De fet, en el pacient 5 (el cas índex d'una família (López-Laso E et al., 2007)), les concentracions de 5HIAA en LCR eren normals en el moment del diagnòstic, cosa que suggereix una alteració mínima o nul·la de l'estat de serotonina cerebral. A més, la majoria dels nostres pacients amb deficiència d'adGTPCH tenien la mateixa mutació lleu (p.Q89X) del gen *GCH1* que el pacient 5, i aquest fet explicaria que els casos d'adults d'aquesta cohort presenten un fenotip molt lleu (o fins i tot sense símptomes) tal com ja s'havia descrit anteriorment ((López-Laso E et al., 2007; Opladen T et al., 2012).

La deficiència de arGTPCH acostuma a presentar PKU i té un inici precoç amb un curs clínic més sever que la deficiència de adGTPCH (Opladen T et al., 2012). En un cas (pacient 14) amb dèficit de arGTPCH els nivells d'aMT6s en orina també van ser normals. Aquest pacient tenia també nivells normals de fenilalanina i un fenotip que s'assemblava a la forma dominant de deficiència de GTPCH, cosa que va suggerir una activitat residual elevada de l'enzim GTPCH.

La deficiència de SR s'hereta de manera autosòmica recessiva. Els pacients presenten un trastorn motor que fluctua diürnament i, en la majoria dels casos, s'associa a un retard cognitiu i a una disfunció neurològica severa. Els tres pacients que es descriuen aquí són germans i van mostrar una important reducció dels nivells de aMT6s (60.3%, 23.8% i 87.3%). En el cas índex (pacient 15), també va ser notable la reducció de 5HIAA en LCR en el moment del diagnòstic (taula 1 de l'article). Aquests tres pacients presentaven un fenotip lleu amb un debut tardà (Arrabal L et al., 2011). A més, només estaven en tractament amb L-dopa/carbidopa, ja que fa uns anys es va provar el 5HTP, però es va suspendre el tractament per efectes secundaris (vòmits i diarrea). Aquestes tres germanes van presentar una nova mutació en el gen *SPR* que afecta els processos de splicing i que es va informar com un canvi lleu (Arrabal L et al., 2011). En la deficiència de SR, tant la via de la dopamina com la de la serotonina solen afectar-se greument (Friedman J et al., 2012), i els nivells baixos d'aMT6 podrien ser un reflex del deteriorament de l'estat cerebral de la serotonina.

El pacient 18, amb una forma greu de dèficit d'AADC (va debutar amb un any d'edat presentant hipotonia, crisis oculogíriques i distònies) va mostrar, com era d'esperar, un valor extremadament baix de aMT6s en orina, que correlacionava amb la dràstica reducció concomitant de les concentracions de 5HIAA en LCR.

S'ha descrit que, en cultius cel·lulars, la teràpia amb L-dopa pot ser tòxica per a les neurones serotoninèrgiques a través de mecanismes oxidatius que produeixen espècies de quinona altament reactives que redueixen les neurones serotoninèrgiques (Stansley BJ & Yamamoto BK, 2013). Aquests descobriments també s'han observat *in vivo* per mecanismes oxidatius similars que produeixen una disminució significativa de la serotonina i del metabolit 5HIAA (Stansley BJ & Yamamoto BK, 2015), a més a més d'afectar en el comportament i les funcions cognitives en models animals (Stansley BJ & Yamamoto BK, 2015). Tanmateix, no es van observar diferències quan es van comparar els valors de aMT6 en orina entre els pacients amb GTPCH sense tractament i els sotmesos a tractament amb L-dopa/carbidopa. És interessant veure que el tractament amb carbidopa (un inhibidor de l'activitat AADC perifèrica) no sembla afectar a l'excreció de aMT6s en orina, posant de manifest que la contribució de la melatonina perifèrica és menys rellevant que la de la glàndula pineal (Claustrat B & Leston J, 2015).

Pel que fa als pacients sotmesos a un tractament serotoninèrgic, tres pacients amb deficiència d'AADC van mostrar concentracions baixes d'aMT6 malgrat el tractament serotoninèrgic. En el pacient 18, l'excreció urinària de aMT6s va augmentar després de 3 mesos de teràpia amb inhibidors de la monoaminooxidasa (IMAO), cosa que suggereix que aquesta teràpia millora l'estat de la serotonina i la melatonina, tot i que el valor aMT6s es trobava encara per sota dels valors normals. Dos pacients (els pacients 19 i 20) amb un fenotip sever, amb valors de 5HIAA en LCR extremadament baixos en el diagnòstic i que estaven en tractament amb IMAO i PLP, van mostrar una excreció baixa de aMT6s en orina, mentre que el pacient 21 amb un fenotip moderat i que també estava en teràpia amb IMAOs i PLP, mostrava valors normals de aMT6s urinaris. Una explicació d'aquestes dades és que l'AADC és la condició més greu que afecta l'estat de serotonina cerebral (Pons R et al., 2004) amb una capacitat molt reduïda de biosíntesi de serotonina i melatonina. Són necessaris més estudis per determinar si el tractament a llarg termini amb IMAOs pot normalitzar l'excreció de aMT6s en pacients amb AADC. El pacient 22 amb un fenotip lleu i que estava en tractament amb IMAOs, inhibidors selectius de la recaptació de la serotonina (ISRS) i melatonina, va mostrar valors més alts de aMT6s que els valors de referència.

Pel que fa a pacients amb defectes relacionats amb la BH_4, el pacient 23 amb deficiència de SR va ser diagnosticat als vint-i-tres mesos d'edat i va presentar el fenotip típic amb retard psicomotor, hipotonia, atàxia i signes extrapiramidals (Arrabal L et al., 2011). En l'estudi de neurotransmissors en LCR va mostrar el patró de deficiència clàssica de SR amb una concentració de 5HIAA extremadament reduïda. L'anàlisi de aMT6s en orina, va mostrar valors molt baixos malgrat que el pacient estigués en tractament amb 5HTP que, previsiblement, hauria d'haver augmentat la síntesi de serotonina i melatonina (Figura 1 de l'article), ja que, n'és un precursor. Els seus pares van comentar problemes d'incontinència urinària nocturna, un fet que podria explicar els valors inesperadament baixos de aMT6s del pacient.

Tots els pacients amb deficiència de DHPR tenien valors normals d'aMT6s en orina. Tot i que aquests pacients estaven sota diferents règims de tractament, tots ells rebien 5HTP, el precursor de la serotonina. El pacient 24 també estava amb tractament des de feia molt temps amb ISRS, àcid fòlic i BH_4. Als dotze mesos d'edat (quan ja estava sota aquest tractament), va presentar valors normals de 5HIAA en LCR. Als cinc anys d'edat, va mostrar valors elevats d'aMT6s en orina, cosa que suggereix que la combinació de tots aquests fàrmacs podria haver donat lloc a aquest resultat. De fet, s'ha suggerit que alguns fàrmacs ISRS poden augmentar els valors de melatonina plasmàtica (Kirecci SL et al., 2014). Els pacients 25 i 26 estaven només en tractament amb 5HTP, i tots dos van mostrar una excreció normal aMT6s, la qual cosa dóna suport a la hipòtesi que aquest precursor de la serotonina és eficaç per augmentar l'excreció urinària aMT6s. Tot i això, el tamany de mostra reduït de la nostra sèrie ens porta a animar el desenvolupament d'assajos clínics per confirmar o descartar aquesta hipòtesi.

El pacient 27 amb una deficiència de PTPS es va diagnosticar a través d'un programa de cribatge neonatal. Amb un any d'edat, quan ja estava en tractament, seguia presentant valors reduïts de 5HIAA en LCR. Actualment, aquest pacient rep 5HTP, BH_4 i folinat de calci com a medicaments serotoninèrgics. En l'última anàlisi de LCR va presentar concentracions normals de neurotransmissor, fet que suggereix una teràpia adequada. Els valors de aMT6s en orina també eren normals. Inesperadament, el pacient 28 tenia valors molt baixos de aMT6s tot i estar en tractament amb 5HTP i BH_4. A més, s'havia sotmès a una anàlisi prèvia de neurotransmissors en LCR que mostrava resultats normals de 5HIAA. En aquest cas, el metge i la família van confirmar que l'orina s'havia

recollit correctament. Una explicació que podem trobar a aquest fet, pot estar relacionada amb variacions individuals de l'activitat de CYP1A2. El CYP1A2 és una de les isoformes principals del citocrom P450 al fetge i s'encarrega de metabolitzar diversos fàrmacs i compostos endògens importants, com la melatonina (Zhou SF et al., 2010). Hi ha una gran variabilitat interindividual en l'expressió i en l'activitat del CYP1A2, causada principalment per factors genètics (molts polimorfismes) i mediambientals (Jin T et al., 2015). Una altra explicació seria una possible deficiència de folat cerebral, ja que, l'hidroxiindol o-metiltransfrasa (HIOMT) necessita SAM com a cofactor per a la biosíntesi de melatonina. Tot i això, els valors de 5MTHF en LCR eren normals en aquest pacient.

Una limitació d'aquest estudi és que la determinació de la aMT6s depèn estrictament d'una recol·lecció adequada de les mostres, que pot ser difícil en nadons o infants amb discapacitat greu. A més, en alguns casos, les variacions interindividuals de l'activitat de CYP1A2 (Zhou SF et al., 2010; Jin T et al., 2015) poden contribuir a variacions inesperades en l'excreció urinària d'aMT6s. Un altre aspecte important de la variació de la melatonina és la llum. La seva síntesi està molt influenciada per la llum tènue mentre que la llum brillant pot bloquejar-ne la producció (Benloucif S et al., 2008). Finalment, s'hauria de fer una mesura simultània de 5HIAA en LCR i d'aMT6s en orina, en sèries més llargues de pacients per establir una correlació entre aquestes dues variables.

4. Avaluar retrospectivament el fenotip clínic i els perfils bioquímics d'amines biògenes en líquid cefalorraquidi de pacients diagnosticats genèticament d'una malaltia mitocondrial.

En aquest treball, avaluem dades clíniques, radiològiques i bioquímics en LCR en una sèrie de pacients amb malalties mitocondrials per proporcionar una major comprensió de les alteracions de les monoamines (dopamina i serotonina). La relació entre la disfunció mitocondrial i la malaltia de Parkinson ha estat àmpliament investigada.

La fallada energètica, el deteriorament en el recanvi i el control de qualitat mitocondrials, la disfunció mitocondrial provocada pel dany oxidatiu causat per l'excés d'espècies reactives d'oxigen, la disminució de la capacitat de contribuir en l'homeòstasi del calci i d'interaccionar correctament amb el reticle endoplasmàtic, la pèrdua de massa mitocondrial i el deteriorament de la dinàmica mitocondrial han demostrat estar involucrats en la fisiopatologia del Parkinson (Haddad D & Nakamura K, 2015).

Els signes i símptomes de les deficiències secundàries de neurotransmissors en malalties mitocondrials són marcadament heterogenis (Kuster A et al., 2018). Des d'un punt de vista clínic, s'han publicat pocs articles sobre alteracions en les monoamines en pacients amb malalties mitocondrials. Tanmateix, s'ha reportat que alguns pacients amb malalties mitocondrials presenten característiques de parkinsonisme (Garcia-Cazorla A et al., 2008a, b; Moran MM et al., 2011; Kuster A et al., 2018). Tzoulis C et al., (2016) mitjançant DATscan en pacients amb malalties mitocondrials va concloure que la denervació del nigrostriatal es produïa exclusivament en el subconjunt de trastorns a causats per un defecte en la replicació i manteniment de l'ADN mitocondrial. En els pacients amb malaltia POLG, la denervació de l'estriatal es pot detectar per exploració DATscan passats els vint-i-cinc anys d'edat i la gravetat del defecte nigrostriatal va mostrar una forta correlació amb l'augment de l'edat. Estudis addicionals han confirmat l'associació entre mutacions de POLG i la deficiència de dopamina (Invernizzi F et al., 2008; Tzoulis C et al., 2013; Miguel R et al., 2014).

A la nostra sèrie, els cinc pacients que van mostrar destacades característiques clíniques de deficiència dopaminèrgica sense tenir lesions de ganglis basals tenien mutacions en diferents gens (*KARS*, *GFM1*, *NFU1*, *POLG* i una deleció de 5q14.3 que inclou el gen *COX7C*) (taula 1 de l'article). L'heterogeneïtat genètica i fisiopatològica

d'aquest subgrup és notable. Kuster A et al., també van reportar característiques clíniques amb deficiència de dopamina en casos de mutacions de *POLG* i *NFU1*, però, en general, les descripcions de la literatura són anecdòtiques. De fet, és difícil diferenciar els signes clínics de deficiència de dopamina dels derivats dels altres mecanismes fisiopatològics implicats en malalties mitocondrials. La falta d'associació observada en el nostre estudi entre els biomarcadors de deficiència de dopamina (concentració d'HVA en LCR) i el fenotip clínic o radiològic, reforça la idea que la recerca de nous biomarcadors és necessària per dilucidar la base fisiopatològica del parkinsonisme i altres característiques relacionades amb la deficiència de dopamina en malalties mitocondrials. La mateixa manca d'associació es va trobar amb els valors de les pterines, ja que, en la majoria de casos aquesta disminució no era massa marcada i, probablement no tindria impacte en la biosíntesi de monoamines. Curiosament, quatre dels 29 pacients mostraven una alta concentració de neopterina en LCR, indicant, com ja s'havia reportat anteriorment (Hasselmann O et al., 2010), la presència d'esdeveniments inflamatoris en casos aïllats amb malalties mitocondrials.

L'única associació estadísticament significativa que vam trobar va ser la de concentracions elevades d'HVA amb una marcada disminució de la concentració de 5MTHF i augment de les proteïnes en LCR, que corresponia a pacients amb mutacions de l'ADN mitocondrial (principalment delecions causants de la KSS i 2 casos més que tenien mutacions en els gens de l'*ATPase* i *POLG* de l'ADN mitocondrial). Alguns autors ja havien descrit anteriorment aquest característic perfil bioquímic (Allen RJ et al., 1983; Dougados M et al., 1983; Pineda M et al., 2006; Ramaekers VT & Blau N 2004; Ramaekers VT et al., 2007; Hasselmann O et al., 2010; Serrano M et al., 2010; Tondo M et al., 2011; Grapp M et al., 2013) associat a mutacions en l'ADN mitocondrial i, en alguns casos a una deficiència de POLG (Kuster A et al., 2018). Com que aquest perfil no s'ha observat en altres malalties mitocondrials o no mitocondrials (Pérez-Dueñas B et al., 2011), una combinació d'aquests biomarcadors pot ser útil en l'estudi de pacients que alberguen mutacions en l'ADN mitocondrial. No obstant això, el mecanisme que explica aquesta associació no s'ha aclarit del tot ni té les conseqüències clíniques que s'esperarien pel que fa als elevats valors d'HVA en LCR. La hipòtesi més consistent és que el plexe coroide pot ser un òrgan diana en algunes síndromes que presenten mutacions en l'ADN mitocondrial.

