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Research Paper

Determination of new biomarkers for diagnosis of pyridoxine dependent epilepsy in human plasma and urine by liquid chromatography-mass spectrometry

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ABSTRACT

Background: Pyridoxine-dependent epilepsy (PDE) is a rare inborn error of lysine metabolism. To date, diagnosis of PDE relies on the quantification of α-AminoAdipic SemiAldehyde (α- AASA), Piperideine-6-Carboxylate (P6C) and Pipecolic acid (PA) in urine or plasma from patients with overt symptoms. However, these biomarkers are not specific, and their biochemical analysis is challenged by their instability and technical limitations. We set-up and validated a method for the quantification of two new biomarkers of PDE (2S,6S- and 2S,6R-oxopropylpiperidine-2-carboxylic acid, 2-OPP, and 6-oxopiperidine2-carboxylic acid, 6-oxoPIP) on human urine and plasma by LC-MS/MS, to overcome the diagnostic and technical challenges of old biomarkers.
Methods: We analysed urine and plasma samples by LC-MS/MS, and validated the method in both biological matrices.
Results: We performed the biomarkers extraction from a 10 μL aliquot of urine or plasma in around 15 min using water 100 % for urine, and a solution of water/methanol 50 % for plasma, respectively. The analytical method was validated and gave good linearity ($r^2 > 0.999$) in the range 0–15 μmol/L for 2-OPP and 0–25 μmol/L for 6-oxoPIP. In both matrices, the biomarkers were stable at different storage temperatures tested.
Conclusions: We set-up and validated a reliable method and confirmed its clinical applicability on real samples from PDE patients. This method could be used as routine test for the diagnosis and monitoring of PDE.

1. Introduction

Pyridoxine-dependent epilepsy (PDE) is a rare autosomal recessive disorder belonging to inborn errors of lysine metabolism and affecting the metabolism of pyridoxal 5′-phosphate (PLP). It can manifest in the pre-, neo- or post-natal period and occasionally in older children, and is defined by an epileptic encephalopathy with intellectual disability, characterized by stubborn, difficult to control seizures, resistant to conventional antiepileptic drugs but responsive to large daily supplementations of pyridoxine (vitamin B6) [1,2].

Current guidelines recommend that all young individuals with an unexplained seizure disorder should be tested for PDE [3]. Diagnostic biomarkers for the disease include α-AminoAdipic SemiAldehyde (α-AASA), Piperideine-6-Carboxylate (P6C) and Pipecolic acid (PA), that are neurotoxic substances which accumulated in many human matrix as a result of a deficiency in the aldehyde dehydrogenase 7 family member A1 (ALDH7A1) enzyme. α-AASA, P6C, and PA can be measured in urine, plasma, blood and cerebral spinal fluid (CSF), mostly by liquid chromatography (LC) tandem mass spectrometry (MS/MS) with either C18 or HILIC columns [4–17] or by gas chromatography-mass spectrometry (GC–MS) for PA only [18,19].

However, these diagnostic markers present several disadvantages, as

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they degrade quickly at all storage/transport temperatures, and it is difficult to purchase or synthesize the labelled internal standards (IS) for α-AASA and P6C [4,5], so their analyses are often semi-quantitative. Moreover, measuring these analytes in LC-MS/MS requires derivatization (e.g. in ButOH HCl 1 N), which is a time-consuming laboratory procedure and potentially dangerous due to its corrosivity; moreover it requires the use of glass vials, thus also increasing the cost of the analysis [4,5].

We aimed to develop and validate a method for the quantification of two novel biomarkers of PDE, 2S,6S- and 2S,6R-oxopropylpiperidine-2-carboxylic acid (2-OPP) and 6-oxopiperidine2-carboxylic acid (6-oxoPIP) on urine and plasma by LC-MS/MS to be employed for diagnostic purposes. This test could represent an alternative method to the current quantification of α-AASA, P6C and PA, from the same biological matrices already used in routine clinical practice. Literature data suggest that these new biomarkers 2-OPP and 6-oxoPIP derive from the reaction of P6C with the ketone body acetoacetate, and from the oxidation of 6-hydroxy-pipecolate (6-OH-PIP), respectively [20–22], and their suitability for diagnostic and therapeutic monitoring of PDE has already been reported in literature [23] (Fig. 1).

The availability of a new method for the detection and quantification of these new biomarkers in urine and plasma will enable a fast and reliable diagnostic and monitoring test for detecting PDE from a small volume of urine or plasma by LC-MS/MS.

2. Material and methods

The study was conducted in the framework of the CHAnging Rare disorders of LysInE metabolism project (CHARLIE), funded by the European Joint Programme on Rare Diseases, 2020 call.

We conducted all experiments in compliance with the Declaration of Helsinki and with National and International guidelines for research studies on human subjects. The Institutional Review Board of Tuscany Region (Italy) approved this study (n. 2021-11.02.2021).

2.1. Blank samples

We prepared the curve calibration and quality controls (QCs) for the analytical validation using matrix blanks, in particular synthetic urine (Surine™ Negative Urine Control, Sigma-Aldrich) and a plasma sample obtained from a single healthy adult donor, not exposed to any pharmacological treatment at time of blood donation. The donor released

