



Quantification of urine and plasma porphyrin precursors using LC–MS in acute hepatic porphyrias: improvement in routine diagnosis and in the monitoring of kidney failure patients

Antoine Poli, Hana Manceau, Anvi Laetitia Nguyen, Boualem Moulouel, Nathalie Dessendier, Neila Talbi, Hervé Puy, Christophe Junot, Laurent Gouya, Caroline Schmitt, et al.

► To cite this version:

Antoine Poli, Hana Manceau, Anvi Laetitia Nguyen, Boualem Moulouel, Nathalie Dessendier, et al.. Quantification of urine and plasma porphyrin precursors using LC–MS in acute hepatic porphyrias: improvement in routine diagnosis and in the monitoring of kidney failure patients. *Clinical Chemistry*, 2023, 69 (10), pp.1186 - 1196. 10.1093/clinchem/hvad117 . cea-04318571

HAL Id: cea-04318571

<https://cea.hal.science/cea-04318571>

Submitted on 1 Dec 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Quantification of Urine and Plasma Porphyrin Precursors Using LC–MS in Acute Hepatic Porphyrrias: Improvement in Routine Diagnosis and in the Monitoring of Kidney Failure Patients

Antoine Poli,^{a,b,c,d} Hana Manceau,^{a,c,e} Anvi Laetitia Nguyen,^d Boualem Moulouel,^b
Nathalie Dessendier,^b Neila Talbi,^{a,b,c} Hervé Puy,^{a,b,c} Christophe Junot,^d Laurent Gouya,^{a,b,c}
Caroline Schmitt,^{a,b,c} and Thibaud Lefebvre^{a,b,c,d,*}

BACKGROUND: The quantification of delta-aminolevulinic acid (ALA) and porphobilinogen (PBG) in urine are the first-line tests for diagnosis and monitoring of acute hepatic porphyrias (AHP). Ion-exchange chromatography (IEC), which is time- and staff-consuming and limited to urine, is still the preferred method in many specialized laboratories, despite the development of mass spectrometry-based methods.

METHODS: We describe a new LC-MS method that allows for rapid and simple quantification of ALA and PBG in urine and plasma with an affordable instrument that was used to analyze 2260 urine samples and 309 blood samples collected in 2 years of routine activity. The results were compared to those obtained with IEC, and urine reference ranges and concentrations in asymptomatic carriers were determined. Plasma concentrations were measured in healthy subjects and subgroups of symptomatic and asymptomatic AHP carriers.

RESULTS: In urine, the clinical decision limits were not impacted by the change of method despite discrepancies in low absolute concentrations, leading to lower normal values. Two-thirds of asymptomatic AHP carriers (with the exception of coproporphyrin carriers) showed an increased urine PBG concentration. Urine and plasma levels showed a good correlation except in patients with kidney disease in whom the urine/plasma ratio was relatively low.

CONCLUSION: We described an LC-MS based method for the routine diagnosis and monitoring of AHP that

allows for the detection of more asymptomatic carriers than the historical method. Blood analysis appears to be particularly relevant for patients with kidney disease, where urine measurement underestimates the increase in ALA and PBG levels.

Introduction

Porphyrias are inherited metabolic diseases caused by a defect in a specific enzyme in the heme synthetic pathway. Three autosomal dominant acute hepatic porphyrias (AHP) are defined by the occurrence of acute neurovisceral attacks: acute intermittent porphyria (AIP), the most frequent, variegata porphyria (VP), and hereditary coproporphyrin (HCP) (1, 2). Acute attacks are characterized by acute autonomic neuropathy with abdominal pain, dysautonomic symptoms, and, in the most severe cases, acute peripheral and central neuropathy (3). Their diagnosis is based on the measurement of 2 biomarkers in urine: delta-aminolevulinic acid (ALA) and porphobilinogen (PBG) (4, 5), which are considered neurotoxic (6). Both metabolites are precursors of porphyrins, the tetrapyrrolic intermediates of heme biosynthesis. ALA results from the condensation of glycine and succinyl-CoA. Two ALA molecules react together to form a PBG molecule. Precursors accumulate strongly in biological fluids during an acute attack of porphyria (AAP) (7). PBG is pathognomonic of acute porphyrias, while ALA concentration can also be increased in lead poisoning (8).

^aUniversité de Paris Cité, INSERM U1149, Centre de Recherche sur l'Inflammation, HIROS Team, F-75018 Paris, France; ^bAssistance Publique-Hôpitaux de Paris, Centre Français des Porphyrries, Hôpital Louis Mourier, F-92700 Colombes, France; ^cLaboratory of Excellence, Gr-Ex, Paris, France; ^dDépartement Médicaments et Technologies pour La Santé (DMTS), Université Paris-Saclay, CEA, INRAE, MetaboHUB, F-91191 Gif-sur-Yvette, France; ^eAssistance

Publique-Hôpitaux de Paris, Laboratoire de Biochimie, Hôpital Beaujon, F-92110 Clichy la Garenne, France.

*Address correspondence to this author at: Centre Français des Porphyrries, Hôpital Louis Mourier, 178 rue des Renouillers, 92701 COLOMBES cedex, France. Tel +33-1-47-60-63-34; E-mail thibaud.lefebvre@aphp.fr.

Received March 8, 2023; accepted July 10, 2023.
<https://doi.org/10.1093/clinchem/hvad117>

Elevated levels of ALA and PBG during AAP are due to hepatic induction of ALA synthase 1 (ALAS1), the first enzyme of the heme synthesis pathway, when heme demand increases. In such circumstances, the activity of the impaired enzyme downstream of ALAS1 in the pathway becomes limiting, leading to an accumulation of the upstream ALA and PBG metabolites (7).

To date, urine ALA and PBG measurements are the front-line tests when AAP is suspected. Historically, measurement was based on the use of a pyrrole-specific reagent, the Ehrlich reagent, allowing detection by colorimetry (9). ALA and PBG are first separated by ion-exchange chromatography (IEC), and then, ALA is converted to a pyrrolic derivative before reacting with Ehrlich reagent. This method is still used in many porphyria reference laboratories around the world but suffers from several limitations. The sensitivity of the method limits its application to urine only, excluding blood samples. This limitation can impact care in situations such as oligo- or anuria or kidney failure which is common in AIP (10).

Over the past 2 decades, liquid chromatography coupled with mass spectrometry (LC-MS) has become widely used in clinical metabolic biochemistry laboratories and has greatly facilitated the development of small-molecule quantitative methods. Several methods of ALA and PBG measurement using LC-MS have been described in the literature (11–14); however, to date, few laboratories have adopted this technology in routine practice (8, 15, 16). Indeed, except in university medical centers, laboratories specializing in porphyria may have limited access to mass spectrometry.

Based on a 2-year experience in the routine medical laboratory at the French Porphyrria Centre, we present here a fast, economical, robust, and easy-to-handle method for the measurement of both ALA and PBG in biological fluids, which can be performed on a single quadrupole LC-MS.

Material and Methods

SAMPLES AND PATIENTS

All procedures were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Clinical and biological data presented in this article were collected as part of patients' routine care, and all samples were anonymized (online [Supplemental Fig. 1](#)).

Urine. Urine samples ($n = 2260$) were collected over a 2-year period (2020 to 2021) from the routine laboratory of the French Porphyrria Centre. Method correlation was assessed using 217 urine samples for which ALA and PBG were measured both by IEC (RECIPE) and LC-MS (see below). In this batch, 40 pathological samples were selected for high PBG ($>9 \mu\text{mol}/\text{mmol}$ creatinine). Reference values were determined on 1565 samples sent to the laboratory for investigation of

possible AAP that did not lead to the diagnosis of porphyria. In asymptomatic porphyria carriers, ALA and PBG levels were determined in 158 urine samples from AIP, 60 from VP, and 14 from HCP. Moreover, 246 urine samples from AIP patients and asymptomatic carriers (AC, patients with an *HMBS* mutation who never had an AAP) were collected along with blood samples.

Blood. For the determination of plasma levels, specimens were taken from 37 healthy donors, 97 asymptomatic AIP carriers, and 116 symptomatic AIP patients. In AIP patients, blood was collected outside of an attack period in 85 AIP patients (104 samples) with a mild phenotype (less than one attack per year) and in 31 with a severe phenotype (at least one attack per year). In the AIP patient group, 30 samples were also collected during an AAP episode. Plasma samples were collected in EDTA tubes (centrifugation $2500\times g$, 10 min at room temperature). Blood samples collected in lithium-heparin, clot activator, and serum gel tubes were also tested during the development phase. All samples collected are summarized in a flowchart ([Supplemental Fig. 1](#)).

REAGENTS

Water and solvent (mass spectrometry, MS, grade) were purchased from Biosolve Chimie. 5-Aminolevulinic acid hydrochloride, 5-aminolevulinic acid- $^{13}\text{C}_5$, ^{15}N hydrochloride, and sulfosalicylic acid (SSA) were purchased from Sigma-Aldrich. Porphobilinogen and porphobilinogen- $^{13}\text{C}_2$ were obtained from Frontier and Isosciences, respectively. The AccQ-Tag Ultra Derivatization Kit Reagent was purchased from Waters.

CALIBRATION AND QUALITY CONTROL

A calibration scale was adapted depending on the biological fluid studied, by diluting stock solutions of ALA and PBG at $2 \text{ mmol}/\text{L}$ in 0.1 M HCl . For the urine assay, the calibration consisted of the following 5 concentrations: 0.5, 1, 5, 100, and $350 \mu\text{mol}/\text{L}$. For blood monitoring, the scale was shifted to lower concentrations: 0.05, 0.1, 0.5, 1, 5, and $25 \mu\text{mol}/\text{L}$. Calibration levels and reagent blanks were diluted in 0.1 M HCl . Calibration curves were built from the instrument response ratios of standards using $1/x$ weighted least-squares regression analysis. Curve fitting was linear for ALA and quadratic for PBG. In urine, 2 levels of Clincheck quality controls (RECIPE) were used: level 1 ALA and PBG (32.3 ; $7.9 \mu\text{mol}/\text{L}$, respectively); level 2 ALA and PBG (142.0 ; $69.7 \mu\text{mol}/\text{L}$, respectively). For plasma, 2 pools of samples were used as control: low normal pool (1.31 ; $0.26 \mu\text{mol}/\text{L}$, for ALA and PBG, respectively) and high pathological pool (13.91 ; $6.75 \mu\text{mol}/\text{L}$ for ALA and PBG, respectively).

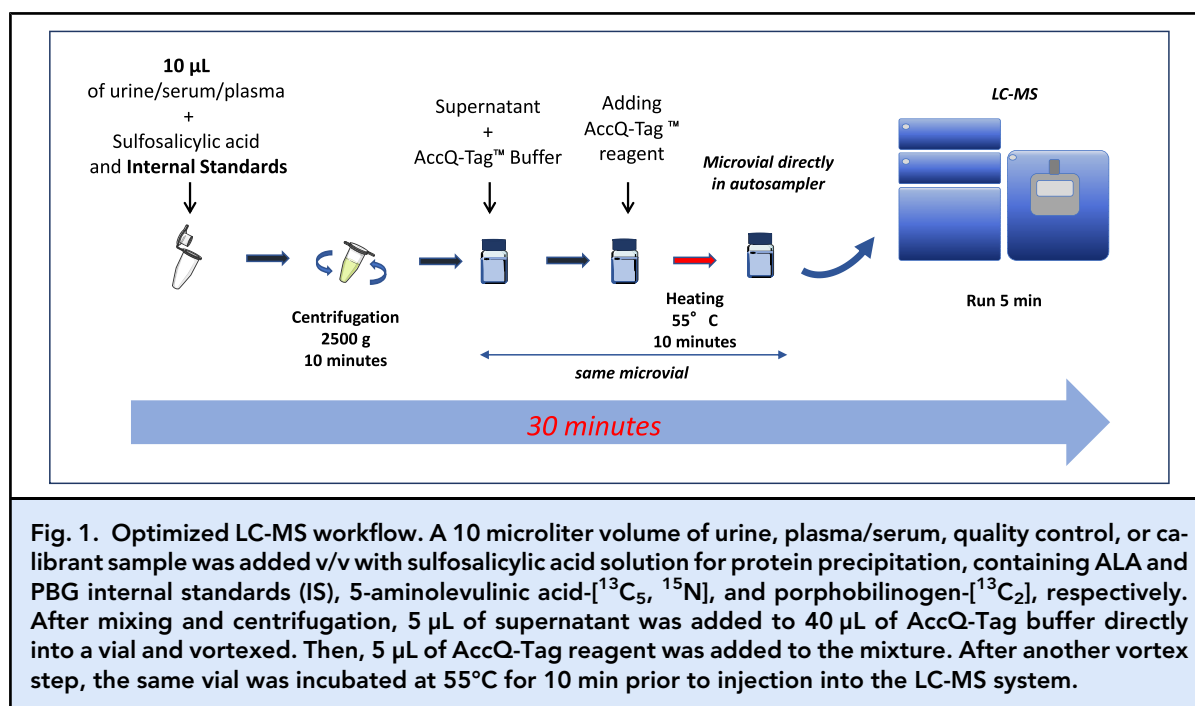


Fig. 1. Optimized LC-MS workflow. A 10 microliter volume of urine, plasma/serum, quality control, or calibrant sample was added v/v with sulfosalicylic acid solution for protein precipitation, containing ALA and PBG internal standards (IS), 5-aminolevulinic acid-[$^{13}\text{C}_5$, ^{15}N], and porphobilinogen-[$^{13}\text{C}_2$], respectively. After mixing and centrifugation, 5 µL of supernatant was added to 40 µL of AccQ-Tag buffer directly into a vial and vortexed. Then, 5 µL of AccQ-Tag reagent was added to the mixture. After another vortex step, the same vial was incubated at 55°C for 10 min prior to injection into the LC-MS system.

LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

The method for urine sample analysis was developed and validated on an H-Class PLUS coupled with a single quadrupole QDa instrument (Waters) and compared to the historical IEC method. For the blood assay, both a single quadrupole and a triple quadrupole mass spectrometer Xevo TQ-MS (Waters) were used.

The analytes were separated on a BEH C18 column (2.1 mm × 50 mm) (Waters) containing particles 1.7 µm in diameter at 45 °C. Mobile phases were composed of 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). After injection of 2 µL of sample, a gradient elution program, detailed in the online [Data Supplement](#), was applied at a constant flow rate of 0.5 mL/min for 5 min ([Supplemental Table 1](#)). Derivatized analytes were detected in positive ionization mode by selected ion recording (SIR) or multiple reaction monitoring (MRM) depending on the kind of mass detector (single or triple quadrupole, respectively). The mass spectrometry parameters are reported in online [Supplemental Table 2](#).

STATISTICAL ANALYSIS

Correlations were assessed using a Pearson test. The methods were compared by a Bland–Altman analysis with determination of 95% limits of agreement. Medians or means were compared using a Mann–Whitney nonparametric test or Student parametric *t*-test when possible. Significance was set as two-sided

$P < 0.05$. All statistical analyses and graphs were performed using Prism 7 (GraphPad software).

Results

DEVELOPMENT AND ANALYTICAL VALIDATION OF ALA AND PBG ASSAYS IN URINE AND PLASMA FOR AAP SCREENING

Pretreatment of urine or plasma samples, to ensure protein precipitation and derivatization, was optimized using the recommendations of the derivatization reagent manufacturer as indicated in [Fig. 1](#), the aim being to achieve sufficient sensitivity and optimize ease of handling. The pipetting volumes, 10 µL and then 5 µL, were optimized to minimize volumes of biofluid and AccQ-Tag reagent, respectively. ALA and PBG concentrations could be easily measured in both urine and plasma within 30 min. This assay was initially developed and validated for urine monitoring on an easy-to-use single quadrupole, the QDa detector (Waters) coupled with an ultra performance liquid chromatography (UPLC). For both ALA and PBG assays, the lower limit of quantification (LLOQ) was determined to be 0.5 µmol/L, and the upper limit of quantification (ULOQ) reached 350 µmol/L ($R^2 > 0.99$ for ALA and PBG). No matrix effect was found when comparing calibration in urine and in 0.1 M HCl (online [Supplemental Table 3](#)). The limit of detection (LOD) was set at 0.1 µmol/L (signal-to-noise ratio, $S/N > 3$). The linearity range was mainly limited by PBG, which exhibits quadratic behavior over a wider

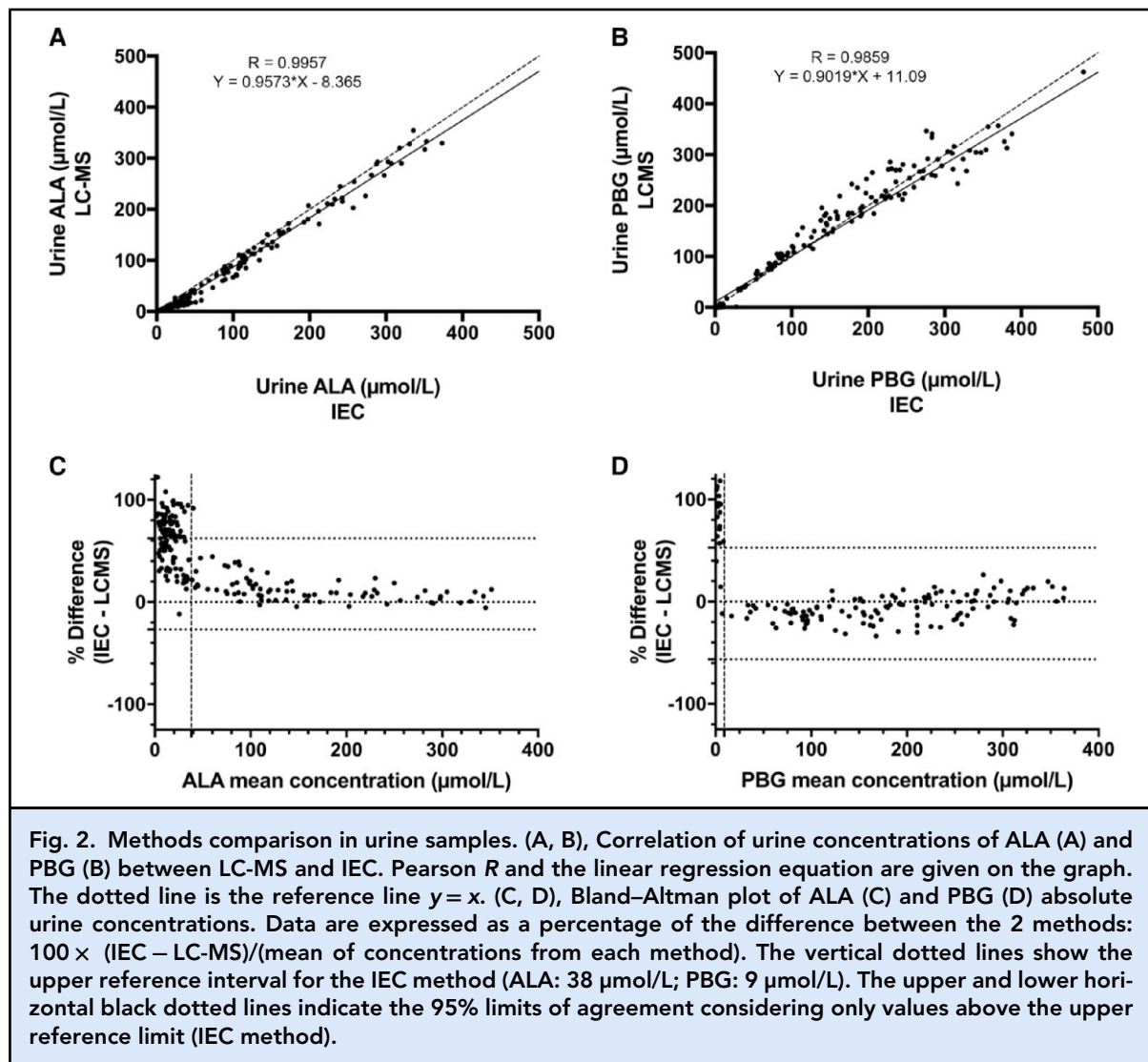
range. However, this range was sufficient to detect the specific rise in ALA (10-fold) and PBG (100-fold) concentrations in an AAP, both in urine and plasma. Intra-assay CVs were $\leq 4.9\%$ (ALA) and $\leq 4.6\%$ (PBG). Inter-assay CVs reached 3.5% and 4.2%, respectively (online Supplemental Table 4). ALA was stable in urine for 8 days at room temperature or 4°C in darkness or natural light. PBG was equally stable only in darkness at 4°C (online Supplemental Fig. 2).

VALIDATION OF ALA AND PBG ASSAYS FOR AHP FOLLOW-UP IN PLASMA

This LC-MS method allows for the measurement of both biomarkers in different matrixes, such as urine, serum, or plasma. To adapt the analytical scale to plasma monitoring, the calibration was shifted to lower concentrations: 0.05 to 25 $\mu\text{mol/L}$ ($R^2 > 0.99$ for ALA and

PBG). As for urine, serum and plasma did not induce matrix effects (Supplemental Table 3). To achieve sufficient sensitivity and validate the method, plasma tests were performed on a triple quadrupole coupled to UPLC. Inter- and intra-assay CVs, assessed in the same way as urine, were determined to be $\leq 7.06\%$ and 5.70% for ALA and $\leq 4.46\%$ and 11.02% for PBG, respectively (online Supplemental Table 5).

ALA and PBG concentrations of samples collected in lithium-heparin, EDTA, or clot activator tubes were not significantly different from the serum-separated tube (SST) with a gel separator (online Supplemental Fig. 3A and B). Regarding stability in whole blood samples, mimicking the shipping conditions, ALA and PBG levels did not show significant differences after storage at 4°C in the dark for 8 days. At room temperature, PBG and ALA concentrations decreased over time. The median

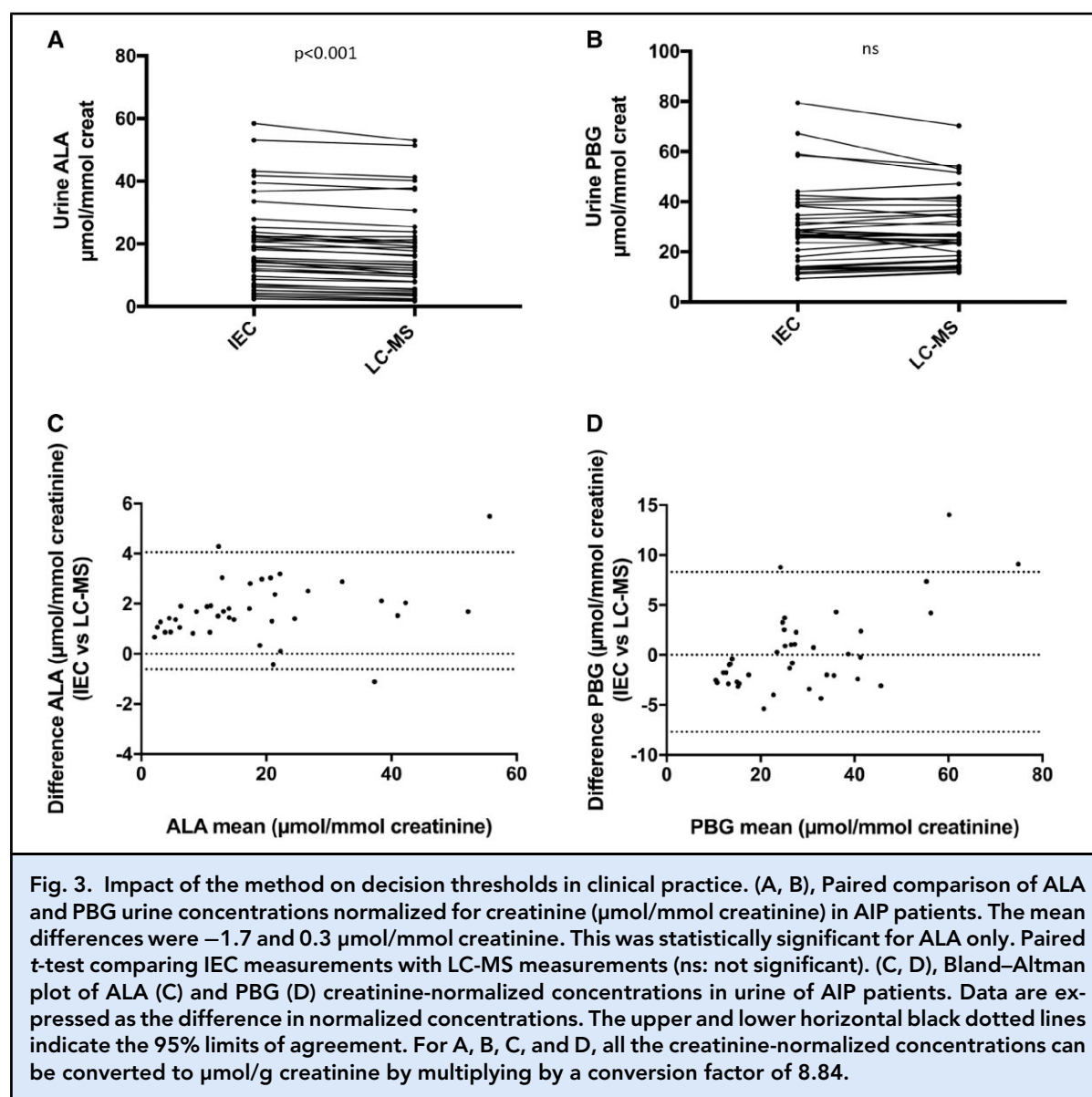


PBG level reached 75% of the initial concentration on Day 3 with or without light protection. ALA was more stable, as the median level decreased below the threshold of 75% on Day 5 (light exposure) or Day 6 (in the dark) at room temperature (Supplemental Fig. 3C–E).

COMPARISON BETWEEN THE LC-MS AND IEC METHODS IN URINE

The LC-MS assay was compared with the standard IEC assay in urine. Absolute ALA concentrations showed a very high correlation ($R > 0.995$). The correlation was slightly lower for PBG ($R > 0.985$) (Fig. 2A and B). Both slopes of the linear regression were close to 1

(>0.9) with a significant bias (−8.365 for ALA, +11.09 for PBG). The Bland–Altman diagram highlighted the limitations of the correlations between the 2 methods. For ALA and PBG, very low concentrations were highly discordant with concentrations determined by IEC, reaching more than double the mean concentration (Fig. 2C and D). This deviation occurred below the upper reference limit of the column test (i.e., ALA: 38 $\mu\text{mol/L}$; PBG: 9 $\mu\text{mol/L}$) and therefore had no impact on interpretation and clinical management. Beyond these extremely low levels, the differences in concentrations were much smaller and more homogenous across the data set for both parameters.



The impact of the change in method on patient care was assessed based on creatinine normalized concentrations within the clinical interpretation range. ALA and PBG measurements were both used to diagnose an acute attack of porphyria and to monitor symptomatic AHP patients. In patients with urine PBG levels greater than 9 $\mu\text{mol}/\text{mmol}$ Cr (79.6 $\mu\text{mol}/\text{g}$ Cr), PBG concentrations did not show a significant difference, and 90% (36/40) of the differences did not exceed 5 $\mu\text{mol}/\text{mmol}$ Cr (Fig. 3A and B). ALA concentrations were significantly higher with the IEC method, but the 95% limits agreement of Bland–Altman analysis was very close, from -0.61 to 4.00 $\mu\text{mol}/\text{mmol}$ Cr (Fig. 3C and D).

REFERENCE INTERVAL OF HEME PRECURSORS IN URINE IN HEALTHY PEOPLE AND ASYMPTOMATIC CARRIERS

Considering the discrepancies observed in the low-concentration ranges between the 2 methods, we report urine ALA and PBG levels measured by LC-MS in a clinical context in which low values are relevant. The 95% range ALA concentrations from 1565 nonporphyric subjects covered 1.2 to 24 $\mu\text{mol}/\text{L}$ and 0.3 to 2.1 $\mu\text{mol}/\text{mmol}$ Cr (2.7 to 18.6 $\mu\text{mol}/\text{g}$ Cr) (Fig. 4A and B). For PBG, normal values were less than 1.4 $\mu\text{mol}/\text{L}$ and 0.2 $\mu\text{mol}/\text{mmol}$ Cr (1.8 $\mu\text{mol}/\text{g}$ Cr), without a detectable lower limit. In AIP AC ($n = 158$), ALA concentrations ranged from 1.68 to 451 $\mu\text{mol}/\text{L}$ with a median of 13.72 $\mu\text{mol}/\text{L}$, or 0.48 to 39.42 $\mu\text{mol}/\text{mmol}$ Cr with a median of 1.595. The distributions were similar in VP AC patients ($n = 60$), with ALA median of 16.6 $\mu\text{mol}/\text{L}$ [1.94–155.4] or 1.68 $\mu\text{mol}/\text{mmol}$ Cr [0.448–9.77]. In HCP patients ($n = 14$), the medians were lower than those in VP and AIP AC patients, with no significant difference from control individuals. In the 3 AHP, the median ALA concentrations of AC subjects were under the upper limit of normal (95%). Urine PBG levels were widely dispersed with a median and range of 5.90 $\mu\text{mol}/\text{L}$ [0–478.3] and 0.64 $\mu\text{mol}/\text{mmol}$ Cr [0–35.5] in AIP patients, 4.27 $\mu\text{mol}/\text{L}$ [0–151.3] and 0.36 $\mu\text{mol}/\text{mmol}$ Cr [0–21.4] in VP patients, and 0 $\mu\text{mol}/\text{L}$ [0–100.5] and 0 $\mu\text{mol}/\text{mmol}$ Cr [0–6.6] in HCP patients (Fig. 4A and B). In contrast to ALA, the median PBG concentrations were above the upper limit of normal, meaning that the majority of AC (65% of AIP, 68% for VP) exhibited high PBG concentrations. For comparison, 181 PBGs measured in AC by IEC during 2018 to 2019 showed that only 21% of the results were above the reference value. PBG levels in HCP carriers were lower, but significantly higher than those in controls (Fig. 4C and D).

ALA AND PBG PLASMA LEVELS: IMPACT OF CKD

Urine and plasma were collected simultaneously from AIP patients. The concentrations in the 2 fluids were

positively correlated with R at 0.78 and 0.70 for ALA and PBG, respectively (Fig. 5A and B). However, the presence of many outliers highlighted the limitations of these correlations. In Fig. 5C, the concentration ratio of ALA and PBG urine/plasma (U/P) is shown according to the stage of chronic kidney disease (CKD) using kidney disease improval global outcome (KDIGO) criteria (17). These results revealed a decrease in the U/P when the glomerular filtration rate drops. With normal renal function, the ratios were 10 and 8 for ALA and PBG, respectively. The decrease was significant from CKD stage 3, with a ratio of approximately 5. These ratios fell below 2 at CKD stages 4/5. Thus, the U/P could be used as a conversion factor (CF) to express a urine equivalent of a plasma concentration in normal renal conditions (glomerular renal function $>90 \text{ mL}/\text{min}/1.73 \text{ m}^2$) according to the formula $[\text{Urine}] = \text{CF} \times [\text{Plasma}]$ (Fig. 5D). These results demonstrate the underestimation of heme precursor levels in CKD porphyria patients when measured in urine.

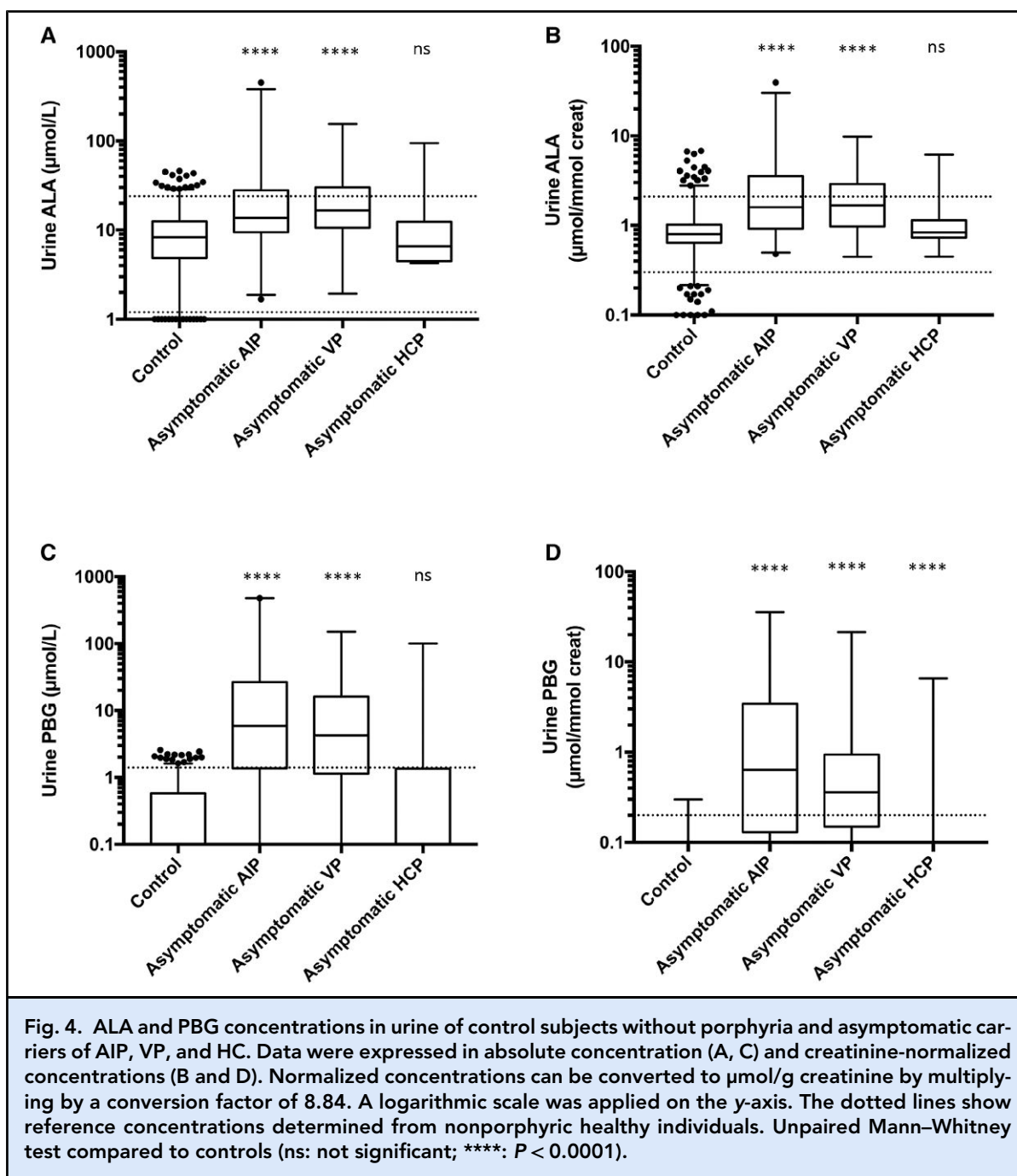
IN AIP PATIENTS, PLASMA LEVELS OF ALA AND PBG IN THE ASYMPTOMATIC PERIOD DEPEND ON THE SEVERITY OF THE DISEASE

In healthy individuals, the plasma concentration of ALA ranged from 0.05 $\mu\text{mol}/\text{L}$ to 0.14 $\mu\text{mol}/\text{L}$ with a median of 0.08 $\mu\text{mol}/\text{L}$. The reference range of 95% of the cohort was from 0.05 to 0.13 $\mu\text{mol}/\text{L}$. There was no detectable PBG in plasma in this control group (Fig. 6).

In patients, plasma ALA concentrations were significantly different between each AIP subgroup studied. The median ALA and PBG concentrations increased with the severity of AIP clinical presentation, with values of 0.38, 1.03, 2.34, and 4.14 $\mu\text{mol}/\text{L}$ (ALA) and 0.80, 2.42, 5.19 and 6.87 $\mu\text{mol}/\text{L}$ (PBG) for AC, mild patients, severe patients, and during an acute attack, respectively (Fig. 6). Unlike ALA, the difference in PBG concentrations between groups was not consistently significant, notably between AC and control on the one hand and severe patients and acute attack on the other hand. Furthermore, we noted that only 61% of AC had PBG concentrations above the upper limit of the normal range.

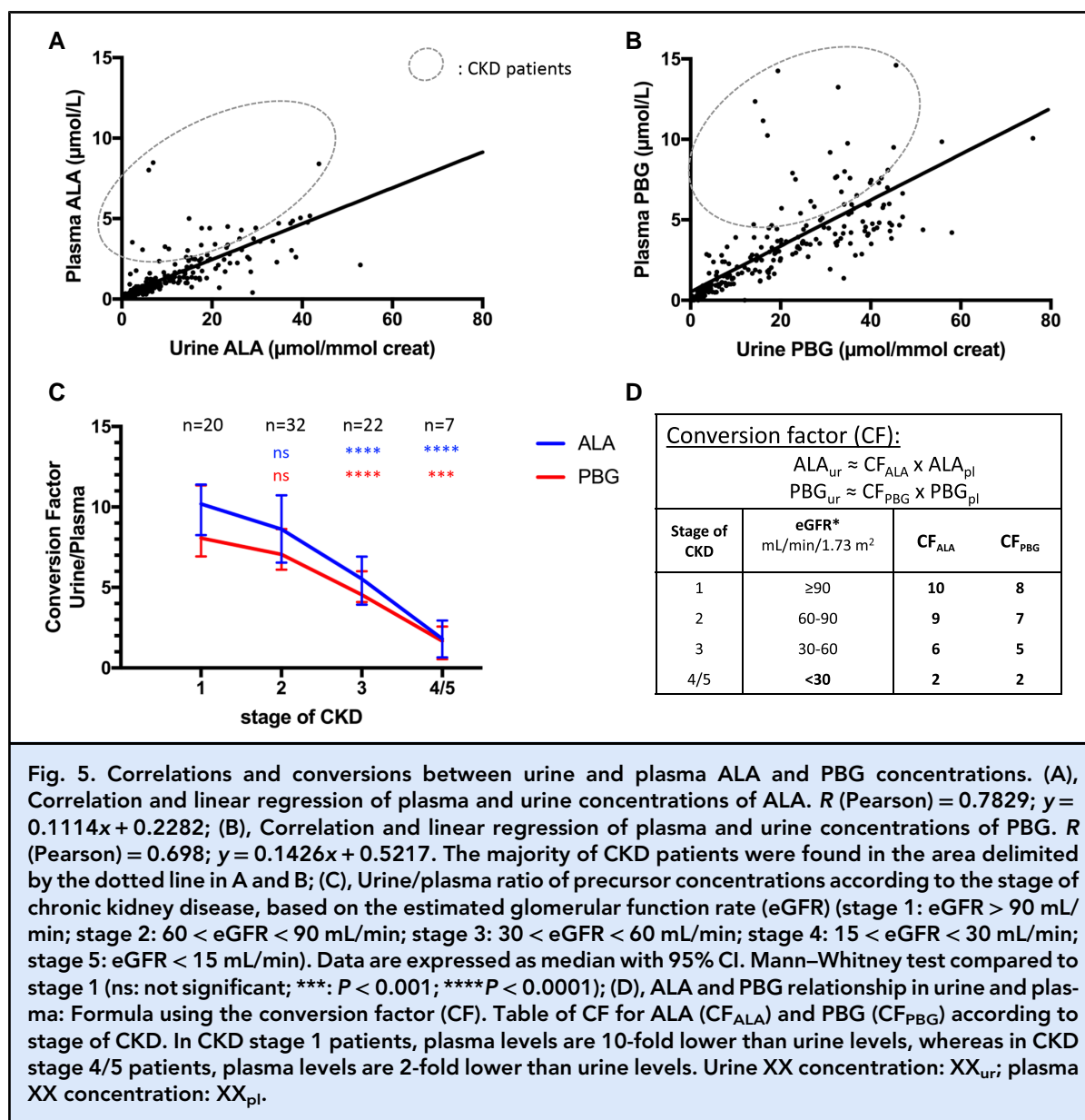
Discussion

Here, we present a rapid and semi-automated method to measure ALA and PBG to meet the daily routine analytical needs of a clinical laboratory. Previously described LC-MS assays highlighted sensitivity and ability to be performed on plasma, but routine clinical laboratory considerations were not taken into account. For



instance, adding a solid-phase extraction step prior to analysis greatly improves sensitivity but makes sample preparation more complex and not suitable for emergency hospital use (12, 18). Hydrophilic interaction liquid chromatography (HILIC) has also been promoted, allowing for the retention of highly polar ALA (13). During our method development, the trade-off between ease of handling and analytical performance led us

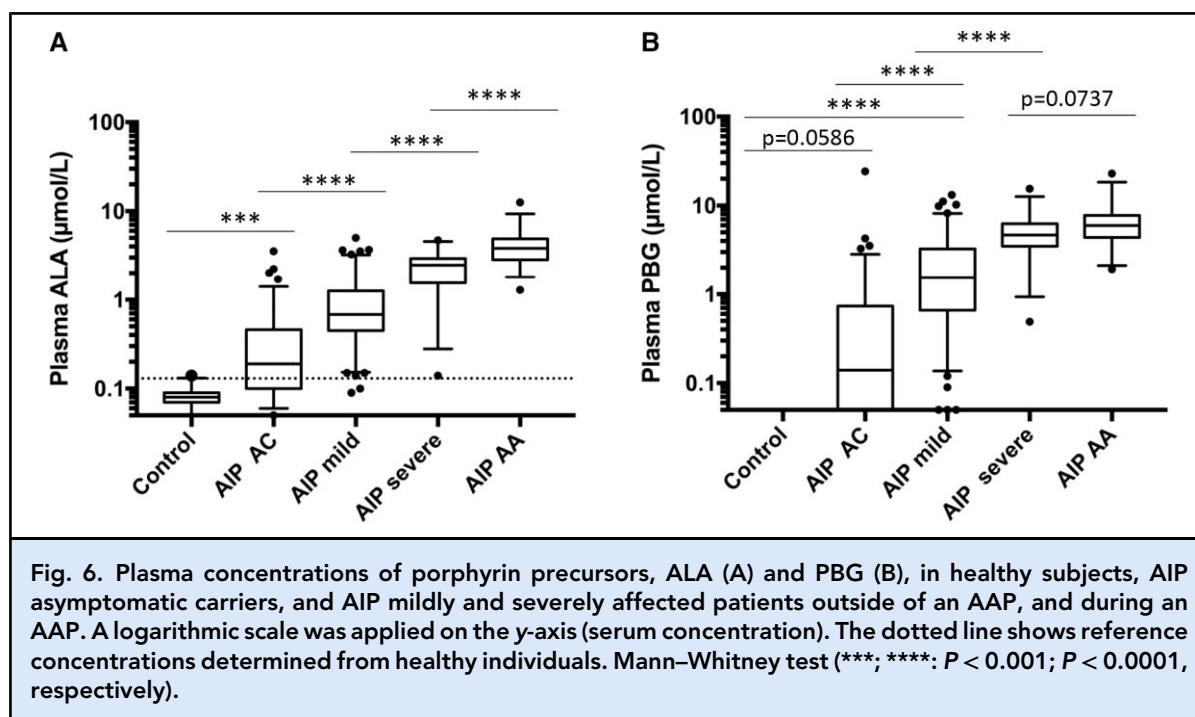
to optimize sample preparation using a protein precipitation step followed by fast but robust derivatization. Thus, ALA can be retained on a conventional reversed-phase separation while maintaining a high level of sensitivity by increasing ionization efficiency. This allowed for the elimination of a time-consuming extraction or classical derivatization (e.g., butylation), including a drying and incubation step. In addition, the coupling



of the UPLC and the easy-to-use MS detector increased the speed of analysis, automation, and connection to the laboratory information management system (LIMS). Finally, this method can be combined with amino acid profiles for which the derivatization process was initially developed (19, 20). In previously published methods, a better sensitivity has been described in urine (e.g., LLOQ = 0.044 $\mu\text{mol/L}$ in Agarwal et al.), but the linearity range of our method was suitable for use in clinical practice, both in urine and plasma samples (18).

Comparison with the historical IEC method showed a strong correlation at clinically relevant concentrations

but displayed discordance at low normal concentrations. This could be explained by the lack of specificity in the Mauzerall and Granick method (9). The switch in method had no impact on the diagnosis and follow-up of patients because concentration differences were negligible. However, this divergence in the lower levels shifted the lower reference interval downwards. Reference concentrations were mainly determined from nonporphyric patients. This reference cohort may appear to be different from healthy volunteers used in other studies. However, in the other published methods, the urine ALA concentrations ranged from 0.152 to 2.30 (18), 0.09 to 3.0 (14), and below 2.6 $\mu\text{mol/mmol Cr}$ (13), and the PBG



concentrations ranged from 0.00234 to 0.185 (18), below 1.1 (14), or below 0.5 $\mu\text{mol}/\text{mmol Cr}$ (13), therefore, very close to our data (ALA: 0.3 to 2.1 $\mu\text{mol}/\text{mmol Cr}$; PBG < 0.2 $\mu\text{mol}/\text{mmol Cr}$).

ALA and PBG are well-recognized biomarkers of an acute attack of porphyria, but we show that this new method allows the detection of 3 times more asymptomatic AIP and VP subjects compared to IEC (65% and 68% above the reference threshold vs 21%, see Results section). In HCP, AC have low ALA and PBG concentrations and cannot be reliably detected in the general population. Agarwal et al. assessed the ability of the upper limit of normal (ULN) to differentiate healthy individuals from AIP patients (18). However, the tested cohort was only composed of asymptomatic chronic high excretors and severe patients. The full spectrum of *HMBS* mutation carriers were not fully represented. Thus, with our method the sensitivity to screen AC is not limited by the analytical sensitivity but by the biological phenotypes of AHP.

The method described here provides the ability to easily measure the concentrations of ALA and PBG in plasma, which provides 2 main advantages. First, blood is easier and faster to collect than urine for emergency screening of AAP. Second, as CKD is a common feature in AHP, measurement in plasma samples allows for screening and/or follow-up in anuric or oligoanuric patients (10). Other studies based on LC-MS have reported the measurement of ALA/

PBG in plasma but only on a small number of patients (11, 21, 22). For the first time, data from a large cohort (300 specimens), including healthy donors, ACs, and patients, during or outside of an AAP, were collected and compared. Plasma ALA and PBG concentrations do not show the same distribution between subgroups. Significant differences in ALA levels between each phenotypic subgroup might reflect different levels of *ALAS1* activation. PBG concentrations during an acute episode were less discriminating, especially between severe patients during or outside of an attack.

Urine was historically used for porphyrin measurement because the IEC method was limited to a noncomplex biological matrix. However, urine can be collected by the patient themselves and directly sent to the laboratory. As porphyria reference laboratories are quite scarce, this advantage makes urine testing a key tool in the diagnosis and monitoring of acute hepatic porphyria. Urine and plasma concentrations are relatively well correlated for both ALA and PBG except in cases of renal failure. A decrease in estimated glomerular filtration rate (eGFR) alters the excretion of ALA and PBG. We showed that the U/P (CF) ratio is influenced by the stage of CKD. In patients with normal renal function, ALA is 10 times and PBG 8 times less concentrated in plasma than in urine. When the CKD reached stage 3 (eGFR < 60 mL/min), the CF was halved and divided 5-fold at CKD stage 4/5. For the first time, we demonstrate an underestimation of heme precursor levels in CKD

AIP patients when using urine measurement. To assess the exposure of the nervous system to ALA and PBG toxicity, the metabolite levels in blood may be more relevant than those in urine, especially in CKD patients. The prevalence of CKD, defined as eGFR < 60 mL/min per 1.73 m², in AIP patients was estimated to be 59% compared to 11% in AC patients (23). Thus, the measurement of circulating ALA and PBG is underestimated in more than half of AIP patients. In addition, during AAP, the occurrence of acute renal failure caused by ALA and PBG toxicity has been suggested (10). Therefore, screening for AAP could be impacted by underestimated precursor exposure. In the case of CKD complicated by acute renal failure during AAP, the usual threshold in urine defining an acute attack would become completely unreliable, and the efficacy of hemin treatment would be more difficult to assess. As serum sodium levels must be monitored in AAP, measurements of ALA and PBG concentrations can be added easily to the same blood sample (4).

Finally, the availability of a new treatment, Givosiran, based on an interfering RNA approach, generates a new requirement for plasma monitoring in the follow-up of treated AHP patients (24). Several patients receiving Givosiran treatment presented with side effects undetected during the phase 3 study (25). These findings led to spacing of the injection based on the individual evaluation of heme precursor concentrations. Plasma monitoring would be more appropriate in those patients treated with Givosiran who frequently have impaired renal function (26). These plasma assays hence contribute to the development of a personalized medicine approach.

The diagnosis and follow-up of AHP can benefit from the spread of mass spectrometry observed in clinical laboratories over the last 20 years. This easy-to-handle and fast LC-MS/MS method, requiring a small sample volume, meets the needs of ALA and PBG assays, which must be performed routinely and often urgently. This method is compatible with amino acid profiles performed on many metabolomic platforms. In addition, mass spectrometry enables plasma testing, allowing a single sample to be used for multiple tests, as well as quantifying ALA and PBG in CKD and oligoanuric patients. The specific ALA and PBG measurements in plasma can be used as a complementary tool in the follow-up and personalized monitoring of severe patients for whom new therapies, such as Givosiran, are indicated.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ALA, delta-aminolevulinic acid; PBG, porphobilinogen; AHP, acute hepatic porphyrias; IEC, ion-exchange chromatography; AIP, acute intermittent porphyria; VP, variegate porphyria; HCP, hereditary coproporphyria; AAP, acute attack of porphyria; AC, asymptomatic carriers; Cr, creatinine; U/P, urine/plasma; CKD, chronic kidney disease; CF, conversion factor.

Human Gene: *HMB*S, hydroxymethylbilane synthase.

Author Contributions: *The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.*

Antoine Poli (Data curation-Lead, Formal analysis-Equal, Investigation-Equal, Validation-Equal, Writing—original draft-Equal, Writing—review & editing-Equal), Hana Manceau (Data curation-Equal, Formal analysis-Equal, Investigation-Equal, Writing—review & editing-Equal), Anvi Laetitia Nguyen (Formal analysis-Equal, Methodology-Equal, Writing—review & editing-Equal), Boualem Moulouel (Data curation-Supporting, Formal analysis-Equal, Investigation-Equal, Writing—review & editing-Supporting), Nathalie Dessendier (Data curation-Equal, Formal analysis-Equal), Neila Talbi (Data curation-Equal, Investigation-Equal, Writing—review & editing-Supporting), Hervé Puy (Conceptualization-Supporting, Formal analysis-Equal, Funding acquisition-Equal, Project administration-Equal, Supervision-Supporting, Writing—original draft-Equal, Writing—review & editing-Equal), Christophe Junot (Funding acquisition-Supporting, Methodology-Supporting, Writing—review & editing-Equal), Laurent Gouya (Conceptualization-Equal, Funding acquisition-Equal, Methodology-Equal, Project administration-Equal, Writing—review & editing-Equal), Caroline Schmitt (Conceptualization-Equal, Data curation-Equal, Formal analysis-Equal, Methodology-Equal, Project administration-Equal, Validation-Equal, Writing—original draft-Equal, Writing—review & editing-Equal), and Thibaud Lefebvre (Conceptualization-Lead, Data curation-Equal, Formal analysis-Lead, Investigation-Equal, Methodology-Equal, Project administration-Lead, Supervision-Lead, Validation-Equal, Writing—original draft-Equal).

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form.*

Research Funding: INSERM and Paris Diderot University, the Laboratory of Excellence, GR-Ex, Paris, France, supported this work. The labex GR-Ex, reference ANR-11-LABX-0051 is funded by the program "Investissements d'avenir" of the French National Research Agency, reference ANR-11-IDEX-0005-02.

Disclosures: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: We would like to thank the HIROS INSERM Unit for methodological assistance and the CFP staff for their excellent

technical work.

References

1. Puy H, Gouya L, Deybach J-C. Porphyrias. *Lancet* 2010;375:924–37.
2. Bissell DM, Anderson KE, Bonkovsky HL. Porphyria. *N Engl J Med* 2017;377: 862–72.
3. Anderson KE, Desnick RJ, Stewart MF, Ventura P, Bonkovsky HL. Acute hepatic porphyrias: “Purple Flags”-clinical features that should prompt specific diagnostic testing. *Am J Med Sci* 2022;363: 1–10.
4. Schulenburg-Brand D, Stewart F, Stein P, Rees D, Badminton M. Update on the diagnosis and management of the autosomal dominant acute hepatic porphyrias. *J Clin Pathol* 2022;75: 537–43.
5. Anderson KE, Lobo R, Salazar D, Schloetter M, Spitzer G, White AL, et al. Biochemical diagnosis of acute hepatic porphyria: updated expert recommendations for primary care physicians. *Am J Med Sci* 2021;362: 113–21.
6. Bissell DM, Lai JC, Meister RK, Blanc PD. Role of delta-aminolevulinic acid in the symptoms of acute porphyria. *Am J Med* 2015;128:313–7.
7. Bonkovsky HL, Dixon N, Rudnick S. Pathogenesis and clinical features of the acute hepatic porphyrias (AHPs). *Mol Genet Metab* 2019;128:213–8.
8. Lefever S, Peersman N, Meersseman W, Cassiman D, Vermeersch P. Development and validation of diagnostic algorithms for the laboratory diagnosis of porphyrias. *J Inher Metab Dis* 2022;45:1151–62.
9. Mauzerall D, Granick S. The occurrence and determination of delta-amino-levulinic acid and porphobilinogen in urine. *J Biol Chem* 1956;219:435–46.
10. Pallet N, Karras A, Thervet E, Gouya L, Karim Z, Puy H. Porphyria and kidney diseases. *Clin Kidney J* 2018;11:191–7.
11. Floderus Y, Sardh E, Möller C, Andersson C, Rejkjaer L, Andersson DEH, et al. Variations in porphobilinogen and 5-aminolevulinic acid concentrations in plasma and urine from asymptomatic carriers of the acute intermittent porphyria gene with increased porphyrin precursor excretion. *Clin Chem* 2006;52:701–7.
12. Ford RE, Magera MJ, Kloke KM, Chezick PA, Fauq A, McConnell JP. Quantitative measurement of porphobilinogen in urine by stable-isotope dilution liquid chromatography-tandem mass spectrometry. *Clin Chem* 2001;47:1627–32.
13. Benton CM, Couchman L, Marsden JT, Rees DC, Moniz C, Lim CK. Direct and simultaneous determination of 5-aminolaevulinic acid and porphobilinogen in urine by hydrophilic interaction liquid chromatography-electrospray ionisation/tandem mass spectrometry. *Biomed Chromatogr* 2012;26:1033–40.
14. Zhang J, Yasuda M, Desnick RJ, Balwani M, Bishop D, Yu C. A LC-MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen. *J Chromatogr B Analyt Technol Biomed Life Sci* 2011;879: 2389–96.
15. Di Pierro E, De Canio M, Mercadante R, Savino M, Granata F, Tavazzi D, et al. Laboratory diagnosis of porphyria. *Diagnostics (Basel)* 2021;11:1343.
16. Woolf J, Marsden JT, Degg T, Whatley S, Reed P, Brazil N, et al. Best practice guidelines on first-line laboratory testing for porphyria. *Ann Clin Biochem* 2017;54:188–98.
17. Levey AS, Eckardt K-U, Tsukamoto Y, Levin A, Coresh J, Rossert J, et al. Definition and classification of chronic kidney disease: a position statement from kidney disease: improving global outcomes (KDIGO). *Kidney Int* 2005;67:2089–100.
18. Agarwal S, Habtemariam B, Xu Y, Simon AR, Kim JB, Robbie GJ. Normal reference ranges for urinary δ -aminolevulinic acid and porphobilinogen levels. *JIMD Rep* 2021;57:85–93.
19. Bruno C, Veyrat-Durebex C, Lumbu Lukuntunda CH, Andres CR, Moreau C, Bendavid C, et al. Validation of plasma amino acid profile using UHPLC-mass spectrometer (QDa) as a screening method in a metabolic disorders reference centre: performance and accreditation concerns. *Clin Biochem* 2021;92:34–45.
20. Narayan SB, Ditewig-Meyers G, Graham KS, Scott R, Bennett MJ. Measurement of plasma amino acids by Ultraperformance® Liquid Chromatography. *Clin Chem Lab Med* 2011;49:1177–85.
21. Benton CM, Couchman L, Marsden JT, Rees DC, Moniz C, Lim CK. Direct and simultaneous quantitation of 5-aminolaevulinic acid and porphobilinogen in human serum or plasma by hydrophilic interaction liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry. *Biomed Chromatogr* 2013; 27:267–72.
22. Sardh E, Harper P, Andersson DEH, Floderus Y. Plasma porphobilinogen as a sensitive biomarker to monitor the clinical and therapeutic course of acute intermittent porphyria attacks. *Eur J Intern Med* 2009;20:201–7.
23. Pallet N, Mami I, Schmitt C, Karim Z, François A, Rabant M, et al. High prevalence of and potential mechanisms for chronic kidney disease in patients with acute intermittent porphyria. *Kidney Int* 2015;88:386–95.
24. Balwani M, Sardh E, Ventura P, Peiró PA, Rees DC, Stölzel U, et al. Phase 3 trial of RNAi therapeutic givosiran for acute intermittent porphyria. *N Engl J Med* 2020;382: 2289–301.
25. Poli A, Schmitt C, Moulouel B, Mirmiran A, Talbi N, Rivière S, et al. Givosiran in acute intermittent porphyria: a personalized medicine approach. *Mol Genet Metab* 2022;135:206–14.
26. Lazareth H, Poli A, Bignon Y, Mirmiran A, Rabant M, Cohen R, et al. Renal function decline with small interfering RNA silencing aminolevulinic acid synthase 1 (ALAS1). *Kidney Int Rep* 2021;6: 1904–11.