A la figura 1 de l'article, es mostren els transportadors de folat (receptor de folat alfa; FRα) i de monoamines (transportador orgànic aniònic tipus 3; OAT3) del plexe coroide. Per controlar l'homeòstasi del LCR i garantir una nutrició i protecció cerebral adequades, se l'ha descrit com un sistema de transport molt complex entre els costats apical i basolateral de les cèl·lules epitelials d'aquest (Spector R, 2010; Spector R & Johanson CE, 2010). Així doncs, el plexe coroide, controla la quantitat i la composició del LCR en secretar molècules en el mateix LCR i excretar-ne d'altres del LCR a la sang (Orešković D et al., 2017). Val la pena esmentar dues característiques addicionals: (1) el plexe coroide està enriquit en mitocondris perquè els sistemes de transport actiu que consumeixen ATP són responsables de l'entrada de folat en el LCR i la sortida de monoamines del LCR a la sang (Alebouyeh M et al., 2003; Mori S et al., 2003), i (2) s'ha suggerit que l'acumulació de mitocondris mutats en el plexe coroide de pacients amb KSS condueix a problemes d'absorció i transport, i/o secreció de proteïnes, folat i monoamines (Tanji K et al., 2000). Així, la manca d'ATP causada pels mitocondris mutats no pot proporcionar l'energia necessària per transportar molècules contra gradient al LCR. Tot i això, aquesta manca d'energia no explica completament la deficiència de folat cerebral que hi ha en els pacients amb KSS ni tampoc en altres malalties mitocondrials (Garcia-Cazorla A et al., 2008a, b). D'altra banda, l'acumulació d'aquests mitocondris mutats al plexe coroide probablement contribueix a una destrucció anatòmica que impedeix el correcte transport de molècules a través del plexe coroide. S'ha descrit que en pacients amb KSS, hi ha una correlació entre les concentracions de 5MTHF i els nivells d'expressió de FRα (Grapp M et al., 2013). Altres mecanismes com l'augment de la generació d'espècies reactives d'oxigen i/o la pèrdua d'antioxidants en el LCR poden ser factors addicionals a considerar pel que fa al desenvolupament d'una deficiència central de 5MTHF en trastorns mitocondrials (Aylett SB et al., 2013).

Les concentracions elevades d'HVA es podrien explicar per una deficiència de l'activitat d'OT3 en el plexe coroide (Spector R, 2010). Per altra banda, el 5MTHF proporciona el grup metil (com a SAM) per a la COMT, necessària pel catabolisme de la dopamina a HVA. Quan hi ha dèficit de folat, l'activitat de COMT és susceptible de veure's compromesa i, per tant, de disminuir la concentració d'HVA al LCR. Per tant, en la KSS, l'augment d'HVA apunta a un mecanisme que implica l'atrapament i l'acumulació

d'HVA al LCR, fins i tot en presència de disminució de la producció per falta d'activitat de COMT.

Els elevats valors d'HVA en LCR també poden reflectir un estat dopaminèrgic deteriorat, com passa en la deficiència genètica de DAT1 (Kurian MA et al., 2011). La pèrdua de la funció del transportador DAT1 en les sinapsis conduiria a una degradació massiva de dopamina en HVA al voltant de l'espai sinàptic, fet que augmentaria la concentració del metabòlit i provocaria un estat deficient de dopamina amb greus conseqüències clíniques. La neurotransmissió dopaminèrgica depèn en gran manera de l'energia i, per tant, es podria veure afectada en els trastorns mitocondrials.

Per tant, una formació excessiva d'HVA, a través de MAO i COMT, podria donar com a resultat un consum excessiu del pool de folat a través de SAM i causar una disfunció en la càrrega de dopamina a les seves vesícules en estats de deficiència energètica. Moy LY et al., (2007) van proporcionar evidència directa que el deteriorament mitocondrial i l'estrès metabòlic poden provocar un flux de sortida de dopamina a nivell estriatal a través del DAT i van suggerir que les interrupcions en l'homeòstasi de dopamina com a resultat del dèficit energètic poden contribuir a la patogènesi de les malalties neurodegeneratives. No obstant això, la manca d'un fenotip dopaminèrgic específic en pacients amb KSS descartaria parcialment aquestes hipòtesis. Encara que és un biomarcador inespecífic, els valors de lactat en LCR no es van associar amb les alteracions clíniques, radiològiques o bioquímiques en aquest estudi.

CONCLUSIONS

1. L'anàlisi metabolòmica dirigida del LCR és factible fins i tot quan es produeix una notable contaminació hemàtica, ja que la centrifugació del LCR per eliminar els eritròcits abans de la congelació impedeix la majoria dels biaixos observats.
2. Tanmateix, les dades s'han d'interpretar amb precaució, especialment per a alguns metabòlits. L'albúmina en LCR, l'hemoglobina i la TDP es poden utilitzar com a biomarcadors secundaris per avaluar el possible efecte de confusió que la contaminació del LCR amb plasma/eritròcits pot provocar a l'hora d'interpretar els resultats.
3. Els procediments descrits en aquest protocol proporcionen mètodes robusts per determinar la concentració de monoamines i els seus cofactors (les pterines i el PLP) en mostres de LCR. No obstant això, es requereix un alt grau d'experiència per interpretar els cromatogrames i identificar les condicions genètiques esmentades en aquesta tesi i els nombrosos trastorns secundaris de la neurotransmissió, de les pterines i del PLP.
4. Es van estudiar 28 pacients amb diferents defectes genètics del metabolisme de la serotonina i es va comprovar que els pacients amb trastorns més greus presentaven una disminució de l'excreció de sulfatoximelatonina. No es van trobar alteracions concluents en els pacients amb dèficit de adGTPCH, que és la malaltia més lleu estudiada aquí.
5. La sulfatoximelatonina en orina pot ser un bon biomarcador no invasiu per estimar l'estat de la serotonina cerebral i poder monitoritzar el tractament.
6. Es van observar valors alts d'HVA en LCR, juntament amb una deficiència cerebral severa de folat, en pacients amb KSS i en altres síndromes amb mutacions en l'ADN mitocondrial, fet que constitueix un perfil molt específic d'aquests trastorns. No hi va haver signes clínics de deficiència dopaminèrgica en pacients amb KSS ni en la majoria dels pacients amb malalties mitocondrials.

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ANNEXES

ANNEX 1

ELSEVIER

Seminars in
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Biochemical Analyses of Cerebrospinal Fluid for the Diagnosis of Neurometabolic Conditions. What Can We Expect?

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In this article, we review the state-of-the-art analysis of different biomarkers in the cerebrospinal fluid for the diagnosis of genetically conditioned, rare, neurometabolic diseases, including glucose transport defects, neurotransmitter (dopamine, serotonin, and gamma-aminobutyric acid) and pterin deficiencies, and vitamin defects (folate, vitamin B_6, and thiamine) that affect the brain. The analysis of several key metabolites are detailed, which thus highlights the preanalytical and analytical factors that should be cautiously controlled to avoid misdiagnosis; moreover, these factors may facilitate an adequate interpretation of the biochemical profiles in the context of severe neuropediatric disorders. Secondary disturbances in these biomarkers, which are associated with other genetic or environmental conditions, are also detailed. Importantly, the early biochemical identification of biochemical disturbances in the cerebrospinal fluid may improve the clinical outcomes of a remarkable number of patients, who may exhibit good neurologic outcomes using the available therapies for these disorders.
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Introduction

After a clinical assessment and the establishment of initial diagnostic hypotheses, the investigation of most neurometabolic conditions discussed in this article depends on the analysis of specific cerebrospinal fluid (CSF) biomarkers. In general, biomarkers in peripheral biological fluids (blood and urine) are not suitable for the diagnosis of most of these conditions. The Table presents the most relevant CSF biomarkers and their expected concentrations in biological fluids according to the different diseases reviewed.

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The type and number of biomarkers that may be analyzed in CSF samples is increasing; however, there are few indications for performing CSF sample collection and analysis compared with blood or urine analyses. Thus, it is not always necessary to routinely collect and analyze CSF because several neurometabolic conditions are easily diagnosed in peripheral fluids. In this article, we would review different biomarkers (metabolites, vitamins, and cofactors) and highlight critical points regarding laboratory analysis and data interpretation.

Biomarkers in CSF

Glucose and Lactate

Associated Diseases

The Table indicates the associated diseases related to glucose and lactate analyses. The glucose transporter 1 (GLUT1) deficiency is caused by mutations in the *SLC2A1* gene (OMIM*138140), which causes a disorder in glucose transport to the brain through haploinsufficiency (ie, only single allele is mutated, whereas the other allele is normal). A low glucose value in the developing brain is the main pathophysiological mechanism in the disease, which is associated with 3 main phenotypes and they are: epilepsy, movement disorders, and

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Table Cerebrospinal Fluid Biomarkers, Associated Diseases and Their Expected Biochemical Profiles in Different Biological Fluids

CSF Marker	Associated Disease *OMIM®/Gene*	Biochemical Profile
Glucose	GLUT1 deficiency 138140/*SLC2A1*	*CSF*: ↓ glucose ↓ or normal lactate ↓ CSF or plasma glucose ratio
Serine	Serine deficiency 606879/*PHGDH* 610936/*PSAT1* 172480/*PSPH*	*CSF*: ↓ Ser and Gly *Plasma*: ↓ Ser (with or without ↓ Gly)
Glycine	Classic NKH 238300/*GLDC* 238310/AMT 238330/ GCSH	*CSF*: ↑↑ Gly *Plasma*: ↑↑ Gly ↑ CSF or plasma gly ratio *Urine*: ↑↑ Gly
	Variant NKH 607031/*LIAS* 609588/*GLRX5* 613183/*BOLA3* 608100/*NFU1* 615316/*IBA57*	*CSF*: ↑ Gly, lactate, and Ala *Plasma*: ↑ Gly *Urine*: ↑ Gly
Biogenic amines (HVA and 5HIAA)	TH deficiency 191290/*TH*	*CSF*: ↓ HVA and HVA/5HIAA ratio
	AADC deficiency 107930/*DDC*	*CSF*: ↓↓ HVA and 5HIAA. ↑↑ 3OMD and 5HTP *Urine*: ↑ vanillactate
	MAO A deficiency 309850/*MAO-A*	*CSF*: ↓↓ 5HIAA and HVA
	DBH deficiency 609312/*DBH*	*CSF*: ↑ HVA, HVA/5HIAA ratio, ↓ MHPG* *Urine*: ↓↓ norepinephrine and epinephrine ↑ Dopamine
	DAT1 deficiency 126455/*SLC6A3*	*CSF*: ↑ HVA and HVA/5HIAA ratio
Pterins (NP, BP, and BH_4)	Dominant GTPCH deficiency 600225/*GCH*	*CSF*: ↓ NP, BP, BH_4, HVA, and 5HIAA
	Recessive GTPCH deficiency 600225/*GCH*	*CSF*: ↓↓ NP, BP, BH_4, HVA, and 5HIAA *Plasma*: ↑ Phe *Urine*: ↓ BP and NP
	PTPS deficiency 612719/*PTS*	*CSF*: ↑ NP. ↓↓ BP, BH_4, HVA, and 5HIAA *Plasma*: ↑ Phe *Urine*: ↓ BP. ↑ NP
	SR deficiency 182125/*SRD*	*CSF*: ↑ BP and SP. Normal NP. ↓ BH_4, HVA, and 5HIAA *Urine*: ↑ SP
	PCD deficiency 126090/*PCBD1*	*CSF*: ↑ Primapterin. ↓ BH_4, HVA, and 5HIAA *Plasma*: ↑ Phe *Urine*: ↑ Primapterin
	DHPR deficiency 612676/*QDPR*	*CSF*: ↑ BP. Normal NP. ↓ BH_4, HVA, and 5HIAA *Plasma*: ↑ Phe *Dried blood spot*: ↓ DHPR activity
Free GABA	GABA-T deficiency 137150/*ABAT*	*CSF*: ↑↑ Free GABA, homocarnosine, and beta-alanine
	SSADH deficiency 610045/*ALDH5A1*	*CSF*: ↑ Free GABA *Plasma and urine*: ↑↑ GHB
5-MTHF	Folate receptor-alpha deficiency	*CSF*: ↓↓ 5MTHF and total folate

Table (*continued*)

CSF Marker	Associated Disease *OMIM*/Gene*	Biochemical Profile
	136430/*FOLR1*	↓↓ CSF 5MTHF or plasma folate ratio *Plasma*: Normal total folate
	PCFT deficiency 611672/*SLC46A1*	*CSF*: ↓↓ 5MTHF and total folate ↓ CSF 5MTHF or plasma folate ratio *Plasma*: ↓↓ total folate.
	MTHFR deficiency 607093/*MTHFR*	*CSF*: ↓↓ 5MTHF. Normal or ↑ total folate Normal CSF 5MTHF or plasma folate ratio *Plasma*: Normal total folate. Severe hyperhomocysteinemia
	DHFR deficiency 126060/*DHFR*	*CSF*: ↓↓ 5MTHF, normal CSF 5MTHF, or plasma folate ratio *Plasma*: Normal total folate and Hcys
PLP	PNPO deficiency 603287/*PNPO*	*CSF*: ↓ PLP, 5HIAA, and HVA. ↑ 3OMD and 5HTP. ↑ Gly, Ser, and Thr Normal CSF or plasma PLP ratio *Plasma*: ↑ Gly, Ser, and Thr *Urine*: ↑ vanillactate
	Antiquitin deficiency 107323/*ALDH7A1*	*CSF*: ↓ PLP, 5HIAA, and HVA. ↑ 3OMD, 5HTP, and X Compound. ↓ GABA. ↑ Gly, Ser, and Thr. ↑ AASA and pipecolic acid ↓ CSF or plasma PLP ratio *Plasma*: ↑ AASA and pipecolic acid *Urine*: ↑ AASA and pipecolic acid
Thiamine isoforms	hTHTR2 deficiency 606152/*SLC19A3*	*CSF*: ↓↓ free thiamine. ↑ lactic acid *Plasma*: ↑ branched amino acids *Urine*: ↑ 2-oxoglutarate
	TPK1 deficiency 606370/*TPK1*	*CSF*: ↑ free thiamine and ↓ TDP *Blood, muscle, and fibroblasts*: ↑ free thiamine and ↓ TDP

* *Expected values*. ↑: increased values compared with reference aged values and ↓: decreased values compared with reference aged values (see Annex).

behavioral disturbances with mental disability.[1] Early diagnosis is of paramount importance, because patients may exhibit a good neurologic outcome after treatment with ketogenic diet.[2]

Biochemical Data Interpretation

For proper data interpretation, it is important to collect the CSF sample after a strict protocol. For a GLUT1 deficiency diagnosis, it is critical to collect blood samples for the analysis of glucose (after a period of 4-6 hours of a fasting state) to rule out hypoglycemia as the cause of hypoglycorrhachia. The CSF should be collected immediately after to analyze glucose and lactate. Low CSF glucose levels, with a low CSF or blood glucose ratio and low-normal lactate values are expected in this disease.[3] New descriptions with low-normal CSF glucose values have been reported depending on the phenotype.[4,5] At this point, it is of utmost importance to rule out several disorders that cause secondary decreased CSF glucose values, such as hypoglycaemia, meningitis (bacterial, but also resulting from other microorganisms), subarachnoid bleeding, sarcoidosis, lupus, status epilepticus, cerebral hypoxia, or energy metabolism defects (eg, mitochondrial disorders).[1] Once low CSF glucose values have been demonstrated, the next step in the diagnosis is typically *SLC2A1* gene sequencing because the biochemical characterization of GLUT1 transporter activity is a complex biochemical test that is only performed in a limited number of laboratories.

CSF lactate determination is important not only for the diagnosis of GLUT1 deficiency but also to support the diagnosis of different mitochondrial disorders (Table) with a primary expression in the central nervous system (CNS).

Amino Acids

The analysis of CSF amino acids based on the quantification of serine, glycine, and alanine, which comprise biomarkers of several conditions that may be misdiagnosed through analysis in peripheral fluids, such as blood and urine.

Associated Diseases

L-serine is a precursor of several important CNS molecules, such as D-serine, glycine, cysteine, 5-methyltetrahydrofolate, sphingomyelin, phospholipids, purines, and thymidine. Serine deficiency disorders are caused by a genetic defect in 1 of the 3 steps of the synthesis pathway and they include 3-phosphoglycerate dehydrogenase (OMIM*606879), phosphoserine aminotransferase (PSAT1; OMIM*610936), and 3-phosphoserine phosphatase (PSPH; OMIM*172480).[6] The first 2 defects lead to severe neurologic symptoms, such as

congenital microcephaly, hypertonia, and epilepsy, whereas the third defect appears to lead to slow head growth and intellectual disability. L-serine supplementation may reverse some neurologic signs.[6]

The glycine catabolic pathway involves glycine cleavage enzyme (GCE), which includes a mitochondrial multienzymatic system composed of 4 proteins such as T-protein, P-protein, H-protein, and L-protein.[7] Deficient GCE activity may primarily be caused by mutations in P or T-proteins, which lead to classic nonketotic hyperglycinemia (NKH; OMIM*238300, *238310, and *238330). NKH may be classified as severe or attenuated forms based on the severity of the neurodevelopmental outcome of the disorder. The H-protein of the GCE has a lipoyl group covalently attached, which is a crucial cofactor for proper functioning and interactions with the other 3 proteins of the GCE. The discovery of the first genetic defect in the lipoylation pathway opened a new avenue in the ethological investigations of patients who present with increased blood and CSF glycine concentrations.[8] The clinical presentation associated with hyperglycinaemia varies from severe, early onset, and epileptic encephalopathy to other CNS signs and symptoms that mimic mitochondrial disorders and, most frequently, severe life-threatening conditions with few therapeutic options.

Data Interpretation

If there is a clinical suspicion, the first step is to perform an amino acid analysis of fasting plasma samples because, in most cases, both serine deficiency and hyperglycinaemia would be present. However, the diagnosis of serine deficiencies may be missed if the amino acid analysis is not performed in the fasting state. If the serine levels are low (regardless of a decreased glycine level), the next step comprises a CSF amino acid analysis. 3-phosphoglycerate dehydrogenase deficiency has been reported in 24 patients, and the biochemical findings included decreased serine and glycine levels in the CSF. These biochemical findings are similar to those patients found with PSAT1 and PSPH deficiencies. A final diagnosis is achieved after the analysis of the 3 genes involved in the serine synthesis pathway.

The expression and catalytic activity of the GCE is only complete in the brain, liver, and placenta. Deficient GCE activity causes glycine accumulation in the blood, urine, and CSF with the peculiarity of an increased glycine ratio between the CSF and plasma.[9] Moreover, deficient GCE activity may also be produced by a deficient synthesis of the lipoate cofactor because of mutations in lipoic acid synthetase (OMIM*607031), which cause the variant NKH. This disorder overlaps with multiple mitochondrial dysfunction syndromes, because all lipoates that contain mitochondrial enzymes are deficient. Moreover, a number of recently identified defects in mitochondrial iron-sulfur cluster synthesis cause a phenotype similar to the lipoic acid synthetase defect[10] (Table). In addition to hyperglycinaemia and increased CSF glycine values, increased plasma and CSF lactate and alanine values may be identified.[11] However, in most cases, hyperglycinaemia is a secondary event that results from different metabolic disturbances, such as the presence of organic acidurias (eg, propionic acidemia, methylmalonic, or isovaleric aciduria, which lead to ketotic hyperglycinaemia because of the secondary inhibition of GCE activity).[9] Nevertheless, brain GCE activity does not appear to be decreased, which results in normal CSF glycine values and a ratio between the CSF and plasma within normal limits. Vitamin B_6 deficiency may secondarily decrease the GCE activity and consequently increase glycine levels. Hypoxic-ischemic events, congenital stroke, or meningitis may also be associated with a transient increase in the CSF or plasma glycine ratio secondary to an impairment of glycine metabolism in the brain during these situations. Other conditions that lead to hyperglycinaemia include protein deficiency and starvation. Treatment with valproic acid inhibits GCE and may increase the plasma, urine, and CSF glycine levels.[9,10]

Regarding alanine, increased CSF values may predict the presence of a mitochondrial disorder, especially when lactate values are concomitantly increased. However, a general impairment in energy metabolism because of environmental conditions, such as hypoxia or ischemic events, may also increase the CSF alanine concentration.

Biogenic Amines and Pterins

Biogenic amines comprise a group of chemically related compounds that are closely related with the neurotransmitters dopamine and serotonin. Figure 1 indicates the metabolic pathways of dopamine and serotonin biosynthesis and its cofactor tetrahydrobiopterin (BH_4). The amino acids tyrosine and tryptophan are the precursors of dopamine and serotonin, respectively. After a common rate-limiting enzymatic step, which is catalyzed by 2 hydroxylases dependent on BH_4, L-dopa, and 5-hydroxytryptophan (5HTP) are synthesized. Then, after a common decarboxylation step, which is catalyzed by L-aromatic amino acid decarboxylase (AADC, whose cofactor is the active form of vitamin B_6), the neurotransmitters dopamine and serotonin are formed. Finally, several catabolic steps lead to the generation of the end-stable metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic (5HIAA) acid, which comprise the most useful biomarkers for the diagnosis of dopamine and serotonin-related disorders.

BH_4 is synthesized from guanosine triphosphate (GTP) in a metabolic pathway that involves GTPciclohydrolase-I (GTPCH-I), 6-pyruvoyltetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) enzymes. The BH_4 salvage pathway is also important, and it includes pterin 4-alpha carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) enzymes (Fig. 1). CSF analysis of pterins may be used to identify genetic conditions that affect the synthesis of the active cofactor BH_4.

Associated Diseases

Overall, 10 disorders of biogenic amine metabolism and 2 disorders related to its transport would be discussed.[12] Of the 10 genetic conditions related to biogenic amine metabolism,

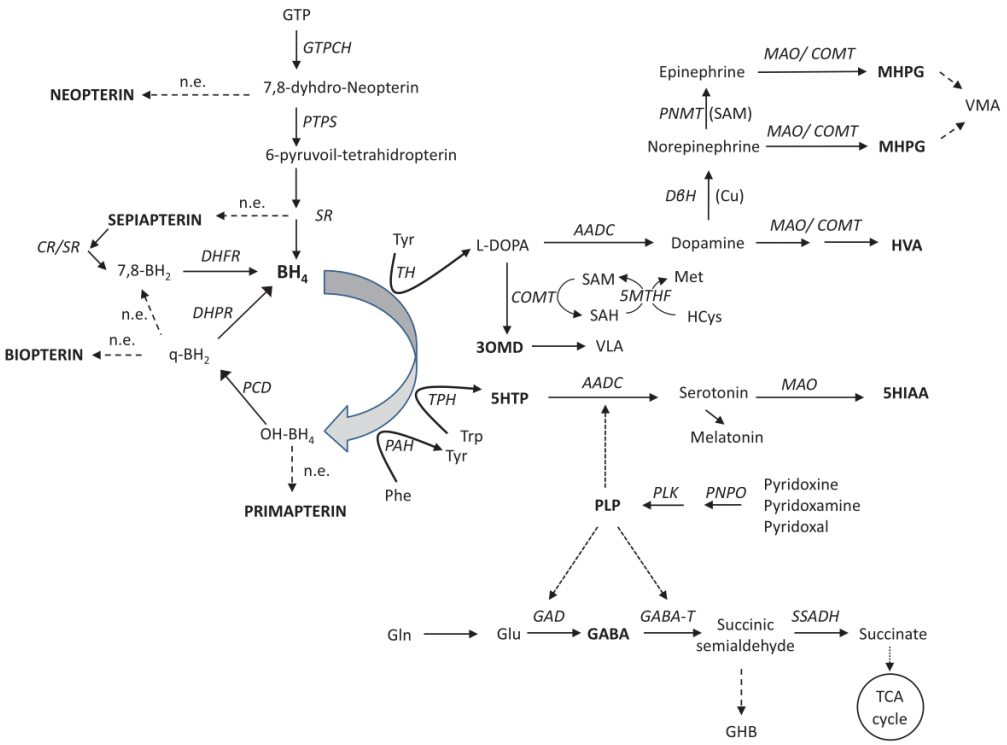

Figure 1 Metabolic pathways for the synthesis and catabolism of monoamines, pterins, GABA, and vitamin B_6. The key metabolites for neurotransmitters and pterins are marked in bold and capital letters. Enzymes appear in italics. AADC, aromatic L-amino acid decarboxylase; 7,8-BH2, 7,8-dihydrobiopterin; BH4, 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., nonenzymatic; 3OMD, 3-O-methyldopa; OH-BH4, hydroxy-tetrahydrobiopterin; PCD, pterin-4a-carbinolamine dehydratase; PLK, pyridoxl kinase; PLP, pyridoxal phosphate; PNMT, phenylethanolamine N-methyltransferase; PNPO, PTPS, 6-pyruvoly-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. (Color version of figure is available online.)

following 2 disorders specifically affect the biosynthesis of dopamine and catecholamines: tyrosine hydroxylase deficiency (TH; OMIM*191290) impairs the synthesis of dihydroxyphenylalanine (L-dopa), which causes dopamine deficiency and a neurologic disease with extrapyramidal signs[13]; and dopamine β-hydroxylase deficiency (OMIM*609312) impairs catecholamine biosynthesis (epinephrine and norepinephrine), and its clinical hallmark is severe orthostatic hypotension with sympathetic failure.[14] Further, 2 other disorders of biogenic amine metabolism involve both dopamine and serotonin metabolism: AADC (OMIM*107930) causes a combined deficiency of dopamine and serotonin, which leads to a complex clinical picture that may include muscle hypotonia, oculogyric crises, movement disorders, and autonomic features (excessive sweating and temperature instability)[15]; and X-linked monoamine oxidase A deficiency (MAO-A; OMIM *309850), which is the only defect in monoamine catabolism, causes isolated, severe, behavioral disturbances.[16]

There are 6 defects in BH_4 cofactor biosynthesis (Fig. 1): the dominant form of GTPCH-I (OMIM*600225)[17] and the recessively inherited SR deficiency (OMIM*182125)[18] primarily cause movement disorders owing to dopamine deficiency, with different degrees of serotonin-related clinical disturbances. BH_4 is also required in the liver for the conversion of phenylalanine to tyrosine in a reaction catalyzed by phenylalanine hydroxylase; thus, several defects that affect BH_4 metabolism lead to hyperphenylalaninemia (PKU). These defects include recessively inherited GTPCH-I, PTPS, PCD, and DHPR deficiencies.[11] These defects also exhibit alterations in the biogenic amine statuses in the CNS; however, they may be detected in neonatal screening programs through

phenylalanine quantification in Guthrie cards, which would not be further discussed in this article.

Totally, 2 genetic conditions that affect synaptic transporter functions have recently been reported and they are dopamine transporter 1 (DAT1; OMIM*126455) deficiency[19] and the vesicular monoamine transporter type 2 (VMAT2; OMIM*193001) defect,[20] which both cause early Parkinsonism-dystonia. DAT1 removes dopamine from the synaptic cleft, which thereby terminates its action on postsynaptic and presynaptic receptors. VMAT2 translocates dopamine and serotonin into synaptic vesicles and is essential for its liberation in the synaptic cleft.

The early identification of these conditions may be critical because some conditions are associated with a good clinical outcome after treatment with neurotransmitter precursors (L-dopa and 5-hydroxytrytophan) or neurotransmitter agonists.

Data Interpretation

In general, the analysis of biogenic amines in urine is not suitable for the diagnosis of these genetic disorders, and only an abnormal profile of organic acids (increased vanillactic acid) may be detected in some patients with AADC and vitamin B_6 deficiencies. The CSF has been identified as the most suitable biological sample for the diagnosis of most biogenic amine disorders and the 2 pterin defects without PKU. The Table indicates the diagnostic value of CSF biogenic amines and pterins for the different genetic conditions previously discussed.

Protocolized lumbar puncture collection is required for the reliable analysis of biogenic amines. Because there is a rostrocaudal gradient (the concentration of some metabolites is higher in the last fractions of the CSF compared with the initially collected samples), it is important to compare the patient's values to his or her own reference values established using the same CSF fraction. Moreover, red blood cell lysis causes the oxidation of metabolites; thus, blood-contaminated samples must be immediately centrifuged, and the clear CSF supernatant must be transferred to a new tube. Sample storage at –80°C is mandatory. The preanalytical conditions for pterin analysis are worthy of consideration. For the measurement of neopterin, biopterin, and sepiapterin, the most common practice includes CSF sample stabilization with manganese dioxide or iodine and sample protection from light.[21] Several protocols have been reported for the investigation of these preanalytical factors.[22,23]

The CSF concentrations of HVA and 5HIAA may be used as indirect markers of dopamine and serotonin metabolic pathway function in the brain. The analyses of 3-ortomethyldopa (3OMD) and methoxyhydroxyphenylglycol (MHPG) (as dopamine and norepinephrine metabolites) and 5-hydroxytryptophan (5HTP), as a serotonin precursor metabolite (Fig. 1), enable the differential diagnosis of biogenic amine disorders after a biochemical analysis. Defects in biogenic amine biosynthesis indicate decreased CSF HVA or 5HIAA or both (TH, AADC, and BH_4 deficiencies, Table), as well as substantially increased precursors for AADC deficiency (3OMD and 5HTP). A dopamine β-hydroxylase defect typically exhibits high HVA values (Fig. 1), whereas an MAO-A defect exhibits substantially low CSF HVA and 5HIAA levels. DAT1 deficiency also exhibits increased CSF HVA values because of an accelerated degradation of dopamine in the synaptic cleft, whereas a VMAT2 defect does not exhibit a clearly impaired biogenic amine profile in the CSF. Thus, variations in HVA values are not always predictive of dopamine deficiency because both low and high HVA values (eg, in TH and DAT1 deficiencies) are indicative of dopamine deficient neurotransmission. In contrast, extremely low CSF HVA and 5HIAA values may indicate dopamine and serotonin "intoxication" because of impaired biogenic amine catabolism, such as in MAO-A deficiency (Fig. 1).

The analysis of urinary pterins is performed in all patients with increased blood phenylalanine levels detected by newborn screening programs to confirm the differential diagnosis of PKU; however, this analysis includes neopterin, biopterin, and primapterin.[24,25] Neopterin, biopterin, and sepiapterin may be analyzed in the CSF, and they are useful biomarkers for the differential diagnosis of genetic disorders related to BH_4 metabolism with normal blood phenylalanine values (SR and the dominant form of GTPCH-I defects) (Fig. 1). GTPCH-I deficiency exhibits partially decreased neopterin and biopterin values, whereas SR deficiency exhibits increased CSF biopterin (because of increased degradation of BH_4) and sepiapterin concentrations.

Similar to other biomarkers, impaired CSF biogenic amine and pterin concentrations are predominately caused by secondary events because primary genetic diseases that lead to dopamine and serotonin deficiencies comprise extremely rare conditions. Thus, impaired HVA and 5HIAA concentrations (increased or decreased CSF concentrations) have been associated with various neurologic disorders (genetic or environmental).[26,27] The causes and consequences of these variations are not always understood, and impairments in monoamine biosynthesis or liberation in the synaptic cleft because of various CNS damaging conditions, accelerated turnover, or impaired elimination of end-stable metabolites have been precluded as the causes of these variations. Interestingly, increased CSF neopterin concentrations because of its synthesis activation using cytokines, such as interferon-alpha, also comprise a powerful tool for the identification of inflammatory and immune conditions in the CNS.[28]

The diagnostic confirmation of genetic conditions that lead to dopamine and serotonin deficiencies is achieved through the genetic analyses of candidate genes. Enzyme activities may also be analyzed, which support the initial diagnosis, especially when data from molecular analyses are inconclusive. Most genes have been investigated in previous years using a single-gene testing approach (Sanger sequencing); however, next-generation sequencing (targeted gene panel diagnosis) has increasingly been used in clinical laboratories, which enables a more rapid diagnosis. Using whole exome and genome sequencing, new genes that cause an impaired monoamine status have been identified.[29] The enormous complexity of synaptic transmission, in which hundreds of proteins with different functions are involved, strongly suggests that massive sequencing of large series of patients with undiagnosed

neurologic diseases would lead to the discovery of novel genes involved in neurotransmission and human diseases.

Gamma-Aminobutyric acid

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, and it naturally occurs in up to one-third of neuronal synapses, predominantly in gray matter. Its major precursor is glutamate, which is converted to GABA using the vitamin B_6 dependent enzyme glutamate decarboxylase. GABA is subsequently catabolized by 2 enzymatic steps, GABA-transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), which yield succinate as the final product of the reactions that enters the Krebs cycle. Through the transamination of α-ketoglutarate, the closed loop of this process returns to glutamate, and its conversion to GABA occurs via glutamate decarboxylase.

Associated Diseases

The inherited disorders of GABA metabolism include GABA-transaminase deficiency (OMIM*137150) and SSADH deficiency (OMIM*610045). GABA-T deficiency has only been described in 3 patients to date. It is associated with developmental delays, hypotonia, pyramidal signs, and refractory seizures. The reported siblings exhibited increased growth hormone levels and growth acceleration.[30] The major diagnostic sign is a substantial increase in free GABA in the CSF, up to 40-fold; however, the diagnosis may also be identified using proton magnetic resonance spectroscopy.[31] Homocarnosine and beta-alanine in the CSF may also be increased (Table).

SSADH deficiency, or 4-hydroxybutyric (GHB) aciduria, is the most prevalent GABA degradation disorder. The clinical presentation of this condition includes intellectual disability, ataxia, epilepsy, and psychiatric symptoms.[32] Because of an enzymatic deficiency, GABA is not converted to succinate, and its metabolite GHB accumulates in biological fluids. The diagnosis is made using an organic acid analysis in urine, which indicates substantially increased GHB excretion compared with healthy controls. CSF analysis in SSADH deficiency indicates an increase in GHB, up to 100-fold, and a mild increase in free GABA, up to 3-fold.[33]

Data Interpretation

Preanalytical conditions in free GABA CSF analysis may represent an important cause of inaccuracy. GABA occurs in the CSF in its free and peptide-linked forms. The hydrolysis of peptide-linked forms of GABA-conjugates yields the free form. After CSF collection and during the storage and thawing of samples, bound GABA may be nonenzymatically hydrolyzed to free GABA, which leads to falsely increased values. To avoid hydrolysis, samples must be frozen at −80°C immediately after collection and maintained in an ice bath during handling. Freeze or thaw cycles must also be avoided to obtain an accurate measurement of free GABA in the CSF.[34]

As previously discussed, GABA is significantly increased in both GABA-T and SSADH deficiencies; thus, CSF measurement has diagnostic importance. Nevertheless, the most prevalent condition (SSADH deficiency) is diagnosed through urinary GHB quantification. A patient's CSF GABA concentrations are compared with reference values, which are age dependent and increase according to age. High CSF GABA concentrations may suggest a defect in the catabolic pathway of GABA; however, elevated levels of free GABA may also be present after several antiepileptic treatments, such as valproic acid and vigabatrin, which modulate GABA metabolism.[35] Thus, CSF GABA analysis may be interesting for increasing knowledge regarding several CNS disorders (such as epilepsy); however, its routine measurement in CSF is not a common practice.

Folate

Folate is a water-soluble vitamin B that is mainly present in the CNS as 5-methyltetra-hydrofolate (5MTHF). 5MTHF is the most important methyl donor, and it is involved in more than 100 methylation reactions via S-adenosylmethionine (SAM) (Fig. 2). Among these reactions, folate is essential for myelin stability, dopamine and serotonin turnover, purine synthesis, and amino acid metabolism (such as homocysteine, methionine, serine, and glycine).[36,37]

Folate requirements must be obtained entirely from dietary sources because mammalian cells cannot synthesize it de novo. Peripheral folate deficiency has been related to megaloblastic anemia, growth retardation, congenital birth defects, pregnancy complications, osteoporosis, cancer, and several neurodegenerative and psychiatric diseases.[38]

Associated Diseases

Cerebral folate deficiency (CFD) is a relatively recently described condition, which is characterized by low 5MTHF in the CSF with normal peripheral folate metabolism and various neurologic symptoms, including white matter disturbances, developmental delays, epileptic seizures, irritability, hypotonia, dyskinesia, autism, spastic-ataxic syndrome, dystonia, catatonic schizophrenia, dementia, or myoclonus.[37,39] CFD has been associated with both genetic (transport across the blood-CSF barrier and folate metabolic pathway defects) and acquired conditions.

Folate transport defects: 5MTHF transport into the CNS primarily occurs in choroid plexus epithelial cells across the high-affinity folate receptor alpha in an adenosine triphosphate-dependent process, which leads to a 1.5-fold or greater concentration of 5MTHF in the CSF compared with plasma (Fig. 2).[37] Mutations in the folate receptor 1 gene (FOLR1), which codes for folate receptor alpha (OMIM*136430), cause cerebral folate transport deficiencies.[40,41] The other genetic condition that affects folate transport in several tissues, including the brain is the proton coupled folate transporter deficiency (OMIM*611672), which leads to hereditary folate malabsorption and causes an early onset-severe disease that encompasses both blood and CSF folate deficiencies.[42] For both conditions, within the CNS,

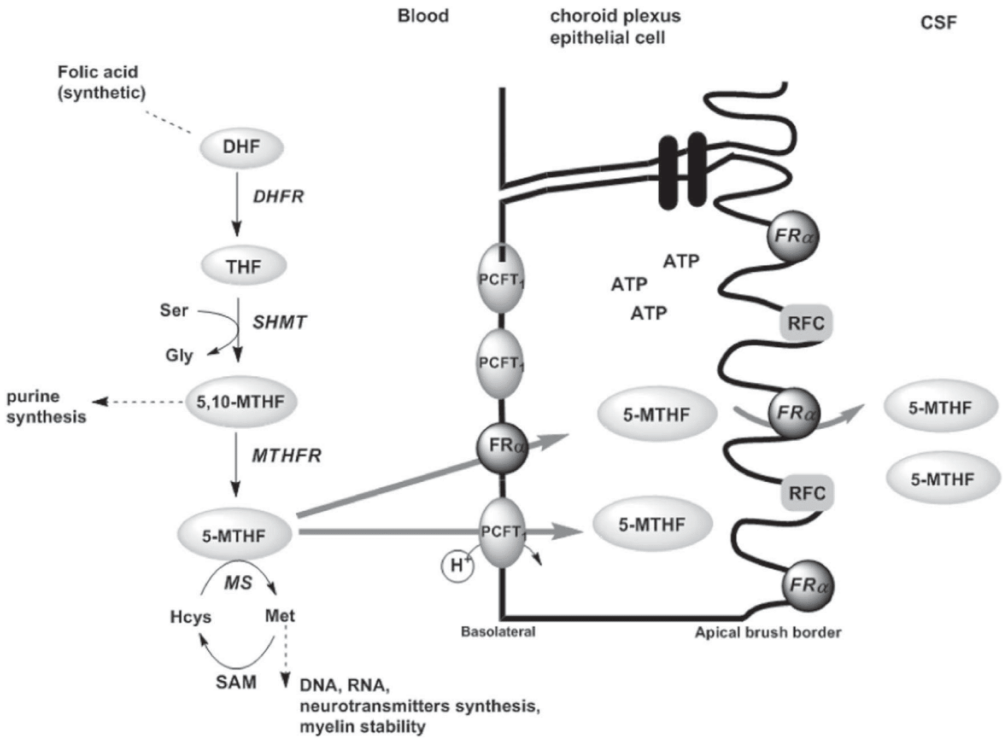

Figure 2 Schematic representation of folate metabolism and transport across the choroid plexus (see Annex).

demyelination and intracranial calcifications are frequent, in combination with developmental delays, mental retardation, seizures, and motor disturbances. Biochemically, the demonstration of an abnormal CSF or serum folate ratio (in the presence or absence of peripheral folate deficiency) is most likely the hallmark of the disorders. The treatment of these patients with folinic acid may partially improve the clinical picture.[41]

Among folate metabolism disturbances, the most profound CSF 5MTHF deficiencies occur in the severe form of methylene-tetrahydrofolate reductase deficiency (MTHFR; OMIM*607093) and dihydrofolate reductase deficiency (OMIM*126060). Biochemically, patients exhibit normal serum total folate levels associated with severe cerebral 5MTHF deficiency in all cases, which are similar to genetic folate transport disturbances.[41]

Regarding acquired conditions, impaired folate transport to the brain is also the cause of some cases of CFD. The presence of autoantibodies against the high-affinity folate receptor has been reported to be one of the main causes of this deficiency.[43] In Kearns-Sayre syndrome (OMIM#530000), the accumulation of mutated mitochondrial DNA copies in the choroid plexus epithelium has been described as the cause of impaired folate transport.[35,44-46] Other conditions associated with CFD include DHPR deficiency, serine biosynthesis defects, Rett syndrome, Aicardi-Goutiere syndrome, other mitochondrial diseases, hypomyelination with atrophy of the basal ganglia syndrome, and catatonic schizophrenia.[37,44] In many cases of CFD, the underlying etiology is not understood.[47]

Data Interpretation

The diagnosis of CFD requires the determination of the CSF 5MTHF concentration and an assessment of peripheral folate and its related metabolites (amino acids [methionine, homocysteine, serine, and glycine] and vitamin B_{12} levels). No special requirements regarding CSF sample collections have been reported. 5MTHF analysis is typically performed in specialized laboratories using high-performance liquid chromatography with fluorescence or electrochemical detection or liquid chromatography-tandem mass spectrometry (LC-MS or LC-MS/MS).[48,49] However, the usefulness of total folate determination (which includes 5MTHF) in the CSF using automated analyzers available in routine clinical chemistry laboratories has been reported to screen for CFD.[48,50] Moreover, most folate compounds in the body exist as 5MTHF; thus, this approach is a good tool for a first-line investigation of folate transport detection in the brain, which may subsequently be confirmed through the specific analysis of 5MTHF. Therefore, the possibility of using an automated procedure to accelerate this diagnostic process is attractive because, in some cases, these diseases are under treatable conditions.

Profound CSF 5MTHF deficiencies ($l < 10$ nmol/L) have been identified in patients with the previously described genetic diseases, thereby leading to impaired transport across the blood-CSF barrier or genetic folate metabolism alterations, whereas in the other secondary CFD forms, 5MTHF levels were, in general, moderately decreased. Of these profound 5MTHF deficiencies, patients with transport defects also

exhibit a profound deficiency in total folate in the CSF, which suggests that impaired transport affects all folate forms.[49] Regarding metabolic alterations, such as MTHFR deficiency, the total folate values in the CSF may be high-normal, which reflects impaired 5MTHF biosynthesis and folate trapping in other metabolic inactive forms of this vitamin. Regarding patients with a mild CFD deficiency, it has been suggested that it may be a secondary event in most diseases and a recurrent finding in many neuropediatric patients. In this sense, the simultaneous measurement of serum folate is mandatory for a better interpretation of CFD because a suboptimal blood folate status would explain most cases that exhibit partially low CSF 5MTHF concentrations.[48,49] Genetic analysis would confirm the etiology of CFD. A thorough clinical investigation of patients is of paramount importance because nonrelated genetic (eg, mitochondrial disorders) and environmental conditions may lead to CFD.

Vitamin B_6

Vitamin B_6 is present in a wide variety of foods, such as meat, milk products, potatoes, beans, nuts, and several fruits and vegetables.[51] The biologically active form of vitamin B_6 is pyridoxal 5-phosphate (PLP), which acts as a coenzyme for more than 100 distinct enzymatic steps. It is closely related to dopamine, serotonin, and GABA biosynthesis (Fig. 1).

Pyridoxal kinase (PLK) converts vitamin B_6 into the corresponding phosphorylated vitamers, whereas pyridox(am)ine 5′-phosphate oxidase (PNPO) converts the latter into PLP. To enter the cells and pass the blood-brain barrier, B_6 vitamin must be dephosphorylated by a membrane associated, tissue-nonspecific alkaline phosphatase and subsequently rephosphorylated inside the cells.[52]

Associated Diseases

The classical forms of vitamin B_6 deficiency, which cause pyridoxine or PLP responsive seizures, have been associated with 2 genetic diseases: PNPO (OMIM*603287) and antiquitin (OMIM*107323) deficiencies. In PNPO deficiency (Fig. 1), impaired PLP biosynthesis leads to decreased PLP levels in both plasma and CSF in most cases, which is the main biomarker of the disease. It is characterized by a therapeutic response to pharmacologic dosages of PLP.[53] Antiquitin deficiency has been associated with classical pyridoxine-dependent epilepsy, and it is caused by an α-Aminoadipic-semialdehyde dehydrogenase (ALDH7A1) deficiency. Antiquitin functions as a dehydrogenase in the lysine degradation pathway, and its deficiency results in the accumulation of α-aminoadipic semialdehyde, piperideine-6-carboxylate (P6C), and pipecolic acid. P6C undergoes chemical condensation with PLP, which results in a functional PLP deficiency.[54]

Data Interpretation

PLP is the metabolite that should be analyzed instead of total vitamin B_6 determination. Preanalytical factors regarding CSF collection are not required beyond sample storage at −80°C. PLP is a cofactor for enzymes involved in the metabolism of several neurotransmitters, such as dopamine, serotonin, and GABA. AADC is a pyridoxine-dependent enzyme that converts L-DOPA and 5HTP to dopamine and serotonin, respectively (Fig. 1). In pyridoxine disorders, the CSF neurotransmitter metabolite profile may mimic AADC deficiency with decreased HVA and 5HIAA and increased 3OMD.[55] Using the same mechanism, CSF GABA concentrations are expected to be low in a PLP deficient state. CSF amino acids may also exhibit increases in glycine, serine, and threonine, because serine and threonine dehydratase enzymes and the GCE are PLP-dependent. In PNPO deficiency, these analytical disturbances may also be identified in plasma samples.[54]

Patients with antiquitin deficiency typically do not exhibit biochemical evidence of PLP deficiency in plasma; however, they may exhibit a reduction in CSF PLP levels. Thus, the depletion of PLP appears to primarily occur in the brain, and the CSF to plasma PLP ratio may represent an adjuvant marker for diagnosis.[54] Therefore, the simultaneous determination of plasma and CSF PLP may be used to distinguish PNPO and antiquitin deficiencies. Moreover, in antiquitin deficiency, the presence of an unknown compound (X compound) is commonly identified (this compound appears during CSF biogenic amine analysis via high-pressure liquid chromatography).

Similar to other vitamin-related disorders, secondary PLP deficiency may be associated with other conditions, such as hyperprolinemia type II (OMIM*606811), mucopolysaccharidosis type III (OMIM*607664, *6097001, *605270, and *610453), and other diseases. Genetic testing is mandatory to elucidate the molecular basis of the different diseases potentially associated with PLP deficiency.

Thiamine

Thiamine, which comprises an essential water-soluble vitamin of the B complex (vitamin B_1), is a key cofactor involved in energy metabolism in brain tissue. Thiamine phosphate derivatives (thiamine monophosphate [TMP], thiamine diphosphate [TDP], and mainly thiamine triphosphate) are involved in several cellular processes. Thiamine isoforms are absorbed in the small intestine by 2 specific transporters and they are thiamine transporter-1 (hTHTR1, encoded by *SLC19A2*) and thiamine transporter-2 (hTHTR2, encoded by *SLC19A3*). After absorption, thiamine is converted to TDP by a specific cytosol kinase (thiamine phosphokinase, TPK). The mitochondrial thiamine pyrophosphate carrier encoded by *SLC25A19* subsequently mediates the uptake of TDP into the mitochondria (Fig. 3). TDP is a cofactor of various enzymes in the cytosol (transketolase), peroxisomes (2-hydroxyacyl-CoA lyase), and mitochondria (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and branched-chain alpha-keto acid dehydrogenase) (Fig. 3).

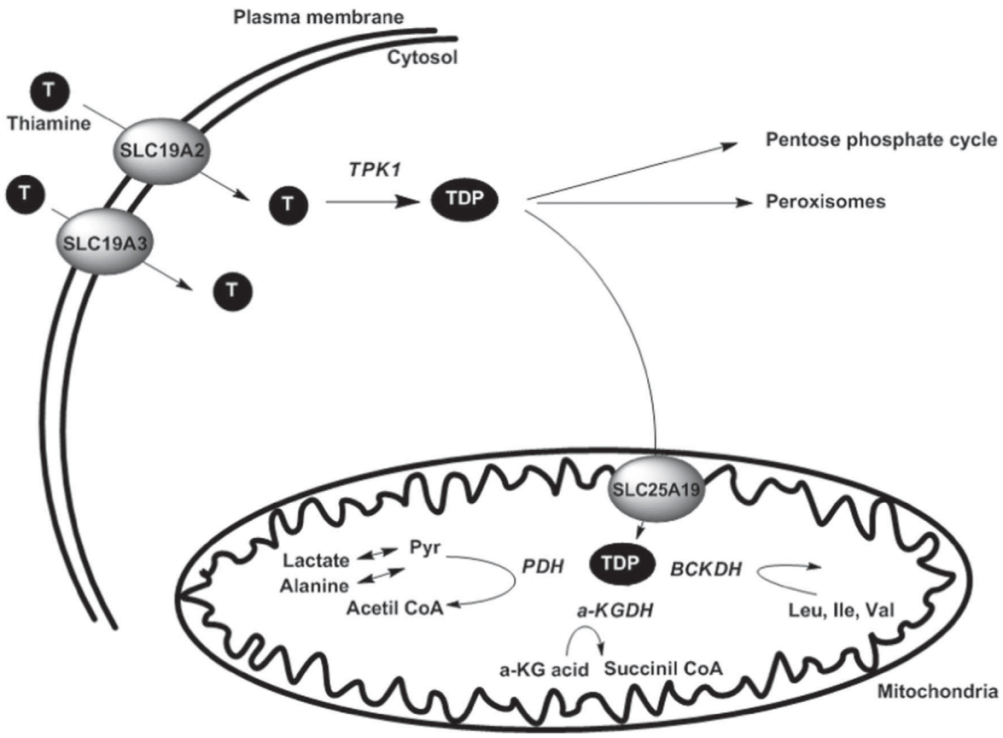

Figure 3 Metabolism and transport of thiamine across cells and mitochondria (see Annex).

Associated Diseases

There are 4 known genetic defects (*SLC19A2*, *SLC19A3*, *SLC25A19*, and *TPK1*) involved in the metabolism and transport of thiamine with variable responses to the administration of thiamine and biotin. The phenotype in *SLC19A2* (OMIM*603941) includes megaloblastic anemia, diabetes, and sensorineuronal deafness. *SLC19A3* (OMIM*606152) presents with a biotin-thiamine-responsive basal ganglia disease, Leigh syndrome, infantile spasms, and lactic acidosis. Patients with *TPK1* (OMIM*606370) exhibit a Leigh syndrome phenotype, whereas patients with *SLC25A19* (OMIM*606521) may exhibit severe congenital microcephaly or striatal bilateral necrosis and axonal polyneuropathy, frequently with 2-oxoglutaric aciduria.

Data Interpretation

The most important preanalytical consideration for thiamine derivative analysis is temperature (samples should be stored at −80°C and maintained on ice during sample preparation). Thiamine derivatives (free thiamine, TMP, TDP, and TTP) may be analyzed in different biological specimens, including whole-blood and CSF.[56,57] Thiamine analyses in CSF have rarely been reported.[56,58] It has recently been demonstrated that free thiamine is the most sensitive form for the identification of hTHTR2 deficiency.[56] Thus, the determination of CSF-free thiamine in patients who had Leigh syndrome with a suspicion of hTHTR2 deficiency may provide rapid evidence of thiamine deficiency and the initiation of early treatment, which may restore the lack of thiamine in the CNS. More importantly, most patients exhibit an excellent neurologic outcome after treatment. Whole-blood thiamine analysis did not indicate consistently decreased values in these patients; however, it may be useful to monitor treatment compliance because repeated lumbar punctures for treatment monitoring are not appropriate because of ethical reasons.[59] Mild CSF thiamine reductions have been identified in patients with Leigh syndrome with no hTHTR2 deficiency. This finding may be explained by an increase in oxidative stress and thiamine turnover.[56]

In TPK-1 deficient patients,[55] low TDP values in the blood, muscle and fibroblasts with normal levels of free-thiamine, and TMP have been reported. Theoretically, the same findings are expected in CSF samples (low TDP and normal-high CSF-free thiamine levels). There are no other CSF studies regarding the other genetic defects in the transport and metabolism of thiamine. hTHTR1 deficiency does not affect the CNS; thus, a lumbar puncture to obtain CSF is not advisable. Regarding the *SLC25A19* defect, there are no studies regarding CSF analyses, and the few reported patients present with lactic acidosis. Thus, other biomarkers may be useful to identify a deficient thiamine status, such as lactate, 2-oxoglutarate or branched-chain amino acids and their metabolites because their metabolism is dependent on thiamine availability (Fig. 3). However, alterations in these biomarkers are not universally identified in patients with thiamine deficiency.

Conclusion

An increasing number of CSF biomarkers have been identified for the diagnosis of different neurometabolic conditions. The main clinical indication for performing these studies is the rapid identification of the different diseases reviewed in this article. Some of these conditions may benefit from treatment based on a remarkable number of cases that exhibit a good neurologic outcome. A genetic diagnosis is ultimately mandatory; however, this process is clearly slower than biochemical analysis. Because of the substantial number of genetic or nongenetic conditions that may impair the status of these biomarkers, a thorough clinical investigation of patients is of paramount importance not only to initiate early treatment but to also confirm the etiologic diagnosis. With the application of novel omics technologies (targeted and untargeted genomic, metabolomic, and proteomic studies), new biomarkers would be identified in the near future.

Annex

3OMD, 3-ortomethyldopa; PHGDH, 3-phosphoglycerate dehydrogenase; PSPH, 3-phosphoserine phosphatase; GHB, 4-hydroxybutyric; 5,10MTHF, 5,10-methylene-THF; THF, 5,6,7,8-tetrahydrofolate; 5HTP, 5-hydroxytryptophan; 5HIAA, 5-hydroxyindoleacetic acids; 5MTHF, 5-methyltetrahydrofolate; PTPS, 6-pyruvoyltetrahydropterin synthase; DHF, 7,8-dihydrofolate; 7,8-BH2, 7,8-dihydrobiopterin; AASA, alpha-aminoadipic semialdehyde; AMT, aminomethyltransferase; AADC, aromatic l-amino acid decarboxylase; AD-GTPCH, autosomal dominant guanosine triphosphate ciclohydrolase-I; AR-GTPCH, autosomal recessive guanosine triphosphate ciclohydrolase-I; BP, biopterin; BOLA3, BolA family member 3; CR, carbonyl reductase; COMT, catechol O-methyltransferase; CNS, central nervous system; CFD, cerebral folate deficiency; CSF, cerebrospinal fluid; Cu, copper; Cys, cysteine; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; l-dopa, dihydroxyphenylalanine; DAT1, dopamine transporter 1; DBH, dopamine β-hydroxylase; FOLR1, folate receptor 1 gene; GABA-T, GABA-transaminase; GABA, gamma-aminobutyric acid; GLUT1, glucose transporter 1; Glu, glutamate; GAD, glutamate decarboxylase; Gln, glutamine; GLRX5, glutaredoxin 5; Gly, glycine; GCE, glycine cleavage enzyme; GSCH, glycine cleavage system h protein; GLCD, glycine decarboxylase; GTPCH-I, GTP ciclohydrolase-I; GTP, guanosine triphosphate; FRα, high-affinity folate receptor α; HPLC, high-performance liquid chromatography; HCys, homocysteine; IBA57, homolog, iron-sulfur cluster assembly; HVA, homovanillic acid; OH-BH4, hydroxy-tetrahydrobiopterin; PKU, hyperphenylalaninemia; NFU1, iron-sulfur cluster scaffold; Ile, isoleucine; Leu, leucine; LIAS, lipoic acid synthetase; LC-MS or LC-MS/MS, liquid chromatography-tandem mass spectrometry; Met, methionine; MHPG, methoxyhydroxyphenylglycol; MTHFR, methylene tetrahydrofolate reductase; MAO-A, monoamine oxidase A deficiency; MAO, monoamino oxidase; NP, neopterin; NGS, next-generation sequencing; n.e., nonenzymatic; NKH, nonketotic hyperglycinemia; PAH, phenylalanine hydroxylase; PNMT, phenylethanolamine N-methyltransferase; PSAT1, phosphoserine aminotransferase; P6C, piperideine-6-carboxylate; PCFT, proton coupled folate transporter deficiency; PCD, pterin 4-alpha carbinolamine dehydratase; PNPO, pyridox(am)ine 5′-phosphate oxidase; PLP, pyridoxal 5-phosphate; PLK, pyridoxal kinase; q-BH2, quinoide-dihydrobiopterin; RFC, reduced folate carrier; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SR, sepiapterin reductase; SHMT, serine-hydroxymethyltransferase; Ser, serine; SSADH, succinic semialdehyde dehydrogenase; BH4, tetrahydrobiopterin; TDP, thiamine diphosphate; TMP, thiamine monophosphate; TPK, thiamine phosphokinase; hTHTR2, thiamine transporter-2; hTHTR1, thiamine transporter-1; TTP, thiamine triphosphate; TCA, tricarboxylic acid cicle; TPH, tryptophan-5-hydroxylase; Trp, tryptophan; Tyr, tyrosine; TH, tyrosine hydroxylase; Val, valine; VLA, vanillactic acid; VMA, vanillmandelic acid; VMAT2, vesicular monoamine transporter type 2; vB_6, vitamin B_6; ALDH7A1, α-aminoadipic-semialdehyde dehydrogenase

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ANNEX 2

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ORIGINAL ARTICLE

A targeted metabolomic procedure for amino acid analysis in different biological specimens by ultra-high-performance liquid chromatography–tandem mass spectrometry

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Abstract

Introduction Amino acid analysis in biological fluids is essential for the study of inborn errors of metabolism (IEM) and other diseases.

Objectives Our aim was to develop a UPLC-MS/MS procedure for the analysis of 25 amino acids and identification of 17 related compounds.

Methods Sample treatment conditions were optimized for plasma, urine, cerebrospinal fluid (CSF) and dried blood spots. Amino acids and related compounds were analyzed on a Waters ACQUITY UPLC H-class instrument with a reversed-phase C-18 column using water and acetonitrile with 0.1% formic acid as the mobile phases (run time = 9 min). The detection was performed with a Waters Xevo TQD triple-quadrupole mass spectrometer using positive electrospray ionization in the multiple reaction monitoring mode.

Results The method linearity, intra-assay and inter-assay precision, detection limit, quantification limit and trueness analysis displayed adequate results in both physiological and pathological conditions. Method comparison was performed between UPLC-MS/MS and ion exchange chromatography (IEC) with ninhydrin derivatization, and the methods showed good agreement, except for 4-hydroxyproline, aspartate and citrulline. Paediatrics age-related reference values in plasma, urine and CSF were established and patients with different IEM were easily identified.

Conclusion We report a modified UPLC-MS/MS procedure for the analysis of 42 amino acids and related compounds in different specimens. The method is fast, sensitive and robust, and it has been validated to be an alternative to the traditional IEC procedure as the routine method used in metabolic laboratories. The method greatly decreases the run time of the analysis while displaying good metrological results.

Keywords Amino acids · Inborn errors of metabolism · UPLC · Tandem mass spectrometry

1 Introduction

Amino acid analysis in different biological fluids is essential for the diagnosis of several inborn errors of metabolism (IEM), but the amino acids can also serve as sensitive biomarkers for the study of other diseases. To date, a large number of IEM related to amino acid metabolism disturbances have been genetically described, with some of them being treatable diseases (DeArmond et al. 2017). In the last few decades, expanded neonatal screening programs have grossly improved, where some amino acids are included. Nevertheless, not all of the amino acids can be analyzed in these newborn screening programs. Thus, sensitive and fast methods for the diagnosis and treatment monitoring of IEM must be adapted in clinical chemistry reference laboratories.

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In addition to the study of IEM, amino acid profiling has become a new tool for studying other diseases. Plasma amino acids have been studied in several types of cancer (Shingyoji et al. 2013; Ma et al. 2014; Miyagi et al. 2011), revealing significant differences in the amino acid profiles between the controls and patients. In other diseases, such as type-2 diabetes (Wang et al. 2011) and Alzheimer's disease (Corso et al. 2017), the predictive values of amino acid profiles have also been demonstrated. Branched-chain amino acids (leucine, isoleucine and valine) have been related to cardiovascular disease in several studies (Fergurson and Wang 2016; Ruiz-Canela et al. 2016).

Quantitative analysis of amino acids have been performed by different technologies: ion exchange chromatography (IEC), reverse phase HPLC (Leah et al. 1986) or gas chromatography-mass spectrometry (Singh and Ashraf 1988). The most widely employed method (reference method) for amino acid quantification and the study of IEM is IEC coupled to post-column ninhydrin derivatization and spectrophotometric detection at two wavelengths (DeArmond et al. 2017; Duran 2009). This quantitative analysis presents some disadvantages, such as a high chromatographic separation time, generally requiring 2–3 h per sample, high sample volumes (> 200 µL) and the detection interfering compounds that react with ninhydrin and cannot be identified with spectrophotometric detection.

Other approaches for amino acid analysis use pre-column derivatization and reversed-phase HPLC separation. The most common derivatization reagents for amino acid analysis include *o*-phthalaldehyde (OPA) and phenylisothiocyanate (PITC) (Fekkes 1996), but display some disadvantages. OPA cannot react with secondary amines, and some derivatives are unstable, such as glycine and lysine (Mengerink et al. 2002). PICT can derivatize secondary amines, but it produces unstable derivatives. Additionally, it is necessary to remove the excess reagent to avoid poor chromatographic resolution and rapid column deterioration (Checa-Moreno et al. 2008), as occurs with other derivatization agents (Uutela et al. 2009).

Compared with reversed-phase chromatograpy, methods with no derivatization have been developed (Piraud et al. 2005; Waterval et al. 2009; Chen et al. 2016), but they display problems regarding retention and resolution due to the high hydrophilicity of amino acids. In order to improve amino acid retention, ion pairing agents are required, resulting in methods with a simple sample pretreatment but requiring long equilibration times and column regeneration to avoid retention time shifts. Decreases in amino acid ionization efficiency has been reported. Hydrophilic interaction chromatography procedures have also been developed (Krumpochova et al. 2015; Prinsen et al. 2016); these methods use no mobile phase modifiers avoiding the problems associated with ion pairing, but they have low separation efficiency when compared with reversed-phase chromatography.

6-Aminoquinolyl-*N*-hydroxysuccinimildyl carbamate (AQC) is a derivatization reagent that requires simple and easy sample pretreatment, and it reacts with both primary and secondary amines. This reaction is a fast process that produces stable derivatives. This reagent has been used in UPLC coupled to fluorescence detection with good results for amino acid analysis (Sharma et al. 2014). The only disadvantage is the long analysis time (30–60 min) required to obtain good resolution of all clinically relevant amino acids. This disadvantage could be overcome by coupling this UPLC procedure to tandem mass spectrometry detection (MS/MS), which offers greater sensitivity and selectivity in the multiple reaction monitoring (MRM) mode (Salazar et al. 2012).

Our aim was to standardize an UPLC procedure using AQC derivatization and MS/MS detection in the MRM mode for amino acid determination in plasma, urine, cerebrospinal fluid (CSF) and dried blood spot (DBS) samples. This method analyzes 42 amino acids and related compounds of clinical interest for the diagnosis and treatment follow-up of IEM of amino acids and other molecules. Comparison of the analytical results obtained with those of the classic IEC method was also performed.

2 Materials and methods

The amino acid analysis method was based on an application developed by Waters. The chromatographic conditions and mass spectrometry parameters were adopted from the application "A validated method for the quantification of amino acids in mammalian urine" (Gray and Plumb 2016). In this application, complete validation for the analysis of 20 proteinogenic amino acids was described. For the other amino acids and related compounds analyzed in this procedure, MS/MS parameters were optimized in our laboratory. Furthermore, optimal pretreatment conditions for other matrices (plasma, CSF and DBS) were also developed.

2.1 Chemicals

The AccQ·Tag Ultra Derivatization Kit was provided by the Waters Corporation. Mixtures of amino acid standards (acidics, neutrals and basics) and glutamine were purchased from Sigma–Aldrich. A mixture of isotope-labeled amino acids was provided by Cambridge Isotope Laboratories, Inc. Amino acid control materials were provided by MCA Laboratory. All mobile phases and wash solutions were prepared using LC-MS grade acetonitrile, methanol, water and formic acid (Merck & Co., Inc.).

2.2 Standards

The amino acid standard solutions contained 2500 µmol/L of each amino acid, except for cystine (1250 µmol/L), and were diluted in 0.1 mol/L HCl. These solutions were further diluted in water resulting in a final concentration of 250 µmol/L for each amino acid, except for cystine (125 µmol/L). The mixture of isotope-labeled amino acids was diluted in water to reach a final concentration of 250 µmol/L for each amino acid, except for cystine ($^{13}C_6$, $^{15}N_2$) (125 µmol/L).

2.3 Sample treatment

Standard, quality control, plasma and urine sample aliquots of 25 µL were vortexed with 25 µL of the internal standard solution and 150 µL of methanol/0.1% formic acid to precipitate the proteins. Then, the samples were centrifuged at 6000×*g* for 10 min. For the derivatization reaction, 5 µL of the supernatant was mixed with 35 µL of borate buffer (pH 8.8) and 10 µL of the AQC solution (3 mg/mL in acetonitrile; AccQ·Tag Ultra Derivatization Kit). Since CSF samples do not need protein precipitation, a 5 µL CSF aliquot was mixed with 5 µL of the internal standard solution, and 5 µL of the mixture was reacted with 35 µL of borate buffer and 10 µL of AQC solution. In DBS, amino acids were extracted from 1 punch of 6 mm in diameter with 130 µL of 3% trichloroacetic acid and 10 µL of the internal standard solution. The mixture was extracted for 1 h at room temperature and centrifuged at 2200×*g* for 10 min. Ten microliters of the supernatant was mixed with 60 µL of borate buffer and 10 µL of AQC solution.

The reaction mixtures were heated at 55 °C for 10 min. After that, the samples were ready to be analyzed.

2.4 Chromatographic conditions

A Waters ACQUITY UPLC H-class was used. Chromatographic separation was performed at 55 °C with a CORTECS C18 2.1×150, 1.6 µm column (Waters). A gradient of water (mobile phase A) and acetonitrile (mobile phase B), both containing 0.1% formic acid, was used at a flow rate of 0.5 mL/min. The gradient program used was as follows: initial 99% A, hold for 1 min; gradient to 87% A in 1 min; gradient to 85% A in 3.5 min; gradient to 5% A in 1 min, hold for 1 min; return to initial conditions in 0.1 min and equilibrate for 1.4 min. The run time was 9 min, and the injection volume was 2 µL.

2.5 Mass spectrometry conditions

The detection was performed with a Waters Xevo TQD triple-quadrupole mass spectrometer using positive electrospray ionization conditions in the MRM mode. The desolvation temperature was 650 °C, and the source temperature was 150 °C. The cone gas flow was 100 L/h, and the desolvation gas flow was 1000 L/h. The capillary voltage was 1 kV. The MRM transitions, cone voltage, collision energy, dwell time and detection window for each amino acid were optimized and are shown in Table 1.

2.6 Validation procedure

The procedure linearity was analyzed using serial dilutions of a spiked plasma sample, covering the range of amino acid concentrations (low to high) currently observed in IEM. For routine amino acid analysis, calibration was practiced daily with a 250 µmol/L amino acid concentration, except for cystine (125 µmol/L).

To assess the intra-assay precision, ten replicates of physiological range control material were derivatized and analyzed. The inter-assay precision was calculated by derivatizing and analyzing two control materials for 20 days, one in the physiological range and the other in the pathological range. The detection limit (LOD) was calculated as three times the signal to noise (S/N) ratio, and the quantification limit (LOQ) was calculated as ten times the (S/N) ratio by analyzing serial dilutions of a CSF sample. To study the trueness of the method in serum, 8 external quality control samples of European Research Network for evaluation and improvement of screening, diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) were analyzed. Method comparison was performed between UPLC-MS/MS and IEC with the ninhydrin derivatization methods. Fifty-six samples (plasma, urine, CSF and ERNDIM samples) were analyzed by both procedures, covering both the physiological and the pathological ranges. Passing-Bablok regression analysis plots (MedCalc Software, Mariakerke, Belgium) were applied to compare both procedures. Reference values were established by analysing samples from 103 control subjects (age range: 0 days–18 years; median: 3.0 years): 60 plasma samples, 80 urine samples and 40 CSF samples. Regarding DBS analysis, correlation between the plasma and DBS amino acids was also studied by UPLC-MS/MS for 6 amino acids (alanine, valine, isoleucine, leucine, tyrosine and phenylalanine) in 10 subjects. To study the accuracy in DBS samples, 20 DBS samples of the Spanish Association of Newborn Screening (AECNE) external quality control scheme were analyzed for phenylalanine and tyrosine, and 8 DBS samples of the ERNDIM external quality control scheme were analized for leucine, isoleucine, valine, phenylalanine and tyrosine.

Table 1 Specific mass transitions, dwell time (DT), cone voltage (CV), collision energy (CE), retention time (RT) and isotope-labeled internal standard (IS) used for the analysis of each amino acid

Amino acid	Precursor ion (m/z)	Product ion (m/z)	DT (s)	CV (V)	CE (eV)	RT (min)	IS
Histidine	326.1	156.0	0.010	20	10	1.42	Histidine ($^{13}C_6$,$^{15}N_3$)
Hydroxyproline	302.1	171.1	0.009	10	22	1.70	Histidine ($^{13}C_6$,$^{15}N_3$)
Asparagine	303.1	171.1	0.009	30	22	2.08	Serine ($^{13}C_3$,^{15}N)
Phosphoethanolamine	312.0	171.1	0.007	30	20	2.28	Serine ($^{13}C_3$,^{15}N)
Arginine	345.2	171.1	0.007	40	25	2.42	Arginine ($^{13}C_6$,$^{15}N_4$)
Taurine	296.1	171.1	0.007	18	15	2.60	Serine ($^{13}C_3$,^{15}N)
Serine	276.1	171.1	0.007	30	20	2.63	Serine ($^{13}C_3$,^{15}N)
Aspartylglucosamine	506.0	171.1	0.007	30	30	2.63	Serine ($^{13}C_3$,^{15}N)
Glutamine	317.1	171.1	0.007	30	24	2.66	Glutamic acid ($^{13}C_5$, ^{15}N)
Glycine	246.1	171.1	0.007	27	21	2.73	Glycine ($^{13}C_2$, ^{15}N)
Aspartic acid	304.1	171.1	0.007	30	22	2.80	Aspartic acid ($^{13}C_4$, ^{15}N)
Citrulline	346.2	171.1	0.007	30	20	2.86	Glutamic acid ($^{13}C_5$, ^{15}N)
Argininosuccinic acid	461.0	171.1	0.007	30	20	2.86	Glutamic acid ($^{13}C_5$, ^{15}N)
Sarcosine	260.1	171.1	0.007	30	21	2.89	Glutamic acid ($^{13}C_5$, ^{15}N)
Sulfocysteine	372.0	171.1	0.007	20	30	2.89	Glutamic acid ($^{13}C_5$, ^{15}N)
Glutamic acid	318.1	171.1	0.007	30	22	2.90	Glutamic acid ($^{13}C_5$, ^{15}N)
β-Alanine	260.1	171.1	0.007	30	21	2.95	Alanine ($^{13}C_3$, ^{15}N)
Threonine	290.1	171.1	0.007	30	20	2.98	Threonine ($^{13}C_4$, ^{15}N)
Saccharopine	447.0	171.1	0.007	30	25	2.99	Threonine ($^{13}C_4$, ^{15}N)
Alanine	260.1	171.1	0.007	30	21	3.09	Alanine ($^{13}C_3$, ^{15}N)
γ-Aminobutyric acid	274.1	171.1	0.007	10	20	3.11	Alanine ($^{13}C_3$, ^{15}N)
Proline	286.1	171.1	0.007	25	21	3.25	Proline ($^{13}C_5$, ^{15}N)
β-Aminoisobutyric acid	274.1	171.1	0.007	10	20	3.25	Proline ($^{13}C_5$, ^{15}N)
Ornithine	237.1	171.1	0.007	30	25	3.26	Glutamic acid ($^{13}C_5$, ^{15}N)
Homocitrulline	360.0	171.1	0.007	30	25	3.26	Glutamic acid ($^{13}C_5$, ^{15}N)
Cystathionine	282.1	171.1	0.007	30	15	3.33	Proline ($^{13}C_5$, ^{15}N)
Hydroxylysine	333.2	171.1	0.007	16	16	3.43	Proline ($^{13}C_5$, ^{15}N)
Cystine	291.1	171.1	0.007	10	12	3.46	Cystine ($^{13}C_6$, $^{15}N_2$)
Anserine	411.0	171.1	0.007	30	20	3.46	Serine ($^{13}C_3$,^{15}N)
α-Aminobutyric acid	274.1	171.1	0.007	10	20	3.47	Lysine ($^{13}C_6$, $^{15}N_2$)
Lysine	244.2	171.1	0.007	30	12	3.48	Lysine ($^{13}C_6$, $^{15}N_2$)
Glycylproline	343.0	171.1	0.007	30	20	3.48	Lysine ($^{13}C_6$, $^{15}N_2$)
Tyrosine	352.1	171.1	0.037	30	24	3.80	Tyrosine ($^{13}C_9$, ^{15}N)
Methionine	320.1	171.1	0.037	30	22	4.06	Methionine ($^{13}C_5$, ^{15}N)
Pipecolic acid	300.0	171.1	0.037	30	20	4.15	Valine ($^{13}C_5$, ^{15}N)
Valine	288.1	171.1	0.037	30	16	4.21	Valine ($^{13}C_5$, ^{15}N)
Homocysteine	305.0	171.1	0.052	30	20	5.57	Isoleucine ($^{13}C_6$, ^{15}N)
Isoleucine	302.1	171.1	0.052	30	20	5.86	Isoleucine ($^{13}C_6$, ^{15}N)
Allo-isoleucine	302.1	171.1	0.052	30	20	5.91	Isoleucine ($^{13}C_6$, ^{15}N)
Leucine	302.1	171.1	0.052	30	20	6.11	Leucine ($^{13}C_6$, ^{15}N)
Phenylalanine	336.1	171.1	0.052	30	22	6.53	Phenylalanine ($^{13}C_9$, ^{15}N)
Tryptophan	375.1	171.1	0.052	30	26	6.76	Phenylalanine ($^{13}C_9$, ^{15}N)

2.7 Patient samples

For method comparison, 90 samples (plasma, urine and CSF) from subjects with different diseases were analyzed. Among them, 18 plasma, urine or CSF samples of patients with known IEM were studied (maple syrup urine disease, phenylketonuria, hyperlisinemia, aspartylglucosaminuria, antiquitin deficiency,

hyperammoniemia-hyperornithinemia-homocitrullinuria syndrome, argininosuccinic aciduria, ornithine transcarbamylase deficiency, citrullinemia type 1, argininemia, lysinuric protein intolerance, prolidase deficiency, molybdenum cofactor deficiency, tyrosinemia, branched-chain ketoacid dehydrogenase kinase deficiency, nonketotic hyperglycinemia and classic homocystinuria). Twenty-seven dried DBS were also analyzed (8 phenylketonuria, 3 maple syrup urine disease, 2 tyrosinemia and 14 control samples).

3 Results

3.1 Analytical validation

Figure 1 shows the mass chromatograms obtained in the MRM mode for each amino acid in a standard sample. All amino acids were separated in less than 7 min, and the total analytical run time was 9 min. Tandem mass spectrometry in the MRM mode allowed the separation of all of the amino acids without isomers, and the chromatographic conditions were optimized in order to resolve the isomer compound groups. Figure 1 shows the separation between sarcosine, β-alanine and alanine (transition 6) and between γ-aminobutyric, β-aminoisobutyric, and α-aminobutyric acids (transition 10) present in the standard mixture. All of the amino acids and related compounds analyzed by this procedure and the optimized mass spectrometric conditions are stated in Table 1.

The analytical parameters obtained in the validation procedure are shown in Table 2. The entire validation procedure was carried out for the 25 amino acids present in the table since only these amino acids are present in the standard mixture as well as in the quality control materials. The LOD and LOQ values are shown in the table, and they ranged from 0.02 to 0.89 µmol/L and 0.05 to 2.97 µmol/L, respectively. These values were low enough to detect different IEM of amino acids. Linear calibration curves with R^2 ranging from 0.9936 to > 0.999 were obtained for each individual amino acid. The linear concentration ranges established were wide enough to accurately quantify decreased and increased amino acid concentrations. The intra-assay precision for physiological concentrations ($n = 10$) ranged from 1.9 to 5.9%, and the inter-assay precision for physiological and pathological control levels ($n = 20$) ranged from 4.1 to 9.5%. All variation coefficients are summarized in Table 2. The accuracy was studied by analyzing 8 serum samples of the ERNDIM external quality control scheme.

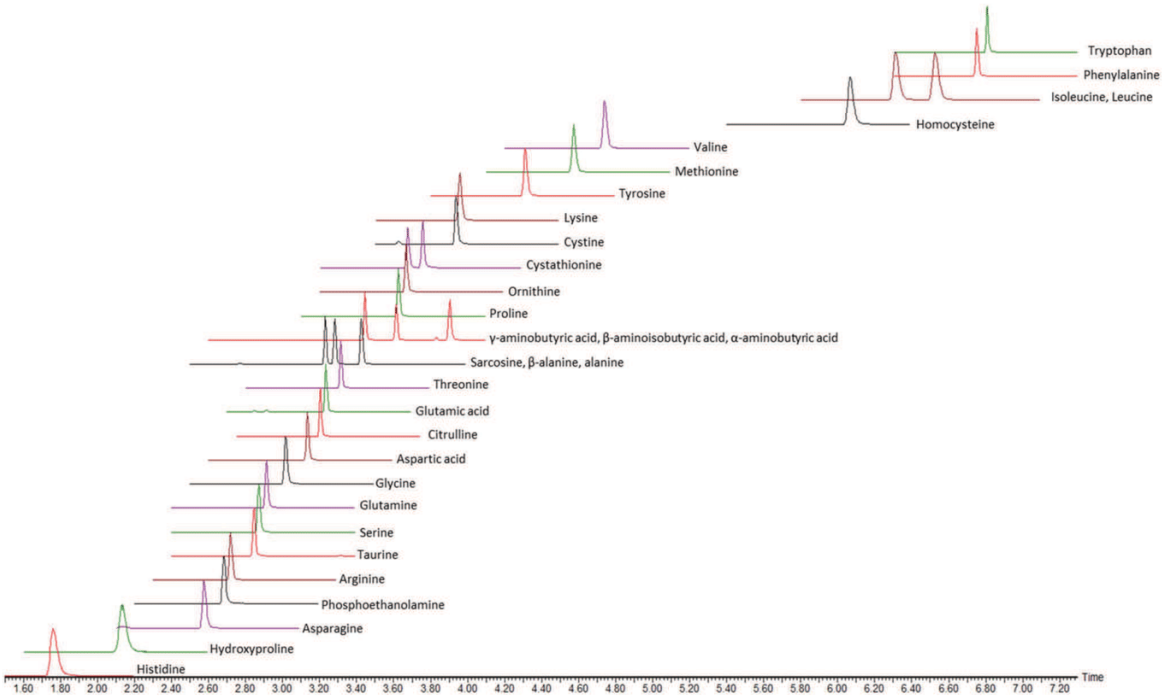

Fig. 1 Mass chromatograms of AQC-derivatized amino acids in a standard mixture obtained in the MRM mode. Specific mass transitions are detailed in Table 1

Table 2 Validation parameters for the quantitative analysis of amino acids

Amino acid	LOD (µmol/L)	LOQ (µmol/L)	Linearity (µmol/L); R^2	Intra-assay variation (%)	Inter-assay variation (%)		Accuracy (%)
					Low level	High level	
Hydroxyproline	0.02	0.05	(0.1–2500); 0.9999	5.9 (52.6)	9.2 (52.6)	6.8 (103)	101.3
α-Aminobutyric acid	0.04	0.12	(0.1–2500); 0.9971	5.8 (31.4)	7.0 (31.4)	8.3 (93.2)	98.5
Alanine	0.15	0.51	(0.5–2500); 0.9999	3.1 (319)	6.4 (319)	7.3 (914)	96.7
Arginine	0.64	2.13	(2.1–2500); 0.9999	4.6 (21.6)	9.1 (21.6)	6.6 (502)	94.3
Asparagine	0.21	0.69	(0.7–2500); 0.9998	3.2 (109.5)	5.9 (109.5)	6.7 (218)	104.1
Aspartic acid	0.10	0.33	(0.3–2500); 0.9995	5.6 (15.1)	9.2 (15.1)	7.6 (97.7)	101.6
Citrulline	0.15	0.51	(0.5–2500); 0.9940	3.4 (6.3)	9.1 (6.3)	9.5 (405)	98.0
Cystine	0.05	0.16	(0.2–1250); 0.9997	4.9 (31.4)	7.3 (31.4)	6.2 (54)	91.9
Glutamic acid	0.46	1.52	(1.5–2500); 0.9998	4.6 (122)	8.6 (122)	6.9 (219)	111.4
Glutamine	0.89	2.97	(3.0–2500); 0.9945	2.8 (598)	8.1 (598)	8.2 (1195)	94.4
Glycine	0.34	1.12	(1.1–2500); 0.9999	1.9 (598)	7.2 (598)	7.8 (1001)	98.7
Histidine	0.10	0.33	(0.3–2500); 0.9999	2.9 (200)	9.1 (200)	4.6 (394)	92.5
Isoleucine	0.05	0.17	(0.2–2500); 0.9999	2.4 (52.6)	6.0 (52.6)	4.9 (398)	98.1
Leucine	0.05	0.16	(0.2–2500); 0.9999	2.7 (26.8)	6.9 (26.8)	5.4 (858)	97.0
Lysine	0.12	0.39	(0.4–2500); 0.9999	2.7 (269)	5.6 (269)	4.1 (527)	97.9
Methionine	0.03	0.11	(0.1–2500); 0.9999	3.2 (80.8)	7.7 (80.8)	7.4 (239)	98.0
Ornithine	0.03	0.11	(0.1–2500); 0.9981	2.5 (157)	7.9 (157)	5.7 (605)	101.4
Phenylalanine	0.06	0.21	(0.2–2500); 0.9990	2.4 (348)	5.6 (348)	5.4 (682)	101.1
Proline	0.23	0.77	(0.8–2500); 0.9997	2.9 (309)	7.2 (309)	6.7 (607)	101.2
Serine	0.28	0.94	(0.9–2500); 0.9999	3.2 (159)	7.3 (159)	8.5 (454)	103.0
Taurine	0.15	0.51	(0.5–2500); 0.9952	3.4 (212)	7.7 (212)	5.6 (411)	92.5
Threonine	0.28	0.92	(0.9–2500); 0.9999	4.3 (206)	6.6 (206)	7.2 (405)	96.8
Tryptophan	0.05	0.17	(0.2–2500); 0.9936	3.1 (116)	5.4 (116)	5.5 (298)	
Tyrosine	0.04	0.14	(0.1–2500); 0.9999	3.2 (235)	6.8 (235)	5.7 (920)	101.7
Valine	0.03	0.09	(0.1–2500); 0.9999	2.9 (430)	5.0 (430)	5.3 (804)	98.8

The concentrations in µmol/L used in the precision studies for each amino acid are shown in brackets

LOD limit of detection, *LOQ* limit of quantification)

The concentrations were compared to the median of all laboratories participating in the scheme, and the recoveries ranged from 91.9% for cystine to 111.4% for glutamic acid. For the other amino acids studied, the recoveries obtained ranged from 92.5 to 104.1%. Results of the external control program ERNDIM 2017 plasma scheme are shown in Supplementary Table 1.

3.2 Method comparison

Results of method comparison are shown in Supplementary Table 2. In this study, the IEC procedure was considered as the reference method. Comparison was performed for 23 amino acids (tryptophan was excluded due to the acidic hydrolysis process that occurs during the sample preparation for the IEC procedure). For the 23 amino acid studied, high correlations were obtained, with intercepts and slopes that included 0 and 1, respectively, except for hydroxyproline, aspartic acid and citrulline.

3.3 DBS analysis

Regarding studies in DBS, the results of method comparison are shown in Supplementary Table 3. Comparisons were performed for six amino acids since these compounds are clinically relevant for maple syrup urine disease or phenylketonuria treatment follow-up. The results obtained for these six amino acids in plasma and DBS samples by UPLC-MS/MS were transferable. Trueness analysis in DBS samples was performed for phenylalanine and tyrosine by analisys of AECNE external quality control scheme samples. Accuracy values were expressed relative to the median of all laboratories participating in the control scheme, with specific procedures to analyze phenylalanine and tyrosine, and the recovery values obtained were 98.3 and 95.9%, respectively. Supplementary Fig. 1 shows the comparison of the results obtained by the UPLC-MS/MS procedure for both amino acids with the median values of all participating laboratories. For leucine, isoleucine, valine, phenylalanine and tyrosine, analisys of ERNDIM external quality control

scheme DBS samples was performed, and results are shown in Supplementary Table 4.

3.4 Pathological samples

Plasma, urine or CSF samples of patients diagnosed with several IEM were analyzed. All pathological samples analyzed showed positive results. Quantitative results are shown in Table 3. In Fig. 2, the mass chromatograms of six IEM examples are shown including the following: Fig. 2a antiquitin deficiency (MIM * 107,323) presenting moderate pipecolic acid accumulation in CSF (5.5 µmol/L); Fig. 2b argininosuccinate lyase deficiency (MIM * 608,310) displaying high plasma and urine argininosuccinic acid concentrations; Fig. 2c molybdenum cofactor deficiency (MIM * 603,707) presenting elevated excretion of urinary sulfocysteine; Fig. 2d *N*-aspartylglycosaminidase deficiency (MIM * 613,228) showing an abnormal peak of *N*-aspartylglucosamine not present in the controls; Fig. 2e typical profile of a maple syrup urine disease patient (OMIM # 248,600) showing increased isoleucine, allo-isoleucine and leucine. the excellent resolution obtained among these three isomers is shown; and Fig. 2f prolidase deficiency (OMIM # 170,100) sample showing the accumulation of glycylproline peptide in the urine. The other IEM samples analyzed showed pathological results (Table 3).

3.5 Reference values

To calculate reference values, 60 plasma, 80 urine and 40 CSF samples from control subjects were analysed. A significant correlation between some amino acids and age was detected (Pearson's test: $p < 0.05$), and significant differences were observed when the data were compared in age groups (Student's t test: $p < 0.05$). In these cases, different age groups were established (Table 4).

Table 3 Amino acids values in different inborn errors of metabolism patients

IEM	Amino acid biomarker	Matrix	Concentration	Age-related reference values
Antiquitin deficiency	Pipecolic acid	Plasma	21	0–5 µmol/L
		CSF	5.5	0–0.2 µmol/L
Argininosuccinate lyase deficiency	Argininosuccinic acid	Plasma	Present	No detectable[a]
	Citrulline		170	8–40 µmol/L
	Glutamine		2128	326–750 µmol/L
Molybdenum cofactor deficiency	Sulfocysteine	Urine	Present	No detectable[a]
N-aspartylglycosaminidase deficiency	*N*-Aspartylglucosamine	Urine	Present	No detectable[a]
Maple syrup urine disease	Isoleucine	Plasma	530	32–90 µmol/L
	Leucine		2186	57–155 µmol/L
	Allo-isoleucine		Present	No detectable[a]
Prolidase deficiency	Glycylproline	Urine	present	No detectable[a]
Phenylketonuria	Phenylalanine	Plasma	1828	40–70 µmol/L
Hyperammoniemia-hyperornithinemia-homocitrullinuria syndrome	Ornithine	Plasma	419	30–109 µmol/L
	Homocitrullina	Urine	Present	No detectable[a]
Ornithine transcarbamylase deficiency	Glutamine	Plasma	3927	326–750 µmol/L
	Citrulline		2	8–40 µmol/L
	Arginine		18	47–122 µmol/L
Citrullinemia type 1	Citrulline	Plasma	1305	8–40 µmol/L
Argininemia	Arginine	Plasma	898	47–122 µmol/L
Lysinuric protein intolerance	Lysine	Urine	472	3–170 mmol/mol creat
	Arginine		88	0–31 mmol/mol creat
	Ornithine		15	0–17 mmol/mol creat
Tyrosinemia	Tyrosine	Plasma	413	41–206 µmol/L
Branched-chain ketoacid dehydrogenase kinase deficiency	Valine	Plasma	41	102–294 µmol/L
	Isoleucine		No detectable	32–90 µmol/L
	Leucine		16	57–155 µmol/L
Nonketotic hyperglycinemia	Glycine	Plasma	841	109–293 µmol/L
		CSF	173	2–18 µmol/L
Classic homocystinuria	Methionine	Plasma	701	12–37 µmol/L

[a]These compounds were identified but not quantified, as no calibrator was available

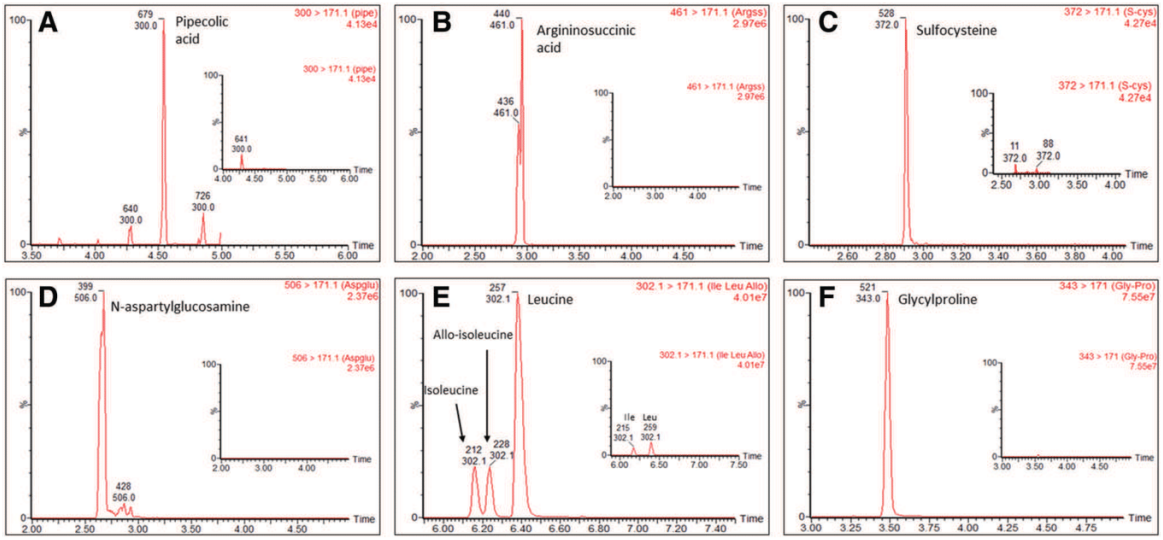

Fig. 2 Examples of the mass chromatograms of plasma, urine or CSF amino acids or related compounds from of patients with different IEM. The small chromatograms show the control pattern. **a** Increased CSF pipecolic acid from a patient with an antiquitin deficiency. **b** Increased urinary argininosuccinic acid from a patient with an argininosuccinate lyase deficiency. **c** Increased urinary sulfocysteine from a patient with a molybdenum cofactor deficiency. **d** Increased urinary *N*-aspartylglucosamine from a patient with an aspartylglucosaminidase deficiency. **e** Increased plasma isoleucine, allo-isoleucine and leucine from a patient with a maple syrup urine disease. **f** Increased urinary glycylproline from a patient with a prolidase deficiency

4 Discussion

In this work, a rapid and accurate method using UPLC coupled to ESI-MS/MS for the quatification of 25 amino acids, which has a potential for further analysis of 17 related compounds in plasma, urine, CSF and DBS samples was developed and validated. This procedure was compared with the IEC method, accredited by ENAC (Spanish Accreditation Agency) according to the ISO 15189 norm for medical laboratories.

The procedure described here is fast (the run time is only 9 min). The sample pretreatment is simple, easy and rapid. A derivatization reaction with AQC has been successfully applied for the determination of amino acids coupled to UV detection (Cohen 2003), and it has been tested as a reagent which generates very stable and well retained derivatives in a fast incubation. Regarding chromatography, the method was shown to be reproducible and reliable with no retention time shift. Excellent chromatographic resolution between isobaric compounds was achieved in only 9 min. In other UPLC-MS/MS methods described with total run times so short, isoleucine and allo-isoleucine are not resolved (Filee et al. 2014; Prinsen et al. 2016). Freeto et al. (2007) developed a method, which was able to separate the isobaric branched-chain amino acids in only 8 min. However, this approach was developed for monitoring maple syrup urine disease, phenylketonuria or tyrosinemia patients and therefore only quantified tyrosine, phenylalanine and branched-chain amino acids. Waterval et al. (2009) developed a procedure for the quantitative analysis of the complete profile of amino acids with good resolution of isoleucine and allo-isoleucine, but an ion pairing reagent was required, resulting in a higher equilibration time between runs and consequently a higher total run time (30 min). Traditional IEC can resolve these three isomers, but the analysis requires at least 2 h per sample, which is an important disadvantage when urgent analysis is required. Besides the run time, the described method has great advantages compared to the IEC method, such as the minimal sample volume required (25 µL vs. > 200 µL) and the upper specificity of LC-MS/MS versus ninhydrin detection. Another important feature of this procedure is its high sensitivity that allows an accurate quantification of CSF samples and DBS extracts. Thus, the method was robust and can be used in different biological specimens. The results obtained in the international quantitative external quality control scheme ERNDIM were highly satisfactory and superior to those obtained by the traditional IEC method. The lower and the higher recoveries obtained for cystine and glutamic acid, respectively, were probably due to artifacts from storage and freeze-thawing. The cystine recovery obtained is lower due to the desulfuration of sulfur-containing amino acids, and the glutamic acid recovery is higher because of the spontaneous conversion of glutamine into glutamic acid.

Table 4 Amino acid age-related reference values in plasma, urine and CSF matrices

Amino acid	Age	Plasma (μmol/L)	Age	Urine (mmol/mol creat)	Age	CSF (μmol/L)
Hydroxyproline	0 days–2 years	12–99	0 days–1 month	4–230	0 days–6 months	0–2.5
	3–18 years	5–40	2 months–6 months	0–143	7 months–18 years	0–1
			4–18 years	0–2		
α-Aminobutyric acid	0 days–18 years	5–31				
Alanine	0 days–1 month	190–337	0 days–1 years	68–334	0 days–6 months	15–59
	2 months–18 years	167–439	2–18 years	14–113	7 months–18 years	7–44
Arginine	0 days–18 years	47–122	0 days–6 months	0–31	0 days–18 years	8–31
			7 months–18 years	0–8		
Asparagine	0 days–18 years	31–120				
Aspartic acid	0 days–18 years	2–20	0 days–18 years	0–9	0 days–18 years	0–4
Citrulline	0 days–18 years	8–40	0 days–12 months	0–10	0 days–6 months	2–5.8
			1–18 years	0–3	7 months–18 years	2–3.8
Cystine	0 days–18 years	15–59	0 days–1 month	2–51	0 days–6 months	0–1.4
			2 months–2 years	2–36	7 months–18 years	0–0.8
			3–18 years	2–17		
Cystathionine			0 days–1 years	0–17		
			2–18 years	0–5		
Glutamic acid	0 days–18 years	5–80	0 days–18 years	0–11	0 days–6 months	0.2–2.6
					7 months–18 years	0.2–1.7
Glutamine	0 days–1 month	326–750	0 days–1 years	12–283	0 days–6 months	309–665
	2 months–2 years	325–676	2–18 years	12–156	7 months–18 years	261–543
	3–18 years	330–632				
Glycine	0 days–18 years	109–293	0 days–1 month	113–1427	0 days–6 months	2–18
			2–12 months	147–570	7 months–18 years	4–12
			1–8 years	66–453		
			9–18 years	51–238		
Histidine	0 days–18 years	45–104	0 days–8 years	11–374	0 days–6 months	2–32
			9–18 years	9–170	7 months–18 years	6–18
Isoleucine	0 days–18 years	32–90	0 days–18 years	0–9	0 days–6 months	3–13
					7 months–18 years	2–9
Leucine	0 days–18 years	57–155	0 days–8 years	0–25	0 days–6 months	8–28
			9–18 years	0–10	7 months–18 years	5–20
Lysine	0 days–1 month	67–252	0 days–1 month	3–170	0 days–6 months	4–41
	2 months–18 years	112–240	2 months–18 years	3–90	7 months–18 years	10–29
Methionine	0 days–18 years	12–37	0 days–8 years	0–33	0 days–6 months	0–9
			9–18 years	0–12	7 months–18 years	0–5
Ornithine	0 days–1 month	30–175	0 days–1 years	0–17	0 days–6 months	0–25
	2 months–18 years	30–109	2–18 years	0–10	7 months–18 years	2–11
Phenylalanine	0 days–18 years	40–70	0 days–1 years	4–30	0 days–6 months	2–21
			2–18 years	5–26	7 months–18 years	5–13
Pipecolic acid	0 days–18 years	0–5			0 days–18 years	0–0.2
Proline	0 days–18 years	90–355	0 days–1 month	8–260	0 days–6 months	0–3.5
			2 months–6 months	0–150	7 months–18 years	0–2
			7 months–18 years	0–23		
Serine	0 days–18 years	92–197	0 days–1 years	39–306	0 days–6 months	25–92
			2–18 years	18–114	7 months–18 years	18–55
Taurine	0 days–18 years	30–150				
Threonine	0 days–12 months	58–292	0 days–12 months	15–136	0 days–6 months	24–94
	1–18 years	78–197	1–18 years	12–67	7 months–18 years	14–40

Table 4 (continued)

Amino acid	Age	Plasma (µmol/L)	Age	Urine (mmol/mol creat)	Age	CSF (µmol/L)
Tryptophan	0 days–18 years	30–85	0 days–12 months	10–40	0 days–18 years	2–5
			1–18 years	4–25		
Tyrosine	0 days–6 months	41–206	0 days–1 years	6–57	0 days–6 months	3–29
	7 months–18 years	39–87	2–18 years	5–34	7 months–18 years	5–17
Valine	0 days–18 years	102–294	0 days–8 years	4–28	0 days–6 months	8–39
			9–18 years	2–12	7 months–18 years	10–24

Regarding method comparison, for most of the amino acids studied, good agreement was observed between both procedures in the different matrixes studied. In the case of hydroxyproline, the low agreement between IEC and UPLC-MS/MS results may be explained by the low sensitivity of the IEC procedure for this amino acid at physiological values, which may generate great imprecision. The quantification of aspartic acid by IEC is also striking since incorrect peak identification could occur due to the presence of the ninhydrin reactive compound that co-eluted with this amino acid. Finally, a suboptimal peak separation between alanine and citrulline can be observed in IEC, thus affecting the quantification of citrulline since this amino acid is present in much a lower concentration that alanine in physiological conditions. This latter observation may be critical for the detection of suboptimal citrulline values in the diagnosis and monitoring of urea cycle disorders and other IEM causing secondary urea cycle defects. Since the agreement tests did not show transferable results for some amino acids, new reference intervals were established for all validated compounds (Table 4). The new reference intervals were very similar to previously calculated for IEC method (Gregory et al. 1986; Parvy et al. 1988; Gerrits et al. 1989) and other UPLC-MS/MS methods reported for paediatric subjects (Filee et al. 2014). Concerning the DBS sample analyses, the high correlation obtained between plasma and DBS samples for the 6 amino acids studied validated the procedure for phenylketonuria or maple syrup urine disease treatment follow-up. Furthermore, the results obtained in the AECNE external quality control scheme for phenylalanine and tyrosine in DBS, and the results obtained in the ERNDIM external quality control scheme for leucine, isoleucine, valine, phenylalanine and tyrosine in DBS were highly satisfactory (Supplemetary Fig. 1 and Supplementary Table 4).

This method is capable of detecting patients affected by different IEM since in all cases studied, pathological values were obtained (Table 3). The 6 examples chosen in Fig. 2. illustrate the differences between IEC and UPLC-MS/MS. In the patient with an antiquitin deficiency, pipecolic acid was mildly increased and could be missed by IEC (Plecko et al. 2007). In argininosuccinate lyase deficiency, arginisuccinic acid was clearly detected with the MS/MS procedure. This metabolite can also be detected by IEC, although it can co-elute with the leucine peak and may be difficult to quantify (Allan et al. 1958). Sulfocysteine is a diagnostic marker for molybdenum cofactor and sulphite oxidase deficiencies (Arnold et al. 1993). Sulfocysteine elevation may be difficult to detect in IEC because of the co-elution that exists between this compound and taurine. *N*-Aspartylglucosamine is highly excreted in urine from patients affected with *N*-aspartylglycosaminidase deficiency (Pollitt et al. 1968). This disorder of glycoprotein degradation could be missed in conventional IEC urine analysis due to the overlap that can exist between this molecule and the huge urea peak. For maple syrup urine disease diagnosis and follow-up, a proper quantification of leucine, isoleucine, allo-isoleucine and valine is mandatory (Morton et al. 2002). The chromatographic method was able to resolve the isomer amino acid group, a critical point for other procedures. Finally, in prolidase deficiency, the biomarker is glycylproline peptide (Powell et al. 1974). In normal urine, small amounts can be detected. The urinary prolidase amino acid profile could be difficult to interpret by IEC since no identification of the compound is possible unlike previous experience.

In this work, we did not study the matrix effect. However, in the 25 compounds analyzed with IS, the matrix effect would be minimized. Moreover, the good agreement observed between UPLC-MS/MS and IEC methods (in this procedure, lower matrix effects when compared with MS/MS are expected), in plasma CSF and urine samples strongly support that matrix effect were not remarkable. Furthermore, the new reference values we have established in the three matrixes are similar to those previously reported.

In summary, we report on a modified UPLC-MS/MS procedure for the quantification of 25 amino acids which has a potential for the analysis of 17 further compounds in plasma, urine, CSF and DBS. The described method is fast, sensitive and robust, and it has been validated to be an alternative to the traditional IEC procedure as the routine method for use in metabolic laboratories. The method significantly

decreases the run time of the analysis while displaying good metrological results.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interests.

Ethical approval The patient samples were collected for diagnostic and treatment monitoring purposes. The study was conducted following the Helsinki Declaration of 1975 as revised in 2013. The ethical committee of the Hospital Sant Joan de Déu approved the study. For genetic diagnosis, informed consent was collected from patients or their guardians.

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