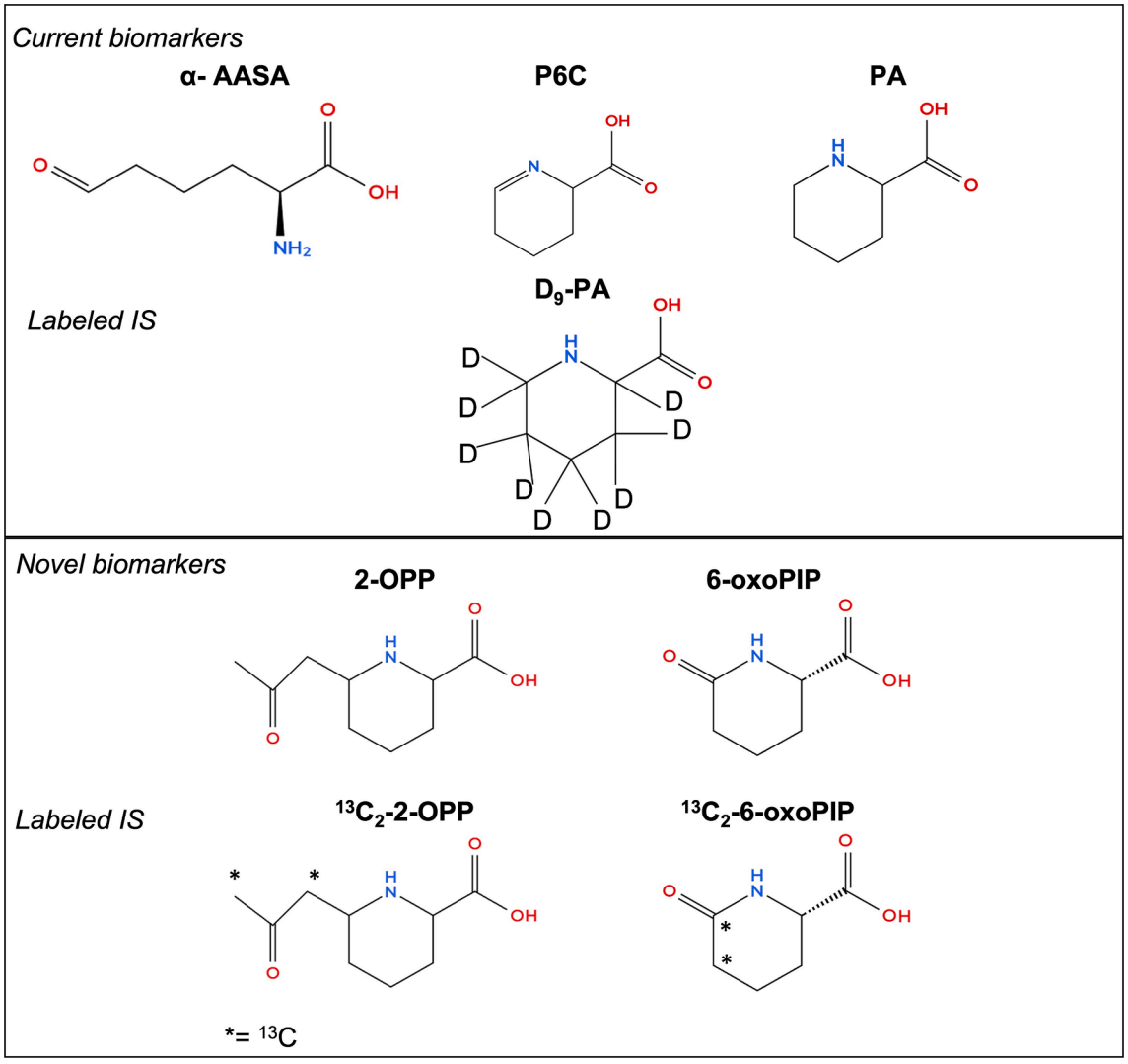

Fig. 1. Chemical structure of the traditional and new biomarkers for PDE and their labelled internal standards. Traditional diagnostic biomarkers for PDE include α-AminoAdipic SemiAldehyde (α-AASA), Piperideine-6-Carboxylate (P6C) and Pipecolic acid (PA); to date, a labelled internal standard is commercially available only for PA. Two novel biomarkers for PDE (2S,6S- and 2S,6R-oxopropylpiperidine-2-carboxylic acid (2-OPP) and 6-oxopiperidine2-carboxylic acid (6-oxoPIP)) have been identified and their labelled internal standards are available.

written informed consent for the study.

2.2. Chemicals and reagents

The chemical standards were: (2S,6R)-6-(2-oxopropyl)-piperidine-2-carboxylic acid (2-OPP, Synvenio, AJ Nijmegen, The Netherlands) and (S)-6-oxopiperidine-2-carboxylic acid (6-oxoPIP, Merck-SigmaAldrich), used to prepare the calibration curve and all QCs; (2S,6R)-6-(2-oxopropyl-1,3-$^{13}C_2$) piperidine-2-carboxylic acid ($^{13}C_2$-2-OPP, Synvenio) and (S)-6-oxopiperidine-2-carboxylic-5,6-$^{13}C_2$ acid ($^{13}C_2$-6-oxoPIP, Synvenio), used as IS. We prepared the stock solutions and subsequent dilutions in water. All solvents used were LC-MS grade and commercially available (Panreac, Barcelona, Spain).

We prepared all stock solutions for both unlabelled and labelled PDE biomarkers (2-OPP and 6-oxoPIP, $^{13}C_2$-2-OPP and $^{13}C_2$-6-oxoPIP) in water at a concentration of 1 mg/mL, and stored at − 20 °C until their use. From these stock solutions, we prepared mix solutions in water at the concentrations reported in Supplementary Table 1. Then we diluted them 1:10 in the matrix of interest (urine and plasma), for the preparation of the points of the external calibration curve, obtaining the final concentrations reported in Supplementary Table 1.

Likewise, we prepared QCs solutions in water at the following concentrations: 2.5 μmol/L, 15 μmol/L, 75 μmol/L for 2-OPP and 5 μmol/L, 75 μmol/L, 200 μmol/L for 6-oxoPIP in water. Then, for each QC level, we diluted the QCs solutions of each biomarker 1:10 in the matrix of interest (urine and plasma), for the preparation of three final levels containing the two biomarkers, chosen as reference: QC Low 0.25 μmol/L for 2-OPP and 0.5 μmol/L for 6-oxoPIP, QC Medium 1.5 μmol/L for 2-OPP and 7.5 μmol/L for 6-oxoPIP, QC High 7.5 μmol/L for 2-OPP and 20 μmol/L for 6-oxoPIP.

2.3. Sample preparation

We obtained one preparation for each matrix, as follows:

- for urine, we combined a 10 μL aliquot of a filtered urine specimen with 10 μL of mix labeled IS in water (containing 2.5 μmol/L $^{13}C_2$-2-OPP and 5 μmol/L $^{13}C_2$-6-oxoPIP) and 200 μL of water, in a 96-well plate, mixed and injected a volume of 1 μL in column;
- for plasma, we combined a 10 μL aliquot of plasma with 10 μL of mix labeled IS in water (containing 2.5 μmol/L $^{13}C_2$-2-OPP and 5 μmol/L $^{13}C_2$-6-oxoPIP) and 200 μL of a solution of water/methanol (50:50), in an Eppendorf tube (capacity 1.5 mL), mixed with vortex, and centrifugated for 10 min at 13200 rpm to precipitate the plasma proteins. Finally, the supernatant was recovered and transferred in a 96-well plate and injected a volume of 0.5 μL in column.

2.4. Validation procedure

We performed validation for the analytical methods on urine and plasma, in accordance with ICH guideline M10 Bioanalytical method Validation and Study Sample Analysis, Guidance for Industry 2022 [24] and with the ICH guideline Q2 (R2) on validation of analytical procedures 2022 [25], as much as possible.

We evaluated selectivity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity of the calibration curve, accuracy and precision, recovery effect (RE%) and matrix effect (ME%), carry-over, storage stability and dilution integrity, by analysing synthetic urine and human plasma from a healthy adult donor.

We conducted validation procedures on calibration points prepared by spiking with known amounts of 2-OPP and 6-oxoPIP into synthetic urine and human plasma.

2.4.1. Limit of detection and limit of quantitation

We determined the LOD and LOQ by preparing an *ad hoc* calibration curve in urine and plasma in the range 0–5 μmol/L for 2-OPP and 0–10 μmol/L for 6-oxoPIP. LOD and LOQ values were calculated based on the standard deviation of a linear response and a slope, estimated from the regression line of these analytes, in accordance with the ICH guideline Q2 (R2) on validation of analytical procedures 2022 [25].

2.4.2. Linearity

We evaluated linearity by constructing a calibration curve with linear regression ($y = ax + b$) in triplicate.

We performed quantification analysis using an external standard calibration curve, covering a concentration range from 0 to 15 μmol/L for 2-OPP and from 0 to 25 μmol/L for 6-oxoPIP.

2.4.3. Accuracy and precision

We determined the accuracy and precision of the developed method by the analysis of urine and plasma samples (QCs) spiked with 2-OPP and 6-oxoPIP at three concentrations: a low concentration (QC Low: 0.25 μmol/L 2-OPP and 0.5 μmol/L 6-oxoPIP), a medium concentration (QC Medium: 1.5 μmol/L 2-OPP and 7.5 μmol/L 6-oxoPIP) and a high concentration (QC High: 7.5 μmol/L for 2-OPP and 20 μmol/L for 6-oxoPIP). We analysed QCs in the same batch for intraday test and in 5 replicates for 10 different batches for interday test.

In particular, we expressed accuracy and precision as percentage difference from nominal concentration (mean measured concentration/nominal concentration x 100) and coefficient of variation (CV%), respectively. We assessed intraday accuracy and precision by analysing 5 replicates of 3 QC (QC Low, QC Medium and QC High) in a single run, and interday accuracy and precision by testing 5 replicates of 3 QCs on different days.

2.4.4. Recovery and matrix effect

We determined the efficiency of the extraction procedure by analysis of urine and plasma samples spiked at two different concentrations, QC Low (0.25 μmol/L 2-OPP and 0.5 μmol/L 6-oxoPIP), and QC High (7.5 μmol/L for 2-OPP and 20 μmol/L for 6-oxoPIP), in matrix, blank matrix with spike, and solvent. Each of them was prepared and acquired in triplicate. We compared the analyte response obtained from extracted spiked samples to a spiked blank matrix sample, and calculated the RE%. We calculated the ME% by comparing the signal of standard in water solvent and spiked blank matrix sample, each at two different concentrations (QC Low and QC High, as above).

2.4.5. Carry-over

We assessed carry-over by injecting blank samples (n = 5) after the highest calibrator of curve (15 μmol/L for 2-OPP and 25 μmol/L for 6-oxoPIP), for both urine and plasma.

2.4.6. Stability and storage

To ensure that patient samples are stable during shipping and storage prior to analysis, we calculated storage stability of the analytes in urine and plasma by testing QCs low, medium and high concentrations in triplicate over one month at + 4 °C and − 20 °C, and over 24 h at room temperature.

2.4.7. Dilution integrity

We evaluated dilution integrity only for plasma, by diluting a blank matrix spiked with the analytes of interest (2-OPP and 6-oxoPIP) at a concentration 2-fold the calibration curve: 30 μmol/L for 2-OPP and 50 μmol/L for 6-oxoPIP. We performed all analyses in triplicate, at the following dilutions 1:2 (15 μmol/L for 2-OPP and 25 μmol/L for 6-oxoPIP), 1:5 (6 μmol/L for 2-OPP and 10 μmol/L for 6-oxoPIP), and 1:10 (3 μmol/L for 2-OPP and 5 μmol/L for 6-oxoPIP).

2.5. Liquid chromatography and mass spectrometry

We performed LC-MS/MS assays in the Newborn Screening, Clinical Biochemistry and Clinical Pharmacy Laboratory of Meyer University

Children's IRCCS Hospital (Florence, Italy), equipped with an ExionLC – QTRAP 6500 mass spectrometer (AB SCIEX, Toronto, Canada) interfaced with a Turbo Ion Spray source and operating in positive and MRM mode.

We acquired data using the ion spray voltage set to 5500 V for positive polarity; the vaporizer temperature was 500 °C, sheath gas and auxiliary gas were both set to 35 (arbitrary units).

The transitions were optimized by infusing the standard solutions (1 μg/mL) and the following were monitored (*m/z*): 144.1 > 98.1 for 6-oxoPIP, 146.1 > 100.1 for $^{13}C_2$-6-oxoPIP, 186.1 > 128.1 for 2-OPP and 188.1 > 128.1 for $^{13}C_2$-2-OPP used as quantifier ions.

MS parameters were the same for all analytes: Collision Exit Potential (CXP) 11 V, Entrance Potential (EP) 10 V, Declustering Potential (DP) 50 V, Collision Energy (CE) 20 V.

Chromatographic separation was achieved using module ExionLC capillary system, operating in gradient mode, coupled with a thermostated autosampler and fully controlled by Analyst Software (Version 1.7.3).

We performed the same chromatographic run for both matrices, using a LUNA Omega C18, 2.1 × 50 mm, 1.6 μm (Phenomenex, Torrance, CA) at a flow rate of 350–400 μL/min, kept in the oven at 40 °C. The eluate was flowed directly into the Electrospray Ionization (ESI) source without splitting.

The mobile phase was composed of water LC MS grade (phase A) and methanol/acetonitrile (50/50) LC MS grade (phase B), both with the addition of 0.1 % formic acid.

Separation was achieved using a linear gradient: we maintained 2 % phase B for 1.4 min at a flow rate of 350 μL/min, at 1.5 min phase B changed from 2 % to 98 % at a flow rate of 400 μL/min and this condition was maintained for another 1.5 min, and finally at 3.1 min the mobile phase was returned to the starting conditions and re-equilibrated for 2.9 min. The total run time was of 6 min.

The injection volumes were 1 μL for urine and 0.5 μL for plasma, and the auto sampler temperature was maintained at 10 °C throughout the analyses.

The data obtained were elaborated using the Analyst software (Version 1.7.3).

2.6. Clinical application

To assess the clinical applicability of this new method, we quantified 2-OPP and 6-oxoPIP on 24 urine samples from 17 pediatric (aged 3–16 years) and 7 adult healthy controls (aged 26–43 years), and on 32 plasma samples from adult healthy controls (aged 18–65 years).

Moreover, we quantified 2-OPP and 6-oxoPIP on three patients with PDE (two provided urine samples and two plasma samples). The two plasma samples are from one patient with neonatal-onset PDE treated with pyridoxine at time of sample collection and analysis (patient 1), and from one late-onset PDE patient treated with antiepileptic drugs at time of sample collection and analysis (patient 2). Patient 2 provided also urine sample. The second urine sample is from a third patient with neonatal-onset PDE, untreated at time of sample collection and analysis (patient 3). Diagnosis of PDE was confirmed on biochemical test of α-AASA, P6C and PA and genetics.

For the three PDE patients, we obtained parents' written authorization to use the biologic samples. All adult healthy controls and pediatric healthy controls' parents provided written informed consent to use the biologic samples.

3. Results

In this work we set-up and validated a simple and rapid method to quantify 2-OPP and 6-oxoPIP in urine and plasma samples by LC-MS/MS, for the diagnosis and monitoring of PDE.

Fig. 2 shows the chromatographic resolution of 2-OPP, 6-oxoPIP, and labelled IS $^{13}C_2$-2-OPP and $^{13}C_2$-6-oxoPIP. The retention time were respectively 0.99 min for 2-OPP and its labelled IS, and 1.49 min for 6-oxoPIP and its labelled IS.

All validation parameters tested for this method were fully satisfactory. Namely, we obtained a good linearity ($r^2 > 0.999$) in the concentration range 0–15 μmol/L for 2-OPP and 0–25 μmol/L for 6-oxoPIP in triplicate.

LOD and LOQ were respectively: for urine, 13 nmol/L and 40 nmol/L for 2-OPP, 18 nmol/L and 55 nmol/L for 6-oxoPIP; for, plasma 30 nmol/L and 90 nmol/L for 2-OPP, 11 nmol/L and 32 nmol/L for 6-oxoPIP.

The variations of intra-day and inter-day for 2-OPP and 6-oxoPIP determinations on urine and plasma are reported in Table 1.

The results obtained for RE% and ME%, calculated for both matrices and for both analytes, fall within the validation acceptability range of ± 15 % (Supplementary Table 2).

For what concerns carry-over, we found that the signal in the blank sample, following calibrator, was not greater than 20 % of the LOQ for both biomarkers.

We showed that the two biomarkers, in both matrices, were stable at different storage temperatures for one month at + 4°C, − 20 °C (Fig. 3) and for 24 h at room temperature (Table 2), with a CV% lower than 15 % for all tested temperature.

For what concerns dilution integrity of the plasma sample, we found that CV for both analytes was ≤ 3.5%.

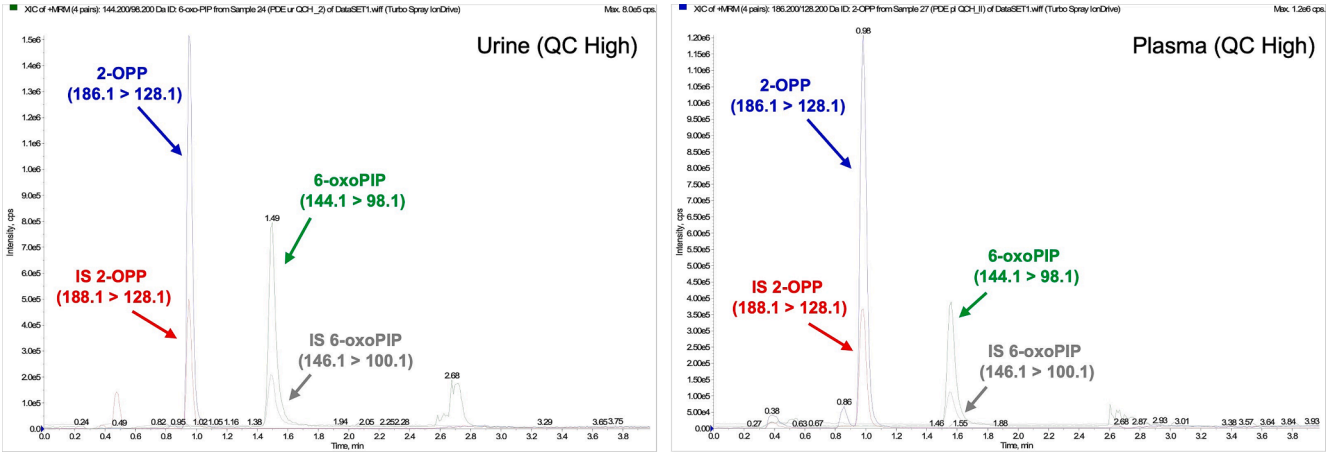

Fig. 2. Chromatographic resolution of 2-OPP and 6-oxoPIP in urine and plasma. The figure shows the chromatographic separation of 2-OPP, 6-oxoPIP, and labelled IS $^{13}C_2$ -2-OPP and $^{13}C_2$ -6-oxoPIP in urine (left panel) and plasma (right panel). The retention time were respectively 0.99 min for 2-OPP and its labelled IS, and 1.49 min for 6-oxoPIP and its labelled IS.

Table 1
Intra- and inter-day precision and accuracy of the assay for 2-OPP and 6-oxoPIP in urine and plasma.

	URINE Media	SDv	CV%	Accuracy (%)	PLASMA Media	SDv	CV%	Accuracy (%)
2-OPP	**Intraday**							
QC Low	0.25	0.00	1.62	99.80	0.25	0.00	0.82	100.64
QC Medium	1.52	0.03	1.78	101.44	1.53	0.02	1.42	101.87
QC High	7.44	0.11	1.44	99.16	7.74	0.16	2.09	103.17
	Interday							
QC Low	0.25	0.01	4.10	100.80	0.25	0.01	4.77	100.79
QC Medium	1.53	0.06	4.06	101.93	1.56	0.02	1.01	103.84
QC High	7.54	0.26	3.42	100.52	7.66	0.13	1.69	102.19
6oxoPIP	**Intraday**							
QC Low	0.48	0.01	2.59	95.07	0.53	0.01	2.05	106.08
QC Medium	7.48	0.06	0.73	99.71	7.62	0.15	1.96	101.60
QC High	20.37	0.14	0.67	101.83	20.54	0.27	1.32	102.70
	Interday							
QC Low	0.52	0.02	4.38	103.40	0.53	0.02	3.38	106.50
QC Medium	7.54	0.07	0.91	100.57	7.56	0.05	0.71	100.80
QC High	20.11	0.35	1.72	100.57	20.21	0.23	1.13	101.10

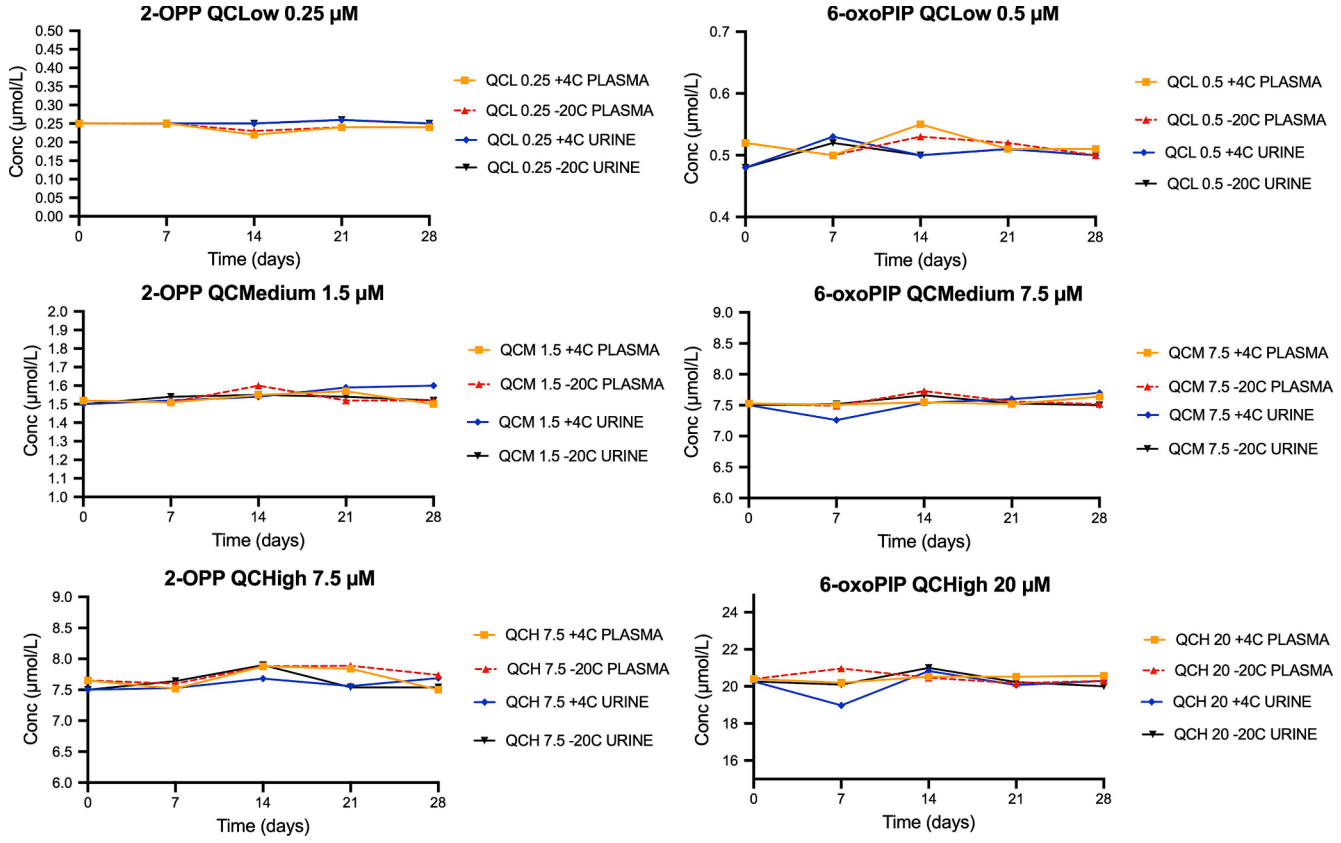

Fig. 3. Stability at + 4°C and − 20 °C for 2-OPP and 6-oxoPIP in urine and plasma. Concentrations of 2-OPP (panels a-c) and 6-oxoPIP (panels d-f) for the 3 QC levels, after 0, 7, 14, 21 and 28 days of storage at + 4°C and − 20 °C.

Table 2
Stability at room temperature for 2-OPP and 6-oxoPIP in urine and plasma.

	Urine 0 h	24 h	Plasma 0 h	24 h
2-OPP				
QC L (0.25 µmol/L)	0.25	0.25	0.28	0.23
QC M (1.50 µmol/L)	1.50	1.54	1.59	1.52
QC H (7.50 µmol/L)	7.50	7.79	7.59	7.54
6-oxoPIP				
QC L (0.50 µmol/L)	0.48	0.55	0.52	0.50
QC M (7.50 µmol/L)	7.50	7.47	7.53	7.54
QC H (20.00 µmol/L)	20.30	20.10	20.47	20.70

3.1. Clinical applicability

After the validation of the method, we tested the clinical applicability of the two biomarkers in urine and plasma samples. By way of example in Figs. 4 and 5, we show increased 2-OPP and 6-oxoPIP concentrations in urine and plasma, respectively, as compared to a healthy control; we obtained a comparable profile for the other patients.

For both matrices, the chromatographic peacks related to unknown interfering compounds can be noted. However, the chromatographic runs had a good resolution for 2-OPP and 6-oxoPIP because their chromatographic peacks had different retention times compared to those of the interfering compounds, and the use of labelled IS for 2-OPP

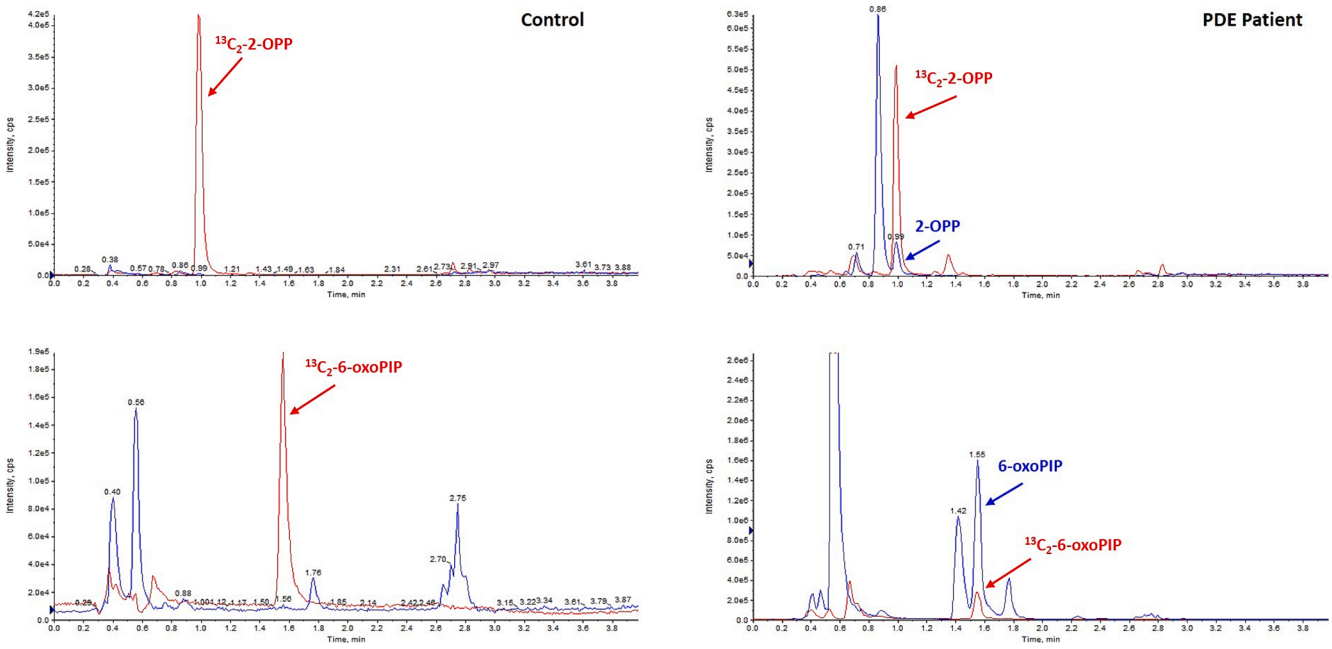

Fig. 4. LC-MS/MS profile in urine sample from a healthy control and PDE patient. Remarkably increased 2-OPP and 6-oxoPIP concentrations can be seen only in the urine from the patient affected by PDE.

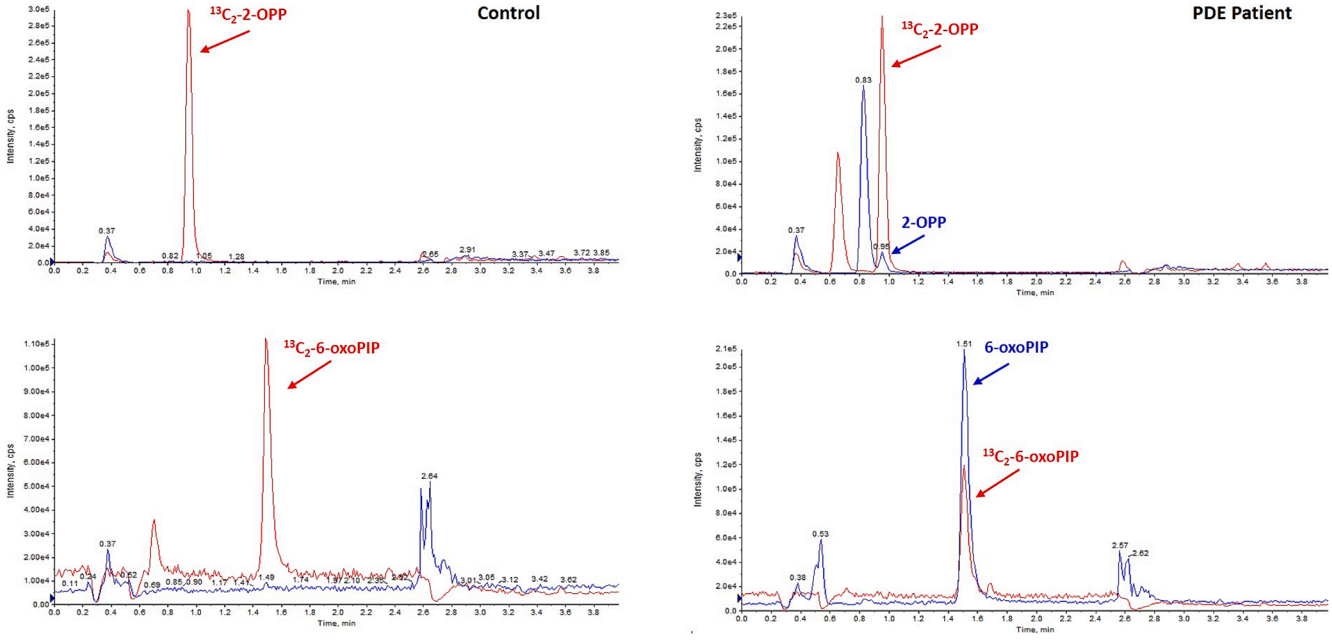

Fig. 5. LC-MS/MS profile in plasma sample from a healthy control and PDE patient. Remarkably increased 2-OPP and 6-oxoPIP concentrations can be seen only in the plasma from the patient affected by PDE.

and 6-oxoPIP confirmed the correct identification of the two biomarkers. Finally, we performed a quantification of 2-OPP and 6-oxoPIP in urine and plasma using calibration curve with IS. Our results reported in Table 3 confirmed increased levels of these biomarkers in all PDE patients for both matrices. Concentrations of 2-OPP and 6-oxoPIP in urines from healthy subjects, resulted $\leq$ 16.0 μmol/mol Creu and $\leq$ 727.6 μmol/mol Creu, respectively.

No significant differences emerged when stratifying urinary levels of these biomarkers according to age groups (pediatrics vs adults) or sex (*data not shown*); however, these findings are limited by the low sample size of these subgroups.

As for plasma, concentrations of 2-OPP and 6-oxoPIP were < LOQ in all tested healthy subjects.

4. Discussion

In PDE, deficiency in the enzyme ALDH7A1, catalysing the third step of the lysine oxidation pathway, causes neurotoxic substances to accumulate in blood, plasma, urine, CSF, and brain, turning them in biomarkers for PDE diagnosis and monitoring [23,26].

To date, biochemical analysis of the metabolites α-AASA, P6C and PA in urine and plasma is performed in patients with overt symptoms suggestive of PDE. However, it must be stressed that these biomarkers are not specific, as α-AASA can also be increased in patients with

Table 3
Quantitative values of 2-OPP and 6-oxoPIP in the urine and plasma samples from healthy controls and three PDE patients.

	Urine 2-OPP (μmol/mol Creu)	6-oxoPIP (μmol/mol Creu)	Plasma 2-OPP (μmol/L)	6-oxoPIP (μmol/L)
Healthy controls (n = 24 for urine, n = 32 for plasma)	n.q. – 16.0*	n.q. – 727.6*	n.q.	n.q.
PDE patient 1 (early-onset, treated with pyridoxine)	–	–	0.20	10.50
PDE patient 2 (late-onset, treated with antiepileptis)	34.56	988.40	0.09	0.50
PDE patient 3 (early-onset, untreated)	2885.30	1974.20	–	–

n.q. not quantifiable.
* minimum- maximum values among healthy controls.

molybdenum cofactor deficiency and isolated sulfite oxidase deficiency, while high PA levels can indicate also peroxisomal disorders [15,27].

Besides these diagnostic issues, their detection and quantification poses several technical challenges: these biomarkers are relatively unstable at room temperature, which requires samples to be frozen prior to shipment to diagnostic laboratories [6]. Even when correctly stored, their quantification is challenged by the difficulty to purchase or synthesize their labelled IS, so their analyses are often semi-quantitative. Moreover, the analytical methods used for their quantification in LC-MS/MS often necessitate of derivatization steps, thus increasing the time and cost of the procedure [4,5].

In 2019, the 6-oxoPIP metabolite was identified in patients with PDE [20], and shortly after the 2-OPP biomarker was also identified [23] (Fig. 6). These novel biomarkers are better candidates for identifying PDE, as they are stable at + 4 °C and − 20 °C for at least one month.

In 2021, Engelke et al. [23] published a paper focused on the identification of biomarkers for PDE (including 2-OPP, 6-oxoPIP and traditional biomarkers) and tested their applicability on animal and human samples from different matrices (including urine, plasma, CSF, and brain tissue). In the present study, we specifically focused on the set-up and validation of an analytical method for 2-OPP and 6-oxoPIP in LC-MS/MS only in human urine and plasma samples, providing for the first time a detailed overview of the analytical validation parameters.

The developed method has proved to be reliable and can serve as an alternative analytical approach for diagnosing PDE after clinical suspicion is formulated, by using the same biological matrices already used in routine clinical practice.

When investigating the clinical applicability of our method to real PDE patients, different concentration of the biomarkers 2-OPP and 6-oxoPIP were observed in our PDE patients with respect to what was reported elsewhere [23]; this could be attributable to biological and clinical variability, as well as to the impact of ongoing pharmacological treatments at time of sample collection. In particular, the two PDE plasma samples quantified in this study presented remarkably lower levels of the two biomarkers as compared to literature data [23], probably due to the fact that they were both under long-term pharmacological treatment and one of them presented with late-onset PDE phenotype (patient 2). Notably, 2-OPP and 6-oxoPIP in plasma were < LOQ in all tested healthy controls.

As for urine, the two samples assessed in our study presented significantly higher levels as compared to what reported elsewhere [23]; this could be due to the fact that one of these patients was an early-onset, untreated PDE patient whose sample was collected and analysed at time of disease onset (patient 3; 2885.30 μmol/mol Creu for 2-OPP and 1974.20 μmol/mol Creu for 6-oxoPIP). Conversely, the other patient (patient 2) was a late-onset, pharmacologically treated PDE patient, whose concentrations of 2-OPP and 6-oxoPIP (34.56 μmol/mol Creu for 2-OPP and 988.40 μmol/mol Creu for 6-oxoPIP) appeared remarkably lower as compared to the early-onset, untreated PDE patient, but still increased as compared to the healthy control samples (≤16.0 μmol/mol Creu for 2-OPP and ≤ 727.6 μmol/mol Creu for 6-oxoPIP). Nevertheless, it cannot be excluded that the differences in 2-OPP and 6-oxoPIP levels in our study as compared to literature findings [23] were related to the different analytical methods; additional comparative analyses on the same biological samples from PDE patients are required to shed light on this aspect.

Further analyses assessing age-related differences in urine and plasma levels of 2-OPP and 6-oxoPIP are advocated, with a particular focus on newborns and infants: indeed, the levels of these biomarkers could significantly differ on that population, as also plasma and urine α-AASA and P6C have been reported to be remarkably higher in infants and later decrease with age [7,22].

For both matrices, the presence of interfering compounds (peaks) in the chromatography does not seem to interfere with the analytical selectivity, given the distinct retention times and the use of labelled IS for the identification and quantification of the two biomarkers of interest. Regarding plasma, these co-eluting peaks are undetectable in the controls, thus we can exclude that the "interference" is related to the plasma matrix or to the analytical procedure. Notably, by comparing the chromatogram from our two real PDE patients with the calibration curve constructed using QCs for plasma, we can exclude that these peacks are related to 2-OPP and 6-oxoPIP per se (e.g dissociated/undissociated forms), as these "splits" are absent in the calibration curve with the IS. Further analysis will clarify the nature of these signals, which are likely to be related to the pharmacological therapies for PDE patients. Conversely, in urines, co-eluting peaks occurred in most healthy controls either before or after the peak of the IS, both for 2-OPP and 6-oxoPIP. Considering that urine is a critical matrix as it accumulates all waste metabolites from all biochemical pathways, it is impossible to define the nature of these peaks, which are probably matrix-related. Further investigation will confirm the origin of these compounds, that are likely to be isobaric compounds of 2-OPP and 6-oxoPIP [23].

Regarding the diagnostic specificity of these biomarkers, as far as we know isolated urinary excretion of 6-oxo-PIP has been reported also in patients with molybdenum cofactor deficiency [22], while no data are available on 2-OPP in patients with molybdenum cofactor deficiency or isolated sulfite oxidase deficiency. Further research is needed to confirm the specificity of 2-OPP for PDE; in the meanwhile, we could speculate on the potential role of 2-OPP and 6-oxoPIP as primary and secondary biomarker of PDE, respectively.

We fully validated the method showing that it is linear, accurate, and precise, and confirmed its clinical applicability for PDE. Validation procedures were conducted by spiking with known amounts of 2-OPP and 6-oxoPIP into synthetic urine and human plasma, rather than by using real human samples from PDE patients. This choice was in line with current guidelines for analytical method validation [25], and was driven by the need to spare real pathological samples for future analyses. For the routine clinical application of the method, further validation procedures might be conducted on real human urine and plasma samples from PDE patients.

An advantage of our method is that small volumes of plasma or urine are required (10 μL aliquot). The labelled IS for 2-OPP and 6-oxoPIP, $^{13}C_2$-2-OPP and $^{13}C_2$-6-oxoPIP, were originally custom made by Synvenio, but they are now both available on the market. The cost is estimated around 0.10 € per test if considering only the reagents, and then acceptable for both research and clinical purposes.

Measuring these two biomarkers is of great importance for both diagnostic and follow-up purposes, particularly considering that new treatment options (including targeted inhibitors, antisense oligonucleotide- and RNA interference-based strategies against AASS, enzymatic replacement and gene therapy) might be released on the market in the

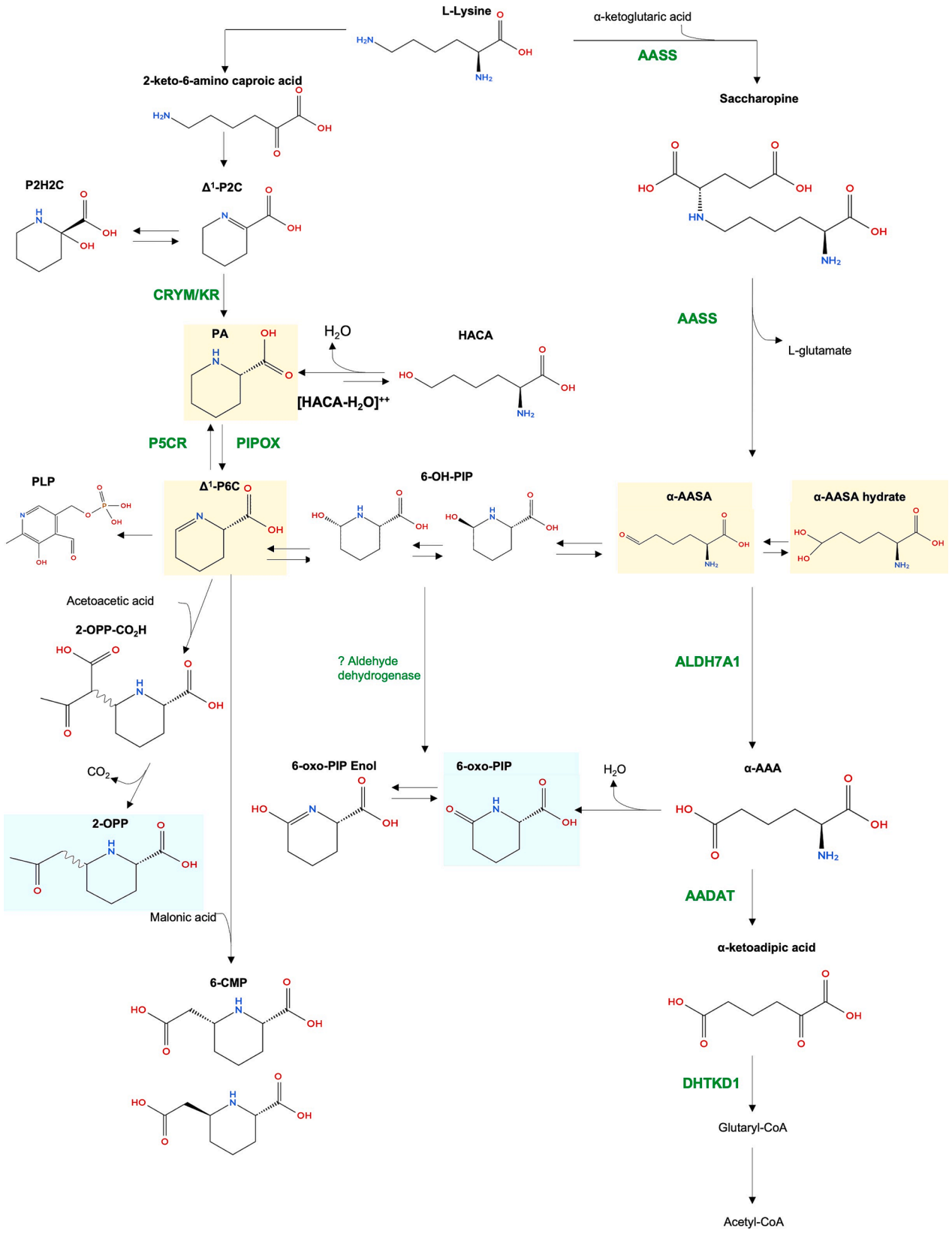

Fig. 6. Schematic representation of the metabolic pathways of L-Lysine involved in PDE. Enzymes are reported in green. Yellow boxes indicate traditional biomarkers for PDE. Light blue boxes indicate new biomarkers for PDE. *2-OPP: (2S, 6S)- and (2S, 6R) 6-(2-oxopropyl)piperidine-2-carboxylic acid; 6-CMP: 6-(carboxymethyl)piperidine-2-carboxylic acid; 6-OH-PIP: 6-hydroxy-pipecolate; 6-oxoPIP: 6-oxopiperidine-2-carboxylic acid; AADAT: aminoadipate aminotransferase; AASS: aminoadipic semialdehyde synthase; ALDH7A1: aldehyde dehydrogenase 7 family member A1; CRYM/KR: μ-crystallin/ketimine reductase; DHTKD1: dehydrogenase E1 and transketolase domain containing 1; HACA: 6-hydroxyl-2-aminocaproic acid; P2H2C: piperidine-2-hydroxy-2-carboxylate; P5CR: piperideine-5-carboxilic reductase; PA: pipecolic acid; PIPOX: pipecolate oxidase; PLP: pyridoxal-5′ phosphate; α-AAA: α-aminoadipic acid; α-AASA: α-aminoadipic-δ-semialdehyde; Δ1- P2C: Δ1-piperideine-2-carboxylate; Δ1-P6C: Δ1-piperideine-6-carboxylate.*

next future.

Future research is required to assess if the method we presented is also feasible on dried blood spot (DBS) specimens, a cost-effective tool for the diagnosis, follow-up and therapeutic monitoring of PDE in routine clinical practice, particularly in the pediatric setting. Also, for the future routine use of this test for diagnostic purposes, further analyses on larger cohorts of pediatric and adult healthy controls covering all ages of potential interest are required, to estimate age-specific reference ranges for the two biomarkers.

5. Conclusions

We set-up and validated a method for the quantification of two new biomarkers of PDE (2-OPP and 6-oxoPIP) on urine and plasma by LC-MS/MS, to overcome the diagnostic and technical challenges related to the analysis of old biomarkers (α-AASA, P6C and PA). The method proved reliable and could also be used as routine test for PDE diagnosis, follow-up and therapeutic monitoring in these biological matrices.

6. Research data

Raw data for the validation procedure are available upon reasonable written request to the corresponding author. For the clinical applicability, raw data for the three patients with PDE and two healthy controls included in the study are provided in the manuscript. Written informed consents from the parents' or from the healthy subjects were obtained.

CRediT authorship contribution statement

Roberta Damiano: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Della Bona:** Validation. **Elena Procopio:** Resources, Investigation. **Serena Gasperini:** Resources, Investigation. **Renzo Guerrini:** Writing – review & editing, Conceptualization. **Alessandra Bettiol:** Writing – original draft, Visualization. **Giancarlo la Marca:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.120111.

Data availability

Data will be made available on request.

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