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URINARY METABOLIC SIGNATURE OF PRIMARY ALDOSTERONISM: GENDER AND SUBTYPE-SPECIFIC ALTERATIONS

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List of abbreviations: PA, primary aldosteronism; BAH, bilateral adrenal hyperplasia, APA, aldosterone-producing adenoma; ARR, aldosterone-to-renin ratio; EH, essential hypertension; CT, computed tomography; AVS, adrenal venous sampling; SLT, salt-loading test; PLS-DA, partial least squares discriminant analysis.

Keywords: hypertension, urinary metabolomics, primary aldosteronism, aldosterone-to-renin ratio (ARR), aldosterone-producing adenoma (APA).

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STATEMENT OF CLINICAL RELEVANCE

Metabolomics is quite a novel approach in the field of hypertension research. We analysed 50 subjects with primary aldosteronism (PA), and 36 patients with essential hypertension (EH) using UHPLC MS. We processed two sample sets, one for biomarker identification and a second one for independent verification. The results demonstrated a strong discrimination between bilateral adrenal hyperplasia (BAH) individuals and all the other tested groups (up to 60-fold against aldosterone-producing adenoma (APA) patients), and striking differences based on gender were noted. Independent verification of preliminary results in a second sample set confirmed that some metabolites (e.g., dAMP, diiodothyronine and 5-methoxytryptophan) deserve further investigation to validate their potential as biomarkers of the BAH subtype. Overall, our results highlight the relevance of state-of-the-art mass spectrometry-based metabolomics technologies in the field of hypertension research. We used these technologies to identify, for the first time, low molecular weight molecular markers of PA and paved the way for follow-up validation studies in larger cohorts.

ABSTRACT

Purpose: The current clinical investigation for primary aldosteronism (PA) diagnosis requires complex expensive tests from the initial suspicion to the final subtype classification, including invasive approaches; therefore, appropriate markers for subtype definition are greatly desirable. The present study performed a metabolomics analysis to further examine specific molecular signatures of PA urines. **Experimental design:** The study considered PA subtype and gender-related differences using two orthogonal advanced UHPLC-MS metabolomics approaches. We investigated patients with essential hypertension (n=36) and PA (n=50) who were referred to the outpatient hypertension clinic and included matched healthy subjects (n=10).

Results: We identified statistically significant changes ($p < 0.05$ ANOVA, $F_c > 1.5$) of metabolites involved in central carbon, energy and nitrogen metabolism, especially purine and pyrimidine nucleosides and precursors, and free amino acids. PLS-DA interpretation provided strong evidence of a disease-specific metabolic pattern with dAMP, diiodothyronine and 5-methoxytryptophan as leading factors, and a sex-specific metabolic pattern associated with orotidine 5-phosphate, N-acetylalanine, hydroxyproline and cysteine. The results were verified using an independent sample set, which confirmed the identification of specific signatures. **Conclusions and clinical relevance:** Metabolomics was used to identify low molecular weight molecular markers of PA, which paves the way for follow-up validation studies in larger cohorts.

INTRODUCTION

Primary aldosteronism (PA) is characterized by inappropriately high aldosterone secretion, and it is the most common secondary form of arterial hypertension (5-15% of the hypertensive subjects) ^[1]. Cardiovascular morbidity and mortality of PA patients is higher than age- and sex-matched patients with essential hypertension (EH) and equivalent blood pressure values ^[1-3].

Adrenal aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH) are common causes of PA ^[1]. Current guidelines for PA require a complex diagnostic work-up of an initial screening test based on the measurement of aldosterone-to-renin ratio (ARR) and, in case of a positive screening test, diagnosis confirmation using a test that documents the autonomous hormone secretion (e.g., intravenous salt-loading test, fludrocortisone suppression test, or captopril challenge test). Finally, all patients with PA should be further classified for subtype (APA or BAH) using adrenal computed tomography (CT) scanning and adrenal venous sampling (AVS) ^[1]. This approach is very important for a tailored therapy of monolateral adrenalectomy in cases of APA or pharmacological treatment in cases of BAH ^[1].

One problem of this diagnostic work-up is the low accuracy of the screening test, which exhibits possible false positives and false negatives ^[1, 4]. AVS is the gold standard for subtype classification, but it is an invasive procedure that is not practicable in settings other than specialist units, and it is

highly dependent on the operator's skills ^[1, 5]. Therefore, the identification of an easy, non-invasive, practical and economical method for subtype classification is highly desirable.

Metabolomics recently emerged as a powerful tool for investigating disease complexity because it provides a particular view of metabolites (as final products of cellular processes) of specific phenotypes and the phenotypic changes associated with environmental factors (e.g., diet, treatments, exercise, etc.). However, metabolites differ greatly in concentration, size and polarity, which makes their detection, quantification and identification technically challenging ^[6]. Urine has gained growing importance in metabolomic analyses, especially clinical practice, because of its non-invasive and easy collection ^[7]. Another advantage of working with urine is that the concentrations of metabolites are often higher than plasma. However, urinary metabolomics remains an emerging approach in the field of hypertension research, and a limited number of human studies are available ^[8].

Our previous studies demonstrated sex-related differences in some proteins related to APA and BAH ^[9], and the present study performed a metabolomics analysis to further identify the specific molecular signatures of PA urine. The study used an advanced UHPLC-MS metabolomics workflow ^[10] to analyse the metabolic profiles of control normotensive subjects, patients with EH, and PA individuals (APA or BAH). The potential biomarkers identified using these untargeted platforms were further verified using an orthogonal semi-targeted approach, and the results pave the way for future validation in larger cohorts using targeted approaches in the near future.

MATERIALS AND METHODS

Selection of subjects

The study was performed according to the principles of the Declaration of Helsinki. Each healthy individual or patient provided written informed consent. The Institutional Review Board Ethical Committee approved the protocol. Second morning urine samples and a blood sample for biochemical routine parameter analysis were collected from each subject. All analysed subjects were in fasting conditions at the time of sampling. Smoking habits were reported.

Subjects in the experimental groups analysed in this study were carefully selected to avoid confounding factors, and all of the procedures for sample handling and storage were highly standardized.

1) Normotensive healthy subjects

Ten normotensive healthy volunteers, five women and five men, were selected for the metabolomic study from healthy volunteers participating in a phase 2 trial study at the Centre for Clinical Research of the Azienda Ospedaliera-Universitaria of Verona, Italy. The following inclusion criteria were used: no history of past, recent or concomitant significant illness; regular menstrual cycles; no drug intake within 4 weeks prior to the examination; no personal or family history of hypertension; and at least two confirmed normal blood pressure (BP) values during the first visit.

2) Hypertensive subjects

Patients were recruited from two different hypertension units (Verona and Turin, Italy). Protocols for sample collection, preparation and storage were the same for both centres.

Patients took no antihypertensive drugs, other than verapamil and/or alpha-blockers, in the previous 4 weeks. Hypokalemia was corrected when present. Hypertensive women receiving oral contraceptive therapy were excluded.

The criteria for PA diagnosis were described previously ^[9, 11]. Briefly, orthostatic ARR higher than the reference value established for each laboratory (32 pg/ml for ratio of aldosterone, expressed as pg/ml, to direct active renin, expressed as pg/ml ^[12]; 40 for ratio of aldosterone expressed as ng/dl to renin activity expressed as ng/ml•h in Turin ^[11]) and positive iv SLT. The test was considered positive when PAC levels were higher than 50 pg/ml at the end of the test ^[11].

The classification for subtypes for PA diagnoses (i.e., BAH or APA) was based on adrenal CT and AVS ^[13]. Other hypertensive subjects, diagnosed as EH, were included in the study, and exhibited orthostatic ARR lower than the reference value for PA screening (low ARR) or an ARR higher than

the reference value but a negative iv SLT (high ARR). These subjects represent a group of patients with hypertension without PA.

We first analysed a sample group composed of 10 BAH and 10 APA subjects, in equal numbers of males and females, and 12 EH subjects (six high ARR and six low ARR) for the metabolomics study. We added an additional group of patients to the study as a verification set, which was composed of PA (n=19; F=8, M=11) and EH (n=7, F= 3, M=4) subjects. We also analysed another group of samples at the Metabolomics Facility of the University of Colorado, Denver using a different high-resolution MS platform for an independent validation of the results.

This new group of samples was subdivided into a training set and test set, and it was composed of the previous cohort and newly enrolled subjects. The training set included, 10 normotensive subjects, 12 APA (F=6, M=6), 12 BAH (F=5, M=7), 10 low ARR (F=5, M=5) and 9 high ARR (F=4, M=5) subjects. The test set was composed of 20 PA patients (APA=13, BAH=7), including PA of undetermined subtype (n=6) and EH subjects (n=17).

Biochemical assay

The Laboratory of the Verona and Turin University Clinical Chemistry Institutes performed measurements of biochemical and hormonal parameters as previously described ^[10, 11].

Metabolomics

Minimally processed urine samples were used for the metabolomics analysis. People who materially performed the assays were unaware of the previous clinical diagnosis of each patient. Samples were stored at -80°C until thawing. Samples were centrifuged at 13500 g at 4°C for 15 minutes to discard the debris. A volume of 0.5 ml of the centrifuged urines was added to 0.5 ml of a -20°C cold methanol/acetonitrile/bidistilled water solution (5/3/2 vol/vol/vol) and subjected to a further centrifugation using the previous conditions. The supernatants were used for the analysis. Samples were loaded onto a rapid resolution HPLC system (LC Packings, DIONEX, CA, USA) for

chromatographic separation of hydrophilic metabolites. The system featured a binary pump and vacuum degasser, well-plate autosampler with a six-port micro-switching valve and a thermostated column compartment. A Phenomenex Luna 3- μ m HILIC 200A (150x2.0 mm) column, protected by a guard column HILIC 4x2.0 mm ID (Phenomenex, CA, USA) was used to perform metabolite separation over a phase B (100% acetonitrile, 10 mM ammonium acetate) to phase A (double distilled 18 m Ω water, 10 mM ammonium acetate) gradient for 35 minutes. Urinary creatinine was obtained for each sample, and samples of less concentrated urine were normalized using a simple preacquisition strategy based on differential injection volumes calibrated by creatinine. This normalization method is more effective compared to other methods^[14]. We set the following LC parameters: column temperature of 25°C and flow rate of 0.3 ml/min. The following multistep gradient was set: 0-5 minutes 100% phase B; 5-15 minutes from 100% phase B to 70% phase B; 15-20 minutes from 70% phase B to 50% phase B; 20-25 minutes from 50% phase B to 0% phase B; 25-30 minutes isocratic at 0% phase B; 30-30.1 minutes return to the initial conditions 100% phase B; and 30.1-35 minutes isocratic column equilibration at 100% phase B.

The eluate from the HPLC system was linked online with a Micro Q-TOF II (Bruker Daltonics, Bremen, Germany) mass spectrometer equipped with an ESI ion source. Instrument calibration was performed externally using a sodium formate solution consisting of 10 mM sodium hydroxide in 50% isopropanol/water (v/v) and 0.1% formic acid. External mass scale calibration was performed twice daily via direct automated injection of the calibration solution from a 6-port divert-valve. Mass spectrometer runs were exported into mzXML files and analysed using the software MAVEN^[15] for correct metabolite assignment, on the basis of absolute intact mass (within a 10 ppm window) against the KEGG database,^[16] and expected retention times on the basis of metabolite chemical properties and in-house available standards (SIGMA Aldrich, St. Louis, MO). We used an automatic research method for each couple of compared groups and allowed the software MAVEN to construct a list of the metabolites that exhibited differential relative abundance (setting the fold-change cut-off >1.5) and statistical significance ($p < 0.05$; FDR < 0.1). Heat maps were generated using the software GENE E (Broad Institute, MA, USA).

Validation analyses

Independent, orthogonal validation analyses were performed at the University of Colorado Denver – Anschutz Medical Campus. Briefly, the analytical platform used a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Samples were resolved over an ACQUITY HSS T3 column (2.1 x 150 mm, 1.8 μ m particle size (Waters, MA, USA) using a gradient from 0- 5% B over 0.5 minutes, 5-95% B over 0.6 minutes, held at 95% B for 1.65 minutes, 95-5% B over 0.25 minutes, held at 5% B for 2 minutes, and flowing at 450 μ l/min at 35°C. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated independently in positive or negative ion mode, and scanning was performed in Full MS mode (2 μ scans) from 150 to 1500 m/z at 70,000 resolution, with 4-kV spray voltage, 45 sheath gas, and 15 auxiliary gas. Automatic dd-MS2 was performed at 17,500 resolution, AGC target = 1e5, maximum IT = 50 ms, and stepped NCE of 25 or 35 for positive mode, and 20, 24, and 28 for negative mode. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data were converted from raw to mzXML file format using Mass Matrix (Cleveland, OH, USA). Samples were analysed in a randomized order with a technical mixture injected incrementally to qualify instrument performance. This technical mixture was also injected three times per polarity mode and analysed using the parameters described above, except CID fragmentation was included for unknown compound identification. Metabolite assignments, isotopologue distributions, and corrections for expected natural abundances of deuterium, ^{13}C , and ^{15}N isotopes were performed using MAVEN (Princeton, NJ, USA), against an in-house library of ~1,000 standards. Discovery mode analysis was performed with standard workflows using Compound Discoverer (Thermo Fisher Scientific, San Jose, CA).

Statistical analysis

The LC/MS data were exported into Microsoft Excel. The resulting data were processed using partial least squares discriminant analysis (PLS-DA) in Multibase software (Numerical Dynamics) and

Metaboanalyst 3.0^[17]. PLS-DA was used to identify differences between the experimental groups. The loading plots, where each metabolite (loading variable) is evidenced by a point, were used to identify the metabolites that were responsible for the separation of the samples on the score plots via sorting metabolites on the basis of loading weights on PC1, PC2 and PC3.

Statistical analysis was performed using SPSS for Windows software (Version 21.0; SPSS, IBM). Independent samples t-test or ANOVA were used to analyse differences between metabolites groups, and a p-value < 0.05 was considered statistically significant. The use of a statistical method was necessary in the search for a key to interpret the large quantity of data from the metabolomic untargeted analysis. Our research involved five different groups of subjects, and the number of possible combinations for couple-comparative studies was rather large. We chose PLS-DA because it relates the data matrix (e.g., multivariate metabolite data) to the response vector (containing the sample class affiliations, e.g., case-control) using a linear regression model. Principal component analysis (PCA), on which PLS-DA is based, reduces the multidimensionality of data sets with a large number of variables (variable=metabolite) in a lower-dimensional space, where the first principal components explain as much of the existing covariance as possible.

Random forest clustering, heat maps, and determinations of VIP scores were calculated and plotted using the software Metaboanalyst 3.0^[17].

Comparisons between the clinical and biochemical characteristics of patients' groups were performed using 2-tailed unpaired Student's t test. Values are expressed as the means $\pm$ standard deviation, and logarithmic transformation was performed on skewed variables to normalize their distribution, which are presented as geometric means (antilogarithms of the transformed means) with estimations of 95% confidence intervals (CIs). Comparisons of proportions were performed using cross-tabulation, Pearson's chi-square and Fisher's exact tests.

RESULTS

Our strategy and experimental workflow involved the use of second morning urine, minimal sample processing to preserve the “original” metabolites, chromatographic separation coupled with mass spectrometry and statistical analysis. The hypertensive population (PA and EH) was further subdivided into BAH and APA for the PA group, and high ARR or low ARR, for the EH group.

Supplemental Tables 1 and 2 report the clinical and biochemical characteristics of the entire population of PA and EH patients and APA and BAH. No significant differences were observed between groups, with the exception of higher aldosterone and lower K^+ values in PA versus EH patients, as expected.

The metabolomics analysis created a list of metabolites that were differentially represented between groups (See Supplemental Table 3 for the list of all metabolites). We identified a panel of highly statistically significant ($p < 0.05$ ANOVA, $F_c > 1.5$ up to 60-fold) changes of metabolites involved in central carbon, energy and nitrogen metabolism. We also performed comparisons based on gender and the specific hypertensive subgroup classification (normal female, normal male, APA female, APA male, BAH female, BAH male, high ARR female, high ARR male, low ARR female, and low ARR male). PLS-DA was performed to determine whether metabolic phenotypes could be used to discriminate between sub-groups (Figure 1A). Notably, principal component 1 (PC1, explaining $>10\%$ of the total variance) discriminated BAH samples from all other groups. PC2 exhibited a signature associated with gender (female vs. male) and hypertension (in the presence or absence of APA and BAH, EH with low ARR and high ARR, of PA traits –Figure 1A). PLS-DA clustering was validated using random permutation, random forest clustering and hierarchical clustering (Figure 1A-E), and all of the algorithms confirmed a distinct signature associated with PA subjects, especially in the female groups. Variables (metabolites) were sorted by fold-change and p values (ANOVA) across groups to determine significant features (Significant Analysis of Microarrays (SAM) plot; Figure 1C) and VIP scores (Figure 1D). The results indicated that pyrimidine nucleoside and precursors (dUMP, orotidine phosphate, thiamine phosphate, UMP, UDP, uracil), purine nucleotides and catabolites (adenosine, uric acid) and free amino acids (histidine, cysteine, N-acetyl-lysine, N-acetyl-glutamine)

were the metabolites with the highest discriminating power among the classes of samples (Figure 1D). Heat map representation of the top metabolic signatures in normal, EH and PA urines revealed further components of this response, such as acetyl-alanine, taurine and hydroxyproline, which is a marker of extracellular matrix remodelling and collagen turn-over ^[18] (Figure 1E). Box and whisker plots for metabolites exhibiting the highest discrimination power (ANOVA) across samples are presented in Figure 2. The subgroups of EH subjects (low ARR and high ARR) always mapped together in the same quadrant in the PLS-DA analyses (Figure 1A), which indicates that no metabolic markers that could significantly discriminate between these subgroups were identified, at least in this preliminary metabolomic characterization.

The comparison of total normal subjects (males and females) with the PA subjects demonstrated that glycolytic intermediates 2/3-phosphoglycerate and dihydroxyacetone phosphate and pyrimidine UMP, both in the ribose and deoxyribose (dUMP) sugar nucleoside form, exhibited the highest discriminatory potential between normal and PA urines (Figure 3A).

A subset of markers reliably discriminated between urines from EH and PA patients (Figure 3B), as extensively detailed in Table 1. Markers included purine nucleosides and related catabolites, deoxyadenosine and uric acid (Figure 3B). Similarly, the adenosine deamination catabolites deoxyinosine, hypoxanthine and IMP, and free amino acids histidine and taurine and the pyrimidine diphosphate CDP exhibited high discriminatory power when comparing APA vs. BAH groups in a gender-independent manner (Figure 3C). The p values of these latter metabolites are also available in Supplementary Table 5. Independent verification of these observations was pursued at the University of Colorado Denver on the same samples and identified similar metabolic specificities across urine samples for sulfurs (methionine, methionine sulfoxide, sulfinoalanine), purines (adenosine, guanosine, guanine, xanthine, hydroxyisourate), and pyrimidines (dihydrouracil, uracil), which confirmed the observations from our preliminary analyses (**Supplementary Figure 1– Training cohort**).

Therefore, we performed a validation study based on a second sample set using targeted relative quantitation of metabolites with the highest loading weights in the PLS-DA analysis (Figure 1D). The

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results confirmed the discriminatory capacity of several metabolites (as gleaned by PLS-DA elaboration, Figure 4A), including but not limited to dAMP (BAH males *vs.* normal males), cysteine (BAH females *vs.* APA females), diiodothyronine (BAH males *vs.* APA males) (Figure 4B and Supplemental Table 4). Elaboration of this verification sample set led to the identification of some metabolites, such as dAMP, methoxytryptophan and diiodothyronine, as discriminating-disease metabolites, and the comparison of normal and BAH males revealed strong differences in the relative abundance of dAMP in the two considered groups, as expected (Figure 4B). Similar trends were observed following the relative quantitation of methoxytryptophan and diiodothyronine in BAH/APA males (Figure 4B). Each discriminating metabolite exhibited similar behaviour in all possible pair-comparisons between clustered groups. Relative P values are also available for these metabolites in Supplementary Table 6A and 6B.

A secondary untargeted metabolomics analysis confirmed and expanded the data from the first analytical site. We detected a dysregulation of urine levels of sulfur-containing metabolites (thiocysteine, homomethionine), purines (S-adenosylhomocysteine, AMP, allantoate, hydroxyisourate), pyrimidines (dihydrothymine, uracil, UDP), arginine metabolism (arginine, creatinine, guanidine oxopentanoate), tryptophan metabolism (hydroxykynurenic acid) and sugars (rhamnose, mannitol) in both cohorts, especially in the EH and BAH groups (**Supplementary Figure 1 and 2 – Training and testing cohort**).

DISCUSSION

Our exploratory investigation highlights, for the first time, the presence of gender and PA subtype-specific alterations in the urinary metabolome. Other investigators previously observed that the careful selection of patients was of crucial importance, especially in multifactorial diseases, such as hypertension^[8]. Our results are consistent with previous reports of an altered energy metabolism associated with hypertension^[8]. The observed gender-related differences are also consistent with our previous study on urinary proteomics in primary aldosteronism^[9] and hormone-dependent changes in females^[19], which confirms a gender-specific signature reflected in a diversified urinary metabolome,

as described in the present results. Our findings on an involvement of purine/pyrimidine pathways suggest a disturbance of this metabolism in hypertensive patients, which is a phenomenon previously described by others in other settings ^[20]. This observation is also consistent with previous data collected from patients who were characterized by increased plasma renin activity in the absence of increased plasma levels of aldosterone following treatment with minoxidil ^[21]. Increased free levels of amino acids, especially N-acetylated forms, suggest augmented proteolysis of N-terminal-acetylated residues ^[22,23]. The presence of metabolites, such as amino acids, was also previously reported to characterize women who develop preeclampsia ^[24]. Metabolites related to altered lipid and carbohydrate metabolism are generally associated with hypertension ^[8]. A very recent report by Zhao and colleagues revealed the involvement of amino acid metabolism and oxidative stress metabolic pathways in a study of urine metabolomics during the course of essential hypertension ^[25].

Gender-specific abnormality previously emerged as a relevant question relevant in the pathogenesis and diagnostic work-up of PA. The recent discovery of somatic mutations on several genes coding for K channels or other molecules involved in cell ion homeostasis clarified the pathogenesis of APA. A recent meta-analysis demonstrated that female gender is largely prevalent (67% vs. 44%, $p < 0.05$) in patients with APA and KCNJ5 mutation, which is the most frequent genetic abnormality recognized in adrenal glands with adenomatous transformation ^[26]. The findings of the largest studies on this topic confirmed this result ^[27-30]. A very recent report further confirmed the female prevalence of the KCNJ5 mutation in APA ^[31]. Williams and colleagues recently discussed a gender-related prevalence in APA patients and other aspects, such as dietary salt intake and cultural behaviours in different parts of the world ^[32].

Possible influences on steroid adrenal production by female sex hormones were hypothesized, but the precise mechanisms underlying this association remains largely unknown and worthy of further specific research.

Gender also affects the diagnostic work-up for PA. We first demonstrated that the initial screening based on the determination of ARR must be differentiated by gender because fertile women may

exhibit sex hormone-dependent changes of ARR, which leads to false positive results more frequently than in males^[33]. Other investigators subsequently confirmed these findings^[1,4].

Therefore, numerous pieces of evidence clearly support gender-specific features in PA patients who must be considered in clinics. The present data on the urinary metabolomics strongly support this view and suggest further investigations to identify new, clinically useful biomarkers.

The application of metabolomics technology in the current exploratory study elucidated peculiar metabolic signatures in the urine of patients diagnosed with different subtypes of PA. The results demonstrated a strong discrimination of BAH individuals from all other groups (up to 60-fold difference from APA patients), and a striking difference based on gender. These results indicate that the urinary metabolome of BAH patients is clearly different from other PA patients and presents gender-specific signatures. Notably, independent verification of preliminary results in a second sample set confirmed that three metabolites, dAMP, diiodothyronine and 5-methoxytryptophan, deserve further investigation to validate their potential as biomarkers of the BAH subtype. However, gender discrimination in the BAH subtype was based on determination of the levels of orotidine 5-phosphate, N-acetylalanine, hydroxyproline and cysteine.

Our study presents elements of strengths and limitations. The characterization by AVS of the “true” diagnosis of PA subtype is surely important because the strict criteria of lateralization allows for clear results and sure definitions of the pathology. The discrimination of the PA subtype was also very recently obtained using LC/MSMS steroid profiling in peripheral and AVS plasma samples, which provided better performance in detecting lateralization than that of aldosterone immunoassay^[34,35]. A second important strength was the use of high-resolution UPLC:MS/MS approaches to obtain further independent validation of the data.

The main limitation of the present study is the relatively limited patient sample size used for the analysis. Therefore, we used a double control of the data with i) a first exploratory sample of subjects, followed by an analysis of validation on a second sample, and ii) a second independent validation

analysis performed at the University of Colorado using another analytical platform. The internal substantial consistency of the results supports their reliability and the use of some metabolites as discriminating factors for the diagnosis of PA or PA subtype. Further and larger studies based on the absolute measurements of selected metabolites are required for clinical application. The possibility to initially separate BAH from APAs would avoid AVS, which is an invasive and difficult procedure that is currently available only in experienced and specialized centres and that fully relies on the technical skills of the operator. Notably, the subtype definition is critical for successful patient treatment and BP normalization.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1. Metabolic contributors to PLS-DA clustering patterns in PA vs. EH. T-test p-values are reported; not all PLS-DA metabolites contributors reached statistical significance.

Metabolite	Pathway and physiological role	p-Value	Fold change
dAMP	Purine pathway	ns	2.18 up, PA
Methoxytryptophan	Trp derivative. Antihypertensive activity ^[36]	ns	2.47 up, PA
Diiodothyronine	Metabolically active iodothyronine; involved in energy metabolism and cellular respiration ^[37] .	ns	4.43 up, PA
Orotidine 5 phosphate	Precursor of UMP	0.016	19.2 up, PA
Hydroxyproline	Associated with a high risk of heart failure ^[38] .	0.05	4.12 up, PA
N acetylalanine	Fundamental in collagen structure. Its high myocardial concentration is related to fibrosis and heart failure ^[39] .	ns	1.60 up, PA
Cysteine	High levels in plasma are related to high risk of coronary heart disease ^[40]	0.02	3.02 up, PA
Xanthosine 5 phosphate	Intermediate in purine metabolism.	0.037	4.39 up, PA
Argininosuccinate	Precursor of fumarate in Krebs cycle; precursor of arginine in urea cycle.	0.026	3.23 up, PA
Uric acid	purine pathway, byproduct of purine breakdown	0.02	2.85 up, PA
deoxyadenosine	purine pathway, catabolite of adenosine.	0.1	3.93 up, PA
CMP	Pyrimidine metabolism. The main source is RNA degraded by RNases, which frees UTP, which is deaminated to CTP.	0.0431	1.75 up, PA
Dihydroxyacetone phosphate	Intermediate in glycolysis, gluconeogenesis and triglycerides synthesis.	ns	1.99 up, PA

Phosphoglycerate	Intermediate of glycolysis.	0.05	1.82 up, EH
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FIGURE LEGENDS

Figure 1. Statistical analyses. (A) PLS-DA was performed to determine whether metabolic phenotypes could discriminate urine samples from male or female subjects who were healthy, hypertensive without primary aldosteronism (PA) and a low/high aldosterone-to-renin ratio (ARR), or with P, divided into bilateral hyperplasia patients (BAH) and adrenal adenoma-producing aldosterone patients (APA). Principal component 1 (PC1) explained ~13% of the total variance and discriminated BAH samples from other groups. PC2 discriminated female (upper) from male (lower quadrant) samples and provided discrimination between normal urine (top quadrant) and hypertensive samples (lower quadrants). (B) Random forest clustering confirmed PLS-DA profiles, and BAH samples exhibited distinct phenotypes compared to other groups. (C) SAM plot identified at least 16 significant features exhibiting discriminating capacity between groups. (D) Significant metabolites with discriminatory capacity between groups were plotted using VIP scores. (E) The same metabolites, and additional metabolites primarily related to purine/pyrimidine metabolism and free/N-acetylated amino acids, were statistically significant ($p < 0.05$ ANOVA) using hierarchical clustering analysis.

Figure 2. Box and whisker plots of metabolites with significant discriminatory capacity between groups. Medians $\pm$ interquartile ranges are shown for each metabolite.

Figure 3. Whisker plots of the metabolites exhibiting discrimination between normal and PA samples (BAH and APA). (A), essential hypertensive (EH) and PA samples (B), and PA subtypes, defined as bilateral hyperplasia patients (BAH) and adrenal adenoma-producing aldosterone patients (APA) (C). Medians $\pm$ interquartile ranges are shown for each metabolite.

Figure 4. Verification of the results was performed via monitoring of the top discriminating metabolites from the first analysis in an independent sample set. (A) A PLS-DA analysis was performed on 11 metabolites of 26 EH or PA (BAH and APA) samples, female or male (detailed in Supplemental Table 2). Verification results confirm the discriminatory capacity of the biomarkers identified in the first analysis, but further, more extensive prospective validation using targeted approaches in a novel cohort is mandatory. **(B)** Box and whisker plots for 10 representative metabolites, as predicted in the first dataset and verified using targeted approaches in a second independent dataset. Medians $\pm$ interquartile ranges are shown for each metabolite. Colour codes are consistent with the box and whisker plots in Figure 2.