STEROID PROFILING BY LC-MS/MS AND PRIMARY ALDOSTERONISM

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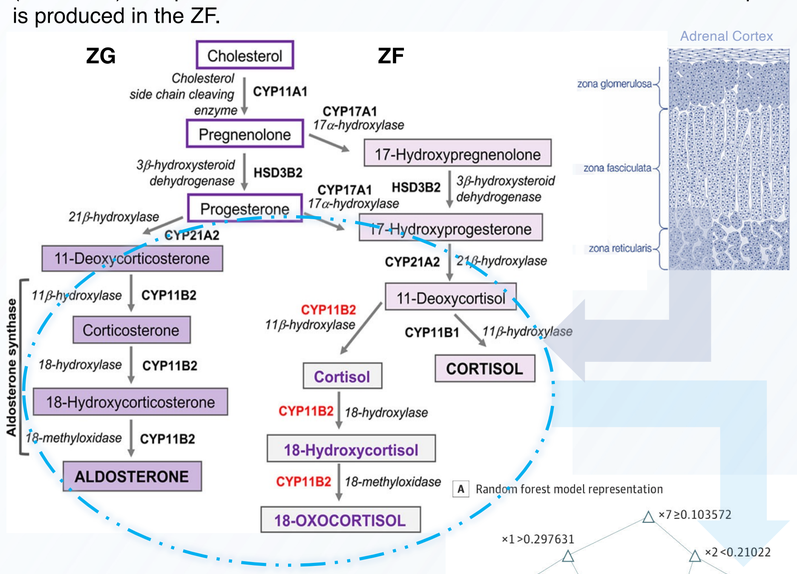
Introduction

Primary aldosteronism (PA) is a common cause of secondary hypertension that requires targeted therapies. Current method of diagnosis involves multiple biochemical tests and invasive procedures, while reliance on aldosterone by immunoassay may result in false-positive confirmation testing and unnecessary procedures in up to 35% of patients[1]. Steroid profiling combined with machine learning (ML) can aid in diagnosis while avoiding the aforementioned disadvantages[2]. An expanded liquid chromatography-tandem mass spectrometry (LC-MS/MS) steroid profile method has been developed to address this clinical need, and to validate diagnostic algorithms locally.

Figure 1a: Steroidogenesis in the zona glomerulosa (ZG) and zona fasciculata (ZF) of the human adrenal cortex[3]. In normal patients, aldosterone synthase (CYP11B2) is expressed only in the ZG, while 17α-hydroxylase (CYP17A1) is expressed in the ZF but not the ZG. Therefore aldosterone is produced only in the ZG while cortisol is produced in the ZF.



In some patients with PA, "hybrid" steroids **18-oxo-cortisol and 18-Hydroxy-cortisol** are produced by adenomas with ZF-like cells that express both CYP11B2 and 17a-Hydroxylase (CYP17A1), resulting in hybrid steroid formation from cortisol.

Elevated hybrid steroid concentrations can help identify patients with a unique subtype of PA without multiple tests / procedures.

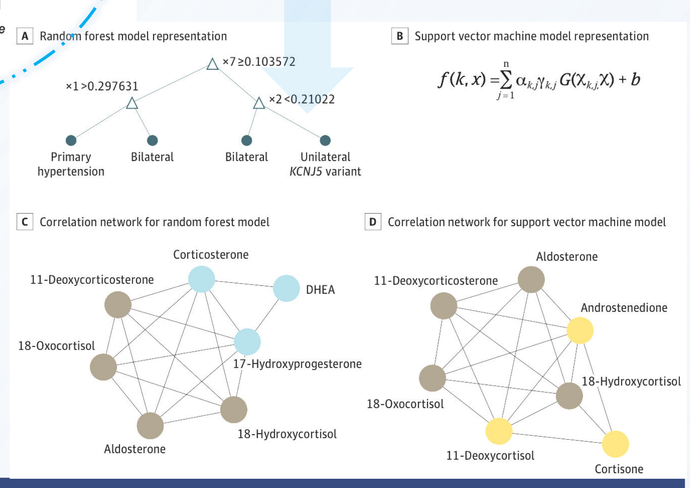


Figure 1b: As demonstrated by Eisenhofer et al.[2], application of multi-dimensional pattern recognition and ML for analysis of steroidomic data in the diagnosis of PA offers the potential for more efficient and effective diagnostic stratification than the traditional series of multiple studies involving single end-point measures in relation to given cutoff values.

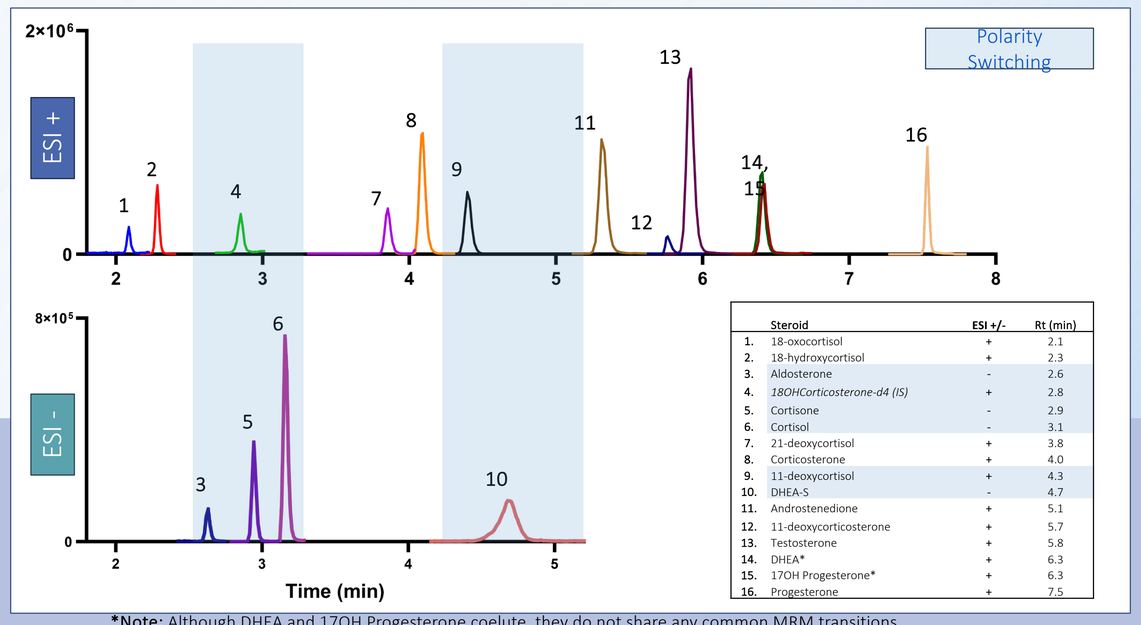
Methods & Materials

Using traceable calibration standards, we developed an LC-MS/MS method that measures 15 endogenous steroids in serum or plasma following supported liquid extraction. The method was validated according to the the principles of CLSI C62-A (2015)[6] and FDA 2018[7]. Method comparisons were performed using patient samples from the international PROSALDO study coordinated by Technische Universität Dresden (TUD), Germany. External quality assurance samples from RCPA QAP and INSTAND were used for assessing trueness. Pooled patient samples were used to measure imprecision.

Table 1. Steroid profile method summary

Specimen	100 μL EDTA plasma or serum (non-gel preferred).
Calibration	Chromsystems combined steroid panels 1 and 2 with spiked 18-Oxocortisol and 18-Hydroxycortisol in Methanol.
Quality control	Pooled patient serum.
Sample preparation	Supported liquid extraction (SLE, Biotage ISOLUTE SLE+ 400μL plate)[4]. Pipetting, premixing and transfer to the SLE plate is programmed on the Tecan Freedom Evo 100 liquid handling robotic system. Samples are reconstituted in 100 μL of 25% methanol. Labelled internal standards are added to an aqueous working internal standard mixture.
Analytical column	Kinetex 2.6 μm EVO C18 150 x 2.1mm 100Å LC analytical column with a 2.0 μm depth filter KrudKatcher maintained at 45 °C.
Mobile phases	Mobile Phase A: 0.2mM Ammonium Fluoride in water; Mobile Phase B: 100% methanol
HPLC method	Gradient elution with 0.8 mL/min flow rate. 20 μL injection.
MS method	35 scheduled MRM transitions in ESI + mode including labelled internal standards, 11 scheduled MRM transitions in ESI - mode including labelled internal standards. Collision energies and source settings are largely consistent with published settings, with some in-house modifications.
Run time	10 minutes

Figure 2. Chromatographic separation of a calibrator, with peaks shown according to their respective ionization modes in scheduled multiple reaction monitoring.



	Steroid	ESI +/-	Rt (min)
1.	18-oxocortisol	+	2.1
2.	18-hydroxycortisol	+	2.3
3.	Aldosterone	-	2.6
4.	*18OHCorticosterone-d4 (IS)*	+	2.8
5.	Cortisone	-	2.9
6.	Cortisol	-	3.1
7.	21-deoxycortisol	+	3.8
8.	Corticosterone	+	4.0
9.	11-deoxycortisol	+	4.3
10.	DHEA-S	-	4.7
11.	Androstenedione	+	5.1
12.	11-deoxycorticosterone	+	5.7
13.	Testosterone	+	5.8
14.	DHEA*	+	6.3
15.	17OH Progesterone*	+	6.3
16.	Progesterone	+	7.5

*Note: Although DHEA and 17OH Progesterone coelute, they do not share any common MRM transitions.

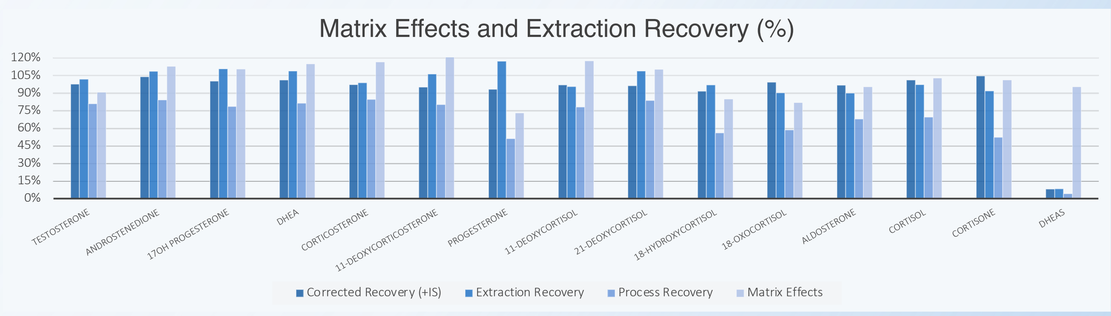
Results: Steroid Profile and ML Validation

Method Validation: Average assay imprecision for all steroids ranged from 2.4 to 9.2% within the analytical measuring range as per CLSI EP05. Using 8 available steroid RCPA QAP LC-MS/MS method medians (N = 27), acceptable average differences ranged from -7.3 to +7.3%. A comparison of 61 samples to the TUD reference LC-MS/MS method gave Passing-Bablok slopes ranging from 0.97 to 1.13 and Bland-Altman relative differences ranging from -1.85 to +13.5%.

Table 2. Some performance characteristics of the steroid profiling method according to the principles of CLSI C62-A (2015) and FDA 2018. *Preliminary LLOQ's due to low %CV's, however in all cases these concentrations are less than calibrator 1. ** DHEA was evaluated by INSTAND (Germany) EQA. [a] Steroids lacking a QAP program were only evaluated by the LC-MS/MS method comparison to TUD-Dresden. N.D. = Not determined.

	Analytical Measuring Range including LLOQ (%CV ≤ 20) Linearity R^2 0.995 - 0.9999 (N = 17 batches)	Average Total Imprecision (%) (N = 30 Low, Med, High Patient Pools)	Linear regression with RCPAQAP (N = 27) and INSTAND (N=12) QAP LC-MS/MS Medians, and TUD-Dresden (N=61)	
	(nmol/L)	%CV	Linear Regression	Ave %Difference
Testosterone	0.058 - 38.5	3.0	y = 1.00x + 0.1	1.8
Androstenedione	0.129 - 45.3	3.6	y = 1.04x - 0.1	1.6
17OH Progesterone	0.047 - 42.3	3.8	y = 1.00x + 0.8	5.1
DHEA	1.295 - 164	8.8	y = 1.01x - 1.5**	-3.1
Corticosterone	*0.653 - 130	3.6	y = 1.05x - 0.0[a]	3.1
11-deoxycorticosterone	0.006 - 4.7	7.8	y = 0.87x + 0.0[a]	7.3
Progesterone	0.078 - 75.7	5.7	y = 1.01x + 0.6	1.2
11-deoxycortisol	*0.013 - 37.7	3.3	y = 1.05x + 0.1	1.5
21-deoxycortisol	0.069 - 16.4	8.1	N.D.	N.D.
18-hydroxycortisol	0.272 - 11.3	8.8	y = 1.05x - 0.2[a]	-7.3
18-oxocortisol	0.014 - 1.15	9.2	y = 1.11x - 0.0[a]	-1.3
Aldosterone	0.022 - 7.1	7.0	y = 1.02x + 0.0	0.4
Cortisol	*5.99 - 721	3.5	y = 0.98x + 8.6	0.3
Cortisone	0.971 - 110	2.4	y = 0.91x + 5.5[a]	6.6
DHEAS	*249.1 - 14649	2.9	y = 0.96x + 69	-2.2

Figure 3. Matrix effects and extraction recovery experiments were carried out according to Matuszewski et al.[5] Apart from DHEA-S, all corrected spike recoveries were within an acceptable range of 100 +/- 15%, as an additional assessment of trueness.



Machine Learning Algorithm Probabilities: Application of support vector machine (SVM) learning algorithms resulted in comparable probabilities to TUD-Dresden for the diagnosis of primary hyperaