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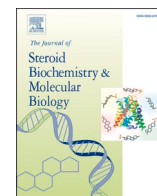
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Urine steroid metabolomics as a diagnostic tool in primary aldosteronism

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ABSTRACT

Primary aldosteronism (PA) causes 5–10% of hypertension cases, but only a minority of patients are currently diagnosed and treated because of a complex, stepwise, and partly invasive workup. We tested the performance of urine steroid metabolomics, the computational analysis of 24-hour urine steroid metabolome data by machine learning, for the identification and subtyping of PA. Mass spectrometry-based multi-steroid profiling was used to quantify the excretion of 34 steroid metabolites in 24-hour urine samples from 158 adults with PA (88 with unilateral PA [UPA] due to aldosterone-producing adenomas [APAs]; 70 with bilateral PA [BPA]) and 65 sex- and age-matched healthy controls. All APAs were resected and underwent targeted gene sequencing to detect somatic mutations associated with UPA. Patients with PA had increased urinary metabolite excretion of mineralocorticoids, glucocorticoids, and glucocorticoid precursors. Urine steroid metabolomics identified patients with PA with high accuracy, both when applied to all 34 or only the three most discriminative steroid metabolites (average areas under the receiver-operating characteristics curve [AUCs-ROC] 0.95–0.97). Whilst machine learning was suboptimal in differentiating UPA from BPA (average AUCs-ROC 0.65–0.73), it readily identified APA cases harbouring somatic *KCNJ5* mutations (average AUCs-ROC 0.79–85). These patients showed a distinctly increased urine excretion of the hybrid steroid 18-hydroxycortisol and its metabolite 18-oxo-tetrahydrocortisol, the latter identified by machine learning as by far the most discriminative steroid. In conclusion, urine steroid metabolomics is a non-invasive candidate test for the accurate identification of PA cases and *KCNJ5*-mutated APAs.

Abbreviations: THAldo, 3α,5β-tetrahydroaldosterone; 18-OH-F, 18-hydroxycortisol; 18-OH-THA, 18-hydroxy-tetrahydro-11-dehydrocorticosterone; 18-oxo-THF, 18-oxo-tetrahydrocortisol; APA, aldosterone-producing adenoma; AUC-ROC, area under the receiver-operating characteristics curve; BPA, bilateral primary aldosteronism; ENSAT, European Network for the Study of Adrenal Tumours; GC-MS, gas chromatography-mass spectrometry; GMLVQ, generalised matrix relevance learning vector quantization; PA, primary aldosteronism; THF, tetrahydrocortisol; THS, tetrahydro-11-deoxycortisol; UPA, unilateral primary aldosteronism.

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1. Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension and is found in 5–10% of patients with hypertension and up to 20% of those with resistant hypertension [1–4]. The main subtypes of PA are unilateral primary aldosteronism (UPA) and bilateral primary aldosteronism (BPA) [1]. In the majority of UPA caused by aldosterone-producing adenoma (APA), somatic driver mutations have been identified in the tumour, mostly affecting ion channels or pumps and eventually resulting in the overproduction of aldosterone [5–9]. Differentiating UPA from BPA as the cause of PA provides the basis for therapeutic stratification: surgery is the preferred treatment option for UPA, whilst patients with BPA typically receive long-term treatment with mineralocorticoid receptor antagonists [1,10]. PA is associated

with an increased risk for cardio- and cerebrovascular complications, which exceeds the risk associated with essential hypertension [11–13]. Furthermore, the prevalence of metabolic comorbidities such as insulin resistance, type 2 diabetes and osteoporosis is higher in patients with PA compared to the general and hypertensive population [14–16], which has been linked to glucocorticoid co-secretion detectable in a large proportion of patients [17]. The current diagnostic workup for PA is complex, expensive and requires repeated visits before the final diagnosis is safely established. Therefore, screening is currently limited to patients with a high degree of suspicion of underlying primary aldosteronism, resulting in many cases remaining undiagnosed [1,18]. Serum multi-steroid profiling has been identified as a promising tool in the diagnostic workup of PA, particularly after the discovery that a subgroup of patients with UPA due to APA secrete high amounts of

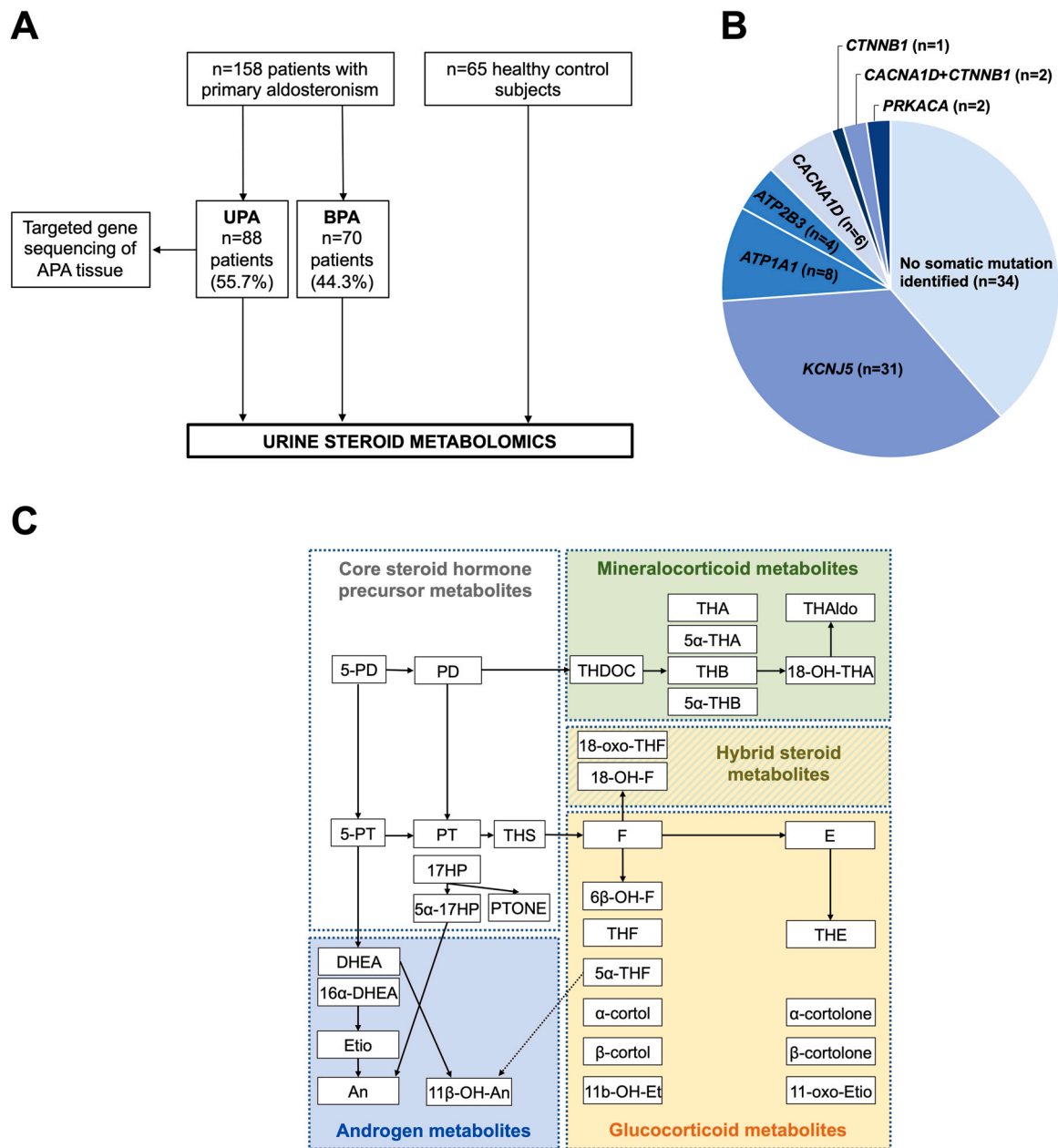


Fig. 1. Study design (A), distribution of the somatic mutations of APAs (B), and schematic overview of urine steroid metabolites analysed in the study participants (C). Steroid metabolites are schematically mapped onto the steroidogenic pathways leading to mineralocorticoid, glucocorticoid, hybrid steroid, and androgen biosynthesis. Arrows do not imply interconversion between metabolites; they represent a simplistic derivation from the synthetic sequential pathway involving hormonal steroid precursors and active hormones. Steroid nomenclature is reported in [Appendix Table 2](#). Abbreviations: APA: aldosterone-producing adenoma; BPA: bilateral primary aldosteronism, UPA: unilateral primary aldosteronism.

“hybrid steroids” (18-oxocortisol and 18-hydroxycortisol), which require enzymatic steps typically restricted to the adrenal zona glomerulosa and the zona fasciculata for their synthesis [19–24]. Here, we explored the performance of 24-hour urine steroid metabolome analysis coupled with machine learning (= urine steroid metabolomics) for diagnosis, subtype differentiation, and identification of APAs harbouring somatic mutations in a large cohort of patients with PA compared to healthy controls.

2. Materials and methods

2.1. Study population

Adult patients (>18 years) with PA were consecutively recruited at four centres participating in the European Network for the Study of Adrenal Tumours, ENSAT (Munich, Würzburg, Nijmegen, Berlin) (Fig. 1A). The diagnosis and subtype differentiation of PA was confirmed according to local protocols in accordance with Endocrine Society guidelines [1]. Patients were studied without antihypertensives during diagnostic procedures whenever possible; interfering medications were paused for at least 1 week before testing (4 weeks or more in the case of mineralocorticoid receptor antagonists). UPA was diagnosed by unstimulated or adrenocorticotrophic hormone 1–24 stimulated adrenal vein sampling, according to local protocols. The study population is a subset of a previously published study assessing the 24-hour urine steroid metabolome in a large cohort of patients with newly diagnosed UPA and BPA [17]. In this study, we included only those UPA cases who also underwent unilateral adrenalectomy and investigation of somatic mutations in the tumour tissue. A group of 65 healthy normotensive controls matched for sex and age to the patients with PA was recruited and provided a 24-hour urine collection. All participants provided written informed consent, and the study was approved by the ethics committee at each participating institution.

2.2. Targeted gene sequencing

DNA was extracted from tumour tissue of patients with UPA after adrenalectomy and subjected to targeted sequencing of genes known to harbour somatic mutations in APAs (*KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, *CTNNB1*, and *PRKACA*), as previously described (Fig. 1B) [25].

2.3. Urine steroid metabolome profiling

All patients with PA collected a 24-h urine sample at the time of diagnosis of PA or at the initial visit for analysis by gas chromatography-mass spectrometry (GC-MS) in selected-ion-monitoring analysis mode, as previously described [26,27]. This included the identification and quantification of 34 different steroid metabolites comprehensively covering net steroid production, including mineralocorticoids, glucocorticoids, hybrid steroids, androgens, and their precursors (Fig. 1C).

2.4. Statistical analysis

Summary statistics were used to describe demographics and steroid metabolome of the study participants. Linear regression models were used to compare the urine steroid profiling data between groups adjusting for age and sex (healthy controls vs. PA, UPA vs. BPA, and *KCNJ5*-mutated APAs vs. non-*KCNJ5*-mutated APAs), and using log-transformed outcome data to reduce the impact of outliers. Separate multiple linear regression models were used for each comparison, using the concentration of each steroid as the dependent variable and age, sex, and the binary comparison of interest (e.g., healthy controls vs. PA) as the independent variables. Associations between the log-transformed outcome data were reported as sympercents (mean percentage change, 95% confidence interval) [28]. The software packages R, Stata® version 16, MATLAB®, and GraphPad Prism 9 (San Diego, CA: GraphPad

Software Inc.) were used for analysis.

2.5. Machine learning analysis of the urine steroid profiling data

We applied two distinct machine learning approaches, generalised matrix relevance learning vector quantization (GMLVQ) [29–31], a variant of LVQ [32], and random forest [33] for the computational analysis of the urine multi-steroid profiling.

The mathematical details of GMLV, a prototype-based classification method, have been previously described [30,31,34] and it has been applied to multi-steroid urine metabolome data in previous studies [26, 35]. In brief, 24-hour excretion values of the 34 steroid metabolites were log-transformed. The resulting set of 34-dimensional vectors $x = (x_1, x_2, \dots, x_{34})$, together with the class membership (healthy controls, PA, UPA, BPA, *KCNJ5*-mutated APA, and non-*KCNJ5*-mutated PA) served as input for the machine learning analysis. The dataset was randomly split into a training and a validation set (90% and 10% of cases, respectively), which were used to evaluate the performance of GMLVQ of each classification experiment. Based on the mean log-transformed excretions in the actual training set and the associated standard deviation, the training and validation sets were subjected to a z-score transformation in each random split. Each experiment was repeated 50 times and the performance was measured as the average area under the receiver-operating characteristics curve (AUC-ROC \pm standard deviation). The three most relevant steroid metabolites were determined as the indices of the highest values of the relevance matrix diagonal on average over the 50 training processes.

To further increase the validity of the computational analysis, we also applied a second machine learning method to the urine multi-steroid data, random forest, as previously described [33]. In order to facilitate the comparison between GMLVQ and random forest the evaluation procedures of the two methods were the same, with the exception of the random forest's hyperparameters. The number of trees in the forest was set to 50 [33].

The evaluation of the performance of the two distinct machine learning approaches was performed in an identical fashion to allow for a fair comparison of GMLVQ and random forest. The training with each of the methods was repeated for 50 randomised runs with 90% of the data used for training and 10% for validation. After each run, the most important features were determined by using the machine learning internal evaluation mechanism.

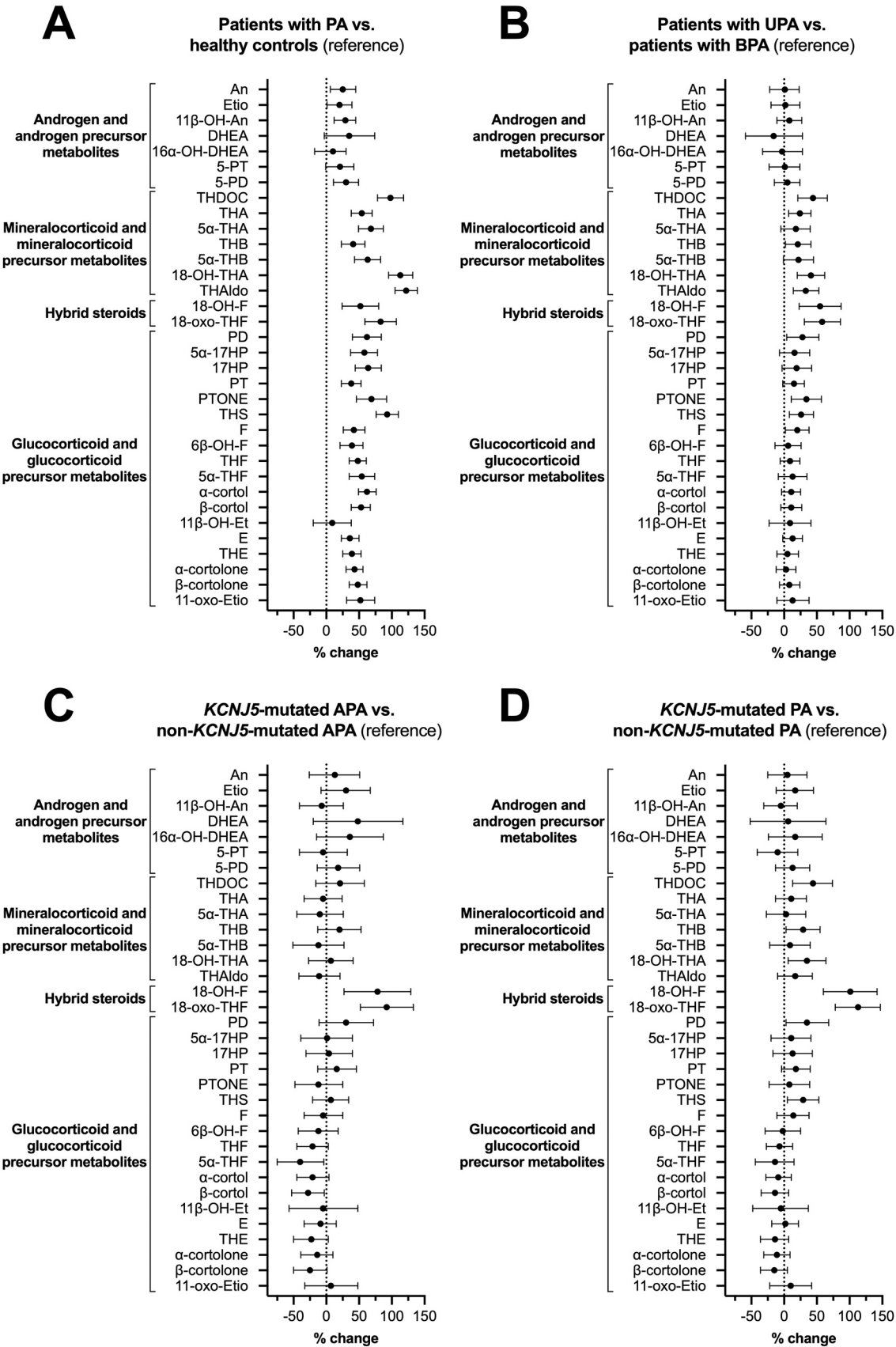
3. Results

3.1. Clinical, biochemical, and genetic characteristics of the study cohort

We included 158 patients with PA (Fig. 1A and Appendix Table 1). Eighty-eight patients (55.7%) were diagnosed with UPA and underwent adrenalectomy, whilst 70 patients (44.3%) were classified as BPA. Somatic mutations were identified in 54 APAs (61.4%); *KCNJ5* mutations were the overall most common finding (35.2% of APAs) (Fig. 1B). The distribution of age and sex was similar between patients with UPA and BPA, although patients harbouring a *KCNJ5* mutation were younger and predominantly women (Appendix Table 1).

3.2. Urine steroid metabolome of primary aldosteronism

Linear regression models adjusted for age and sex showed significantly higher urinary excretion of the major aldosterone metabolite 3 α ,5 β -tetrahydroaldosterone (THAldo) and other mineralocorticoid metabolites in patients with PA compared to healthy controls (Fig. 2A and Appendix Table 2). PA cases also showed a significantly higher excretion of most glucocorticoid and glucocorticoid precursor metabolites, the hybrid steroid 18-hydroxycortisol (18-OH-F) and its metabolite 18-oxo-tetrahydrocortisol (18-oxo-THF), and core steroid precursor metabolites (Fig. 2A and Appendix Table 2). A heatmap visualization



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Fig. 2. Urinary steroid excretion of patients with primary aldosteronism by subtype. Multi-steroid profiling of 24-hour urine samples was carried out by gas chromatography-mass spectrometry with quantification of 34 distinct steroid metabolites, representative of the production of androgens, glucocorticoids, hybrid steroids, and their precursors. The urinary excretion of each steroid metabolite was compared to the reference group using a linear regression model with the log-transformed steroid metabolite as the outcome (adjusted for age and sex). Associations between the log-transformed outcome and the variable of interest are reported as sympercents (mean percentage change, 95% confidence interval). Panel A: Comparison of the steroid excretion in patients with primary aldosteronism (PA, $n = 158$) to that observed in 65 healthy normotensive subjects. Panel B: Comparison of the steroid excretion of the steroid excretion in unilateral primary aldosteronism (UPA, $n = 88$) to that of bilateral primary aldosteronism (BPA, $n = 70$). Panel C: Comparison of the steroid excretion of *KCNJ5*-mutated aldosterone-producing adenomas (APAs, $n = 31$) to non-*KCNJ5*-mutated APAs ($n = 57$). Panel D: Comparison of the steroid excretion of *KCNJ5*-mutated primary aldosteronism cases ($n = 31$) to non-*KCNJ5*-mutated cases, including both UPA and BPA ($n = 127$). The quantitative excretion of each metabolite is reported in [Appendix Table 2](#).

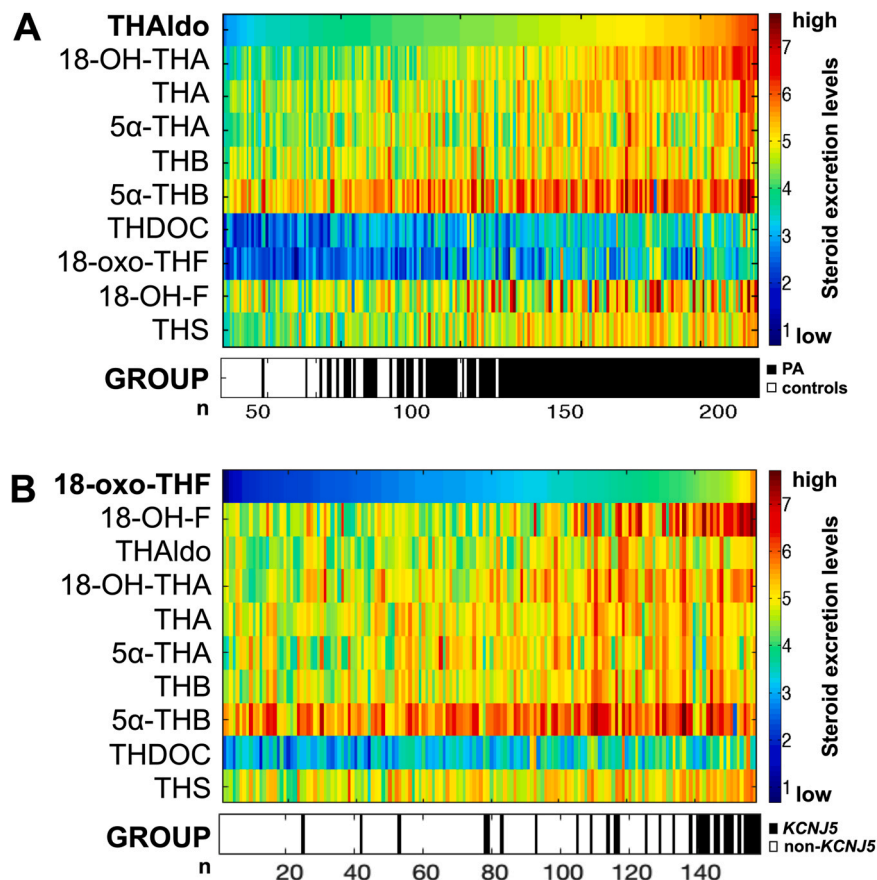


Fig. 3. Heatmap visualization of selected urine steroid metabolites. Panel A: Heatmap depicting log-transformed steroid metabolite excretion values of patients with primary aldosteronism (PA, $n = 158$, black) compared to healthy normotensive controls ($n = 65$, white). Mineralocorticoid metabolites (18-hydroxy-tetrahydro-11-dehydrocorticosterone, 18-OH-THA; THA; 5α-THA; tetrahydrocorticosterone, THB; 5α-THB; tetrahydro-11-deoxycorticosterone, THDOC), the hybrid steroid 18-hydroxycortisol (18-OH-F) and its metabolite 18-oxo-tetrahydrocortisol (18-oxo-THF), and the glucocorticoid precursor metabolite tetrahydro-11-deoxycortisol (THS) are ordered according to increasing excretion of 3α, 5β-tetrahydroaldosterone (THAlDo), the main metabolite of aldosterone. Panel B: Heatmap depicting log-transformed steroid metabolite excretion values of patients with PA harbouring *KCNJ5* mutations ($n = 31$, black) compared to non-*KCNJ5*-mutated cases, including both unilateral PA (UPA) and bilateral PA (BPA) ($n = 127$, white). The hybrid steroid 18-OH-F, mineralocorticoid metabolites, and THS are ordered according to increasing excretion of 18-oxo-THF.

ordered by increasing excretion of THAlDo demonstrated, as expected, a clustering of the PA cases to the right, consistent with increased aldosterone secretion (Fig. 3A). A similar gradient could be observed for other mineralocorticoid metabolites, the hybrid steroids, and the glucocorticoid precursor metabolite tetrahydro-11-deoxycortisol (THS), indicating gradually increased excretion in patients with PA.

Patients with UPA had higher urinary excretion of mineralocorticoid metabolites, hybrid steroids, and glucocorticoid precursor metabolites than those with BPA (Fig. 2B and [Appendix Table 2](#)). The differences observed in the hybrid steroids were driven by patients with APAs harbouring *KCNJ5* mutations, who had more than twofold higher excretion levels than those without mutations (Fig. 2C-D and [Appendix Table 2](#)). Accordingly, a heatmap visualization ordered by increasing excretion of 18-oxo-THF showed a clustering of the *KCNJ5*-mutated

cases to the right, consistent with increased hybrid steroid secretion (Fig. 3B).

3.3. Urine steroid metabolomics for the diagnosis of primary aldosteronism

GMLVQ revealed an excellent performance when using all 34 steroid metabolites for the identification of the PA cases vs. healthy normotensive controls (AUC-ROC 0.97 ± 0.03) (Fig. 4A-B). The analysis of the corresponding relevance matrix identified THAlDo, THS, and 18-hydroxy-tetrahydro-11-dehydrocorticosterone (18-OH-THA) as the three most discriminative steroid metabolites (Fig. 4C and [Table 1](#)); their 24-hour urinary excretion interquartile ranges did not overlap between patients with PA and healthy controls (Fig. 4D-F). Applying GMLVQ

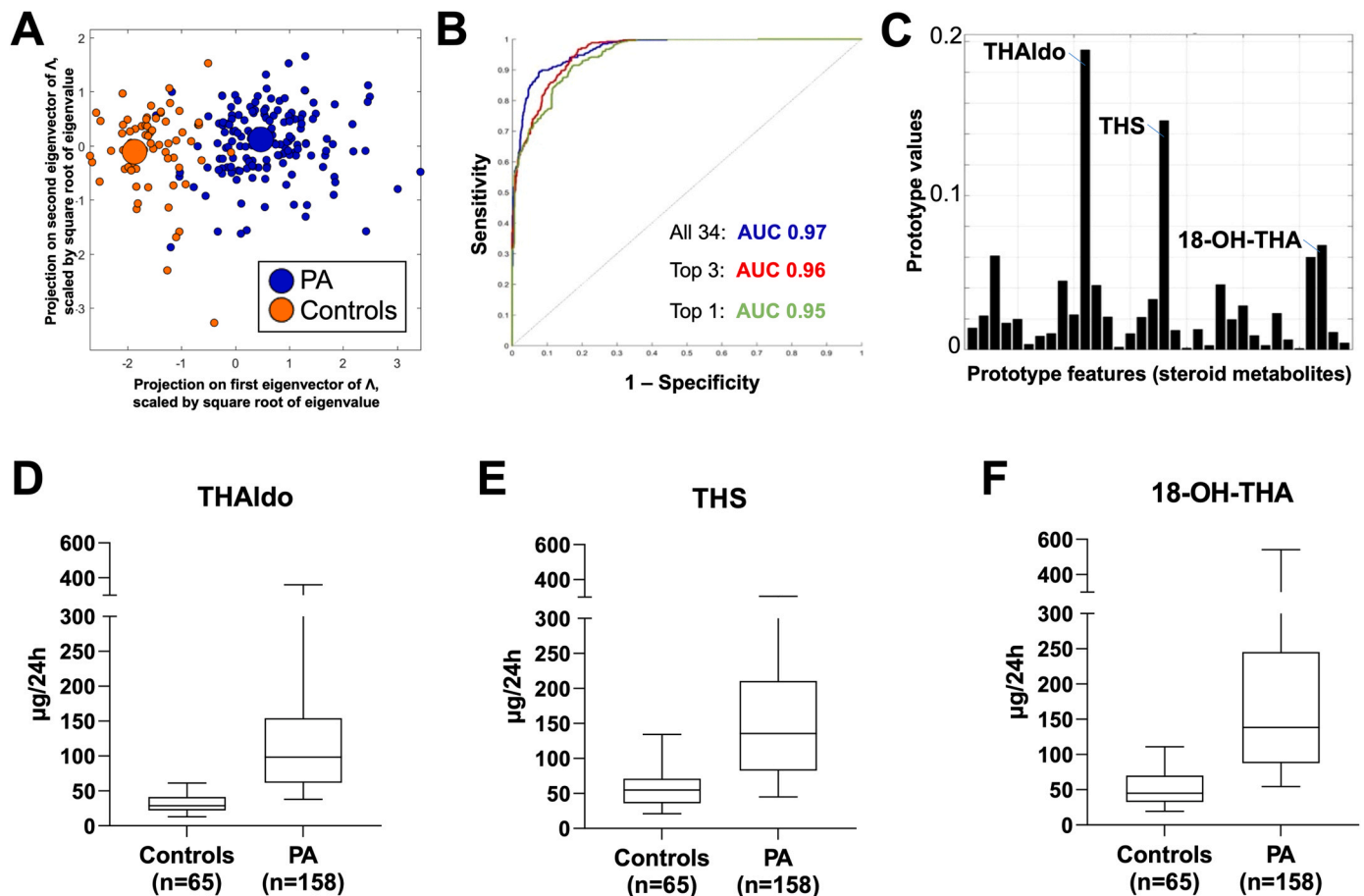


Fig. 4. Discrimination of patients with primary aldosteronism from healthy controls by GMLVQ analysis of the urine steroid metabolome. Panel A: 2D-plot demonstrating the separation of patients with primary aldosteronism (PA, blue) from healthy controls (orange) based on the urine steroid metabolome. Panel B: AUC-ROC curve for all 34 steroid metabolites (blue), the 3 most important markers (red) and the top marker (green) for discriminating PA cases. Panel C: Diagonal of the relevance matrix showing the relevance of each single steroid marker; the 3 most relevant markers to separate PA cases from healthy controls are 3 α ,5 β -tetrahydroaldosterone (THAlDo), tetrahydro-11-deoxycortisol (THS), and 18-hydroxy-tetrahydro-11-dehydrocorticosterone (18-OH-THA). Panels D-F: 24-hour urinary excretion of the 3 top markers identified by GMLVQ; boxes represent the median and interquartile range, whiskers 5th and 95th centiles.

when using only the three most relevant steroid metabolites for the classification rather than all 34 or even only the most discriminative steroid THAlDo, yielded similarly remarkable results for the identification of patients with PA (AUC-ROC 0.96 ± 0.03 and 0.95 ± 0.03 , respectively) (Fig. 4B). Random forest results were comparable to GMLVQ (Table 1). THAlDo was also the top marker selected by random forest for the classification of PA cases whilst, contrary to GMLVQ, 18-OH-THA was more relevant than THS (Table 1).

3.4. Urine steroid metabolomics for discrimination of primary aldosteronism subgroups

First, we tested whether machine learning analysis applied to the urine steroid metabolome would also be informative for the differentiation of UPA from BPA; however, we found a suboptimal performance with an average AUC-ROC of 0.65 for both GMLVQ and random forest (Table 1 and Appendix Fig. 1).

Assuming that this result may be due to the large heterogeneity within the UPA and BPA populations, we undertook an analysis aiming to differentiate patients with APAs and confirmed somatic *KCNJ5* mutations from other PA patients. For this purpose, we used GMLVQ to compare the urine steroid metabolome of APA cases with somatic mutations in *KCNJ5* vs. a combined group of all remaining UPA and BPA cases. The ROC curve for all 34 steroid markers demonstrated good

performance (AUC-ROC 0.83 ± 0.16) (Fig. 5A-B). The corresponding relevance matrix identified 18-oxo-THF, 18-OHF, and tetrahydrocortisol (THF) as the top 3 markers, with 18-oxo-THF having by far the highest discriminatory power (Fig. 5C). The 24-hour urine excretion of these steroids is shown in Fig. 5D-F; in line with the GMLVQ findings, the interquartile ranges of 18-oxo-THF 24-hour urinary excretion did not overlap between PA cases with and without somatic *KCNJ5* mutations. Linear regression confirmed that patients with somatic *KCNJ5* mutations had significantly higher urinary excretion of 18-oxo-THF and 18-OH-F than healthy controls and patients with non-*KCNJ5*-mutated PA (Fig. 3C and Appendix Table 2); the observed changes in THF, on the other hand, were not significant after adjustment for age and sex. Whilst the excretion of the main aldosterone metabolite THAlDo was significantly increased in patients with PA, it did not prove useful to identify *KCNJ5* mutations (Fig. 5G and Appendix Table 2). The performance of GMLVQ improved even further when only looking at the top three steroid markers (AUC-ROC 0.85 ± 0.16) to identify *KCNJ5*-mutated cases (Table 1). The performance was slightly reduced when only relying on the top marker, 18-oxo-THF (AUC-ROC 0.81 ± 0.18) (Fig. 5B).

We observed similar results when applying random forest to the steroid metabolome data, and this method selected the same three steroid metabolites as GMLVQ as the most discriminative to identify APAs harbouring somatic *KCNJ5* mutations (Table 1).

Table 1

Machine learning analysis of the 24-hour urine steroid metabolome by generalised matrix relevance learning vector quantization (GMLVQ) and random forest for diagnosis and subtype differentiation of primary aldosteronism. The experiments represent the comparisons between patients with primary aldosteronism (PA) and healthy controls, and between different subgroups of PA: unilateral PA (UPA), bilateral PA (BPA), and aldosterone-producing adenomas harbouring somatic *KCNJ5* mutations. Each experiment was repeated 50 times and the performance was measured as the average area under the receiver-operating characteristics curve \pm standard deviation (AUC-ROC \pm SD). Results are reported for the entire urine steroid metabolome and the top 3 steroid metabolites (ordered by relevance).

Experiment	GMLVQ		Random Forest	
	AUC-ROC \pm SD	Top 3 steroid metabolites (ordered by relevance)	AUC-ROC \pm SD	Top 3 steroid metabolites (ordered by relevance)
PA vs. healthy controls				
- All 34 steroid metabolites	0.97 \pm 0.03		0.96 \pm 0.04	
- Top 3 steroid metabolites	0.96 \pm 0.03	THAldo, THS, 18-OH-THA	0.95 \pm 0.05	THAldo, 18-OH-THA, THS
UPA vs. BPA				
- All 34 steroid metabolites	0.65 \pm 0.15		0.65 \pm 0.13	
- Top 3 steroid metabolites	0.73 \pm 0.11	PTONE; THDOC;18-oxo-THF	0.72 \pm 0.13	PTONE; THDOC;18-oxo-THF
<i>KCNJ5</i> vs. non-<i>KCNJ5</i> PA^a				
- All 34 steroid metabolites	0.83 \pm 0.16		0.82 \pm 0.17	
- Top 3 steroid metabolites	0.85 \pm 0.16	18-oxo-THF, THF, 18-OH-F	0.79 \pm 0.22	18-oxo-THF, THF, 18-OH-F

^a Combination of aldosterone-producing adenomas without *KCNJ5* mutation and BPA.

4. Discussion

In this study, we showed that urine steroid metabolomics, the combination of mass spectrometry-based steroid profiling and data analysis by machine learning, is highly accurate for the detection of PA and identification of APAs harbouring somatic *KCNJ5* mutations.

The diagnosis and subtyping of PA currently rely on a burdensome multi-step process that typically requires several screening blood samples to measure the aldosterone-to-renin ratio, a confirmatory test such as the saline infusion test or oral salt-loading test, cross-sectional imaging of the adrenal glands, and adrenal vein sampling to differentiate UPA from BPA [1,4]. There are several caveats and confounding factors that affect the interpretation of these tests, and adrenal vein sampling is an invasive procedure that is not standardised and with limited availability [1,36]. Not surprisingly, PA is considerably undiagnosed despite being the most common cause of secondary hypertension [37], leaving many patients without appropriate targeted management and at increased risk of adverse cardiometabolic outcomes [11–13,17]. Novel tools are urgently needed to streamline the detection of patients with PA and improve their clinical outcomes.

Our data show that the computational analysis of the urine steroid metabolome achieved an excellent separation of PA cases from healthy normotensive controls with high accuracy. Patients with PA had increased urinary excretion of mineralocorticoid, glucocorticoid, and glucocorticoid precursor metabolites indicating that glucocorticoid co-secretion is common [17]. The main aldosterone metabolite THAldo had the highest discriminatory power, providing substantive evidence for the previous proposal of urinary THAldo as a reliable screening marker for the detection of PA [38].

Patients with UPA had significantly higher urinary excretion of mineralocorticoid metabolites than those with BPA, which is consistent with the fact that they often present with a more severe phenotype [23, 39]. However, there was considerable overlap between the steroid excretion of the two cohorts and urine steroid metabolomics could not reliably differentiate between the two; based on our findings, urine steroid metabolomics cannot currently obviate the need for cross-sectional imaging and adrenal vein sampling. A study investigating the diagnostic performance of machine learning, applying random forest and support vector machines to multi-steroid serum profiling of patients with PA, showed similar results to our approach using random forest and GMLVQ, with suboptimal sensitivity and average AUC-ROCs ranging between 0.66 and 0.70 [22].

Patients with UPA had higher urinary levels of the hybrid steroid 18-OH-F and its metabolite 18-oxo-THF. We found that the increased excretion of these hybrid steroids was positively and strongly associated with somatic *KCNJ5* mutations in the resected tumour tissue. In line

with this, machine learning identified 18-oxo-THF as the top steroid metabolite discriminating *KCNJ5*-mutated APAs and yielded consistently good discriminative results. Our data are in agreement with other studies showing higher serum levels of hybrid steroids in patients harbouring somatic *KCNJ5* mutations, which have been linked to the co-expression of aldosterone synthase and 17 α -hydroxylase/17,20-lyase in APAs [20–22,40]. Furthermore, a recent prospective study on 143 patients with PA found that higher ratios of urinary 18-OH-F/F could reliably identify *KCNJ5*-mutated cases [41]. Our results are highly relevant for the subtype differentiation of PA: somatic *KCNJ5* mutations are the most common genetic abnormality in UPA (up to 80% of cases) [42] and are associated with better clinical outcomes than wild-type *KCNJ5* cases after adrenalectomy [22,41,43]. Hence, the non-invasive detection of mutant *KCNJ5* by urine steroid metabolomics can potentially prove unilateral adrenal mineralocorticoid excess and facilitate swift surgical treatment.

Strengths of our study include the large number of consecutively recruited patients with PA and healthy controls, the possibility to correlate the somatic tissue genotype of APAs with the urine steroid metabolome, and the use of two distinct machine learning approaches which gave comparable and consistent results. To our knowledge, this is the first study to combine urine steroid metabolome analysis and machine learning to investigate the potential of urinary multi-steroid profiling for the diagnosis and subtype differentiation of PA. Previous studies have investigated the use of serum multi-steroid profiling with a similar aim [19–23]; however, the analysis of 24-hour urine samples is more attractive because it is a non-invasive test that provides a comprehensive overview of the adrenal steroid output throughout the day and is not affected by differences in seated vs. supine blood sampling, which impact on the serum metabolome [22].

A weakness of our study is the selective recruitment of patients with PA and the absence of a comparator cohort of patients with essential hypertension and ruled-out PA. The workup of PA and subtype differentiation of UPA and BPA relied on local protocols in the four recruiting centres; this could have introduced bias in patient classification. A potential further source of patient inclusion bias is linked to the recruitment by highly specialised centres, and our results need to be validated in a larger population of unselected hypertensive subjects with varying degrees of PA. The performance of urine steroid metabolomics would also need to be compared with the current reference standard tests for PA diagnosis (renin, aldosterone, and confirmatory tests), which was not possible in the present study. We could not assess the discriminating power of urine steroid metabolomics on somatic mutations other than *KCNJ5* because of the low prevalence of other somatic mutations. The targeted sequencing for somatic mutations in our study was not CYP11B2 immunohistochemistry-guided and did not include *GNAS*,

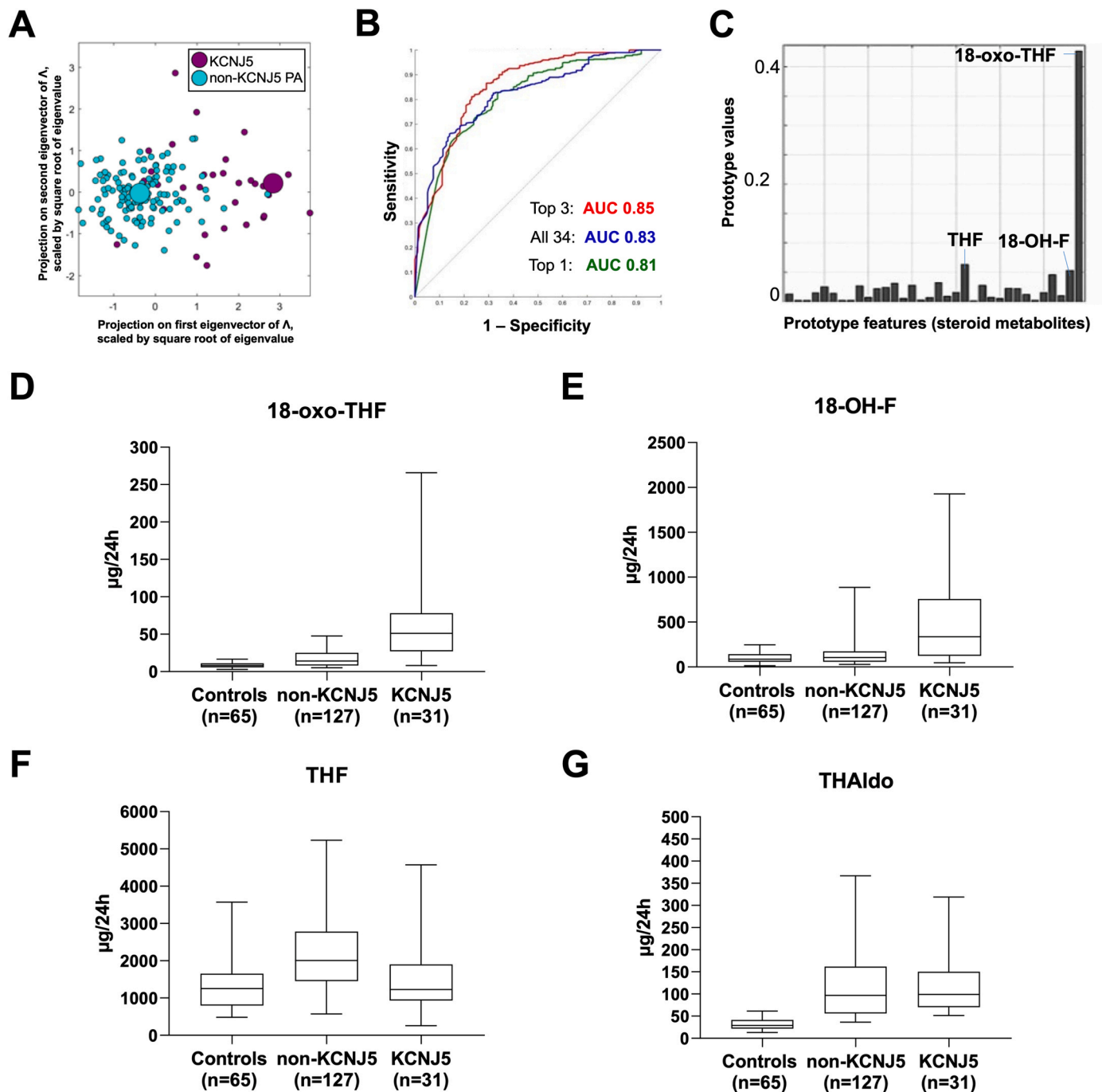


Fig. 5. Discrimination of aldosterone-producing adenomas harbouring *KCNJ5* mutations by GMLVQ analysis of the urine steroid metabolome. Panel A: 2D-plot demonstrating the separation of patients with *KCNJ5* mutations (turquoise) from the rest of patients with primary aldosteronism (purple) based on the urine steroid metabolome. Panel B: AUC-ROC curve for all 34 steroid metabolites (blue), the 3 most important markers (red) and the top marker (green) for discriminating *KCNJ5*-mutated cases. Panel C: Diagonal of the relevance matrix showing the relevance of each single steroid marker; the 3 most relevant markers are 18-oxo-tetrahydrocortisol (18-oxo-THF), 18-hydroxycortisol (18-OH-F), and tetrahydrocortisol (THF). Panels D-G: 24-hour urinary excretion of the 3 top markers identified by GMLVQ and 3 α ,5 β -tetrahydroaldosterone (THAlDo), the main metabolite of aldosterone; boxes represent median and interquartile range, whiskers 5th and 95th centiles.

CACNA1H, or *CLCN2* mutations, and this yielded lower coverage compared to recently published series [44]; this could have affected the subtyping of UPA cases.

5. Conclusions

Our study demonstrates that patients with PA have increased urinary excretion of mineralocorticoid metabolites and glucocorticoid precursor

metabolites that can accurately differentiate them from healthy controls. In addition, urinary hybrid steroid excretion can be employed to reliably identify *KCNJ5*-mutated APAs, which may obviate the need for adrenal vein sampling in a subset of patients with unilateral adenomas. By applying machine learning to the urine steroid metabolome profiling data, we were able to identify the top three steroids for patient classification. Using this much smaller subset of steroid biomarkers for machine learning analysis had equal performance in the identification of

PA cases and *KCNJ5* mutations. This readily facilitates the transfer of this approach to high-throughput liquid chromatography-tandem mass spectrometry, which is increasingly available in clinical practice. Urine steroid metabolomics, therefore, holds the promise of becoming an accurate and non-invasive test that can be used in the clinic for the identification and subtype differentiation of PA. Future prospective studies are needed to validate its use as a diagnostic test, including comparator groups of subjects with low-renin hypertension and excluded PA.

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CRediT authorship contribution statement

Alessandro Prete: Conceptualization, Formal analysis, Data curation, Writing – original draft, Visualization, Funding acquisition. **Katharina Lang:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Funding acquisition. **David Pavlov:** Methodology, Formal analysis, Review and editing of the original draft. **Yara Rhayem:** Investigation, Review and editing of the original draft, Funding acquisition. **Alice J. Sitch:** Conceptualization, Formal analysis, Review and editing of the original draft. **Anna S. Franke:** Investigation, Review and editing of the original draft. **Lorna C. Gilligan:** Methodology, Formal analysis, Investigation, Review and editing of the original draft. **Cedric H.L. Shackleton:** Conceptualization, Review and editing of the original draft. **Stefanie Hahner:** Investigation, Review and editing of the original draft. **Marcus Quinkler:** Conceptualization, Investigation, Review and editing of the original draft. **Tanja Dekkers:** Conceptualization, Investigation, Review and editing of the original draft. **Jaap Deinum:** Conceptualization, Investigation, Review and editing of the original draft, Funding acquisition. **Martin Reincke:** Conceptualization, Investigation, Review and editing of the original draft, Funding acquisition. **Felix Beuschlein:** Conceptualization, Investigation, Review and editing of the original draft, Funding acquisition. **Michael Biehl:** Conceptualization, Methodology, Writing – original draft, Supervision. **Wiebke Arlt:** Conceptualization, Formal analysis, Writing – original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests in relation to this work.

Data availability

Data will be made available on request.

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None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106445.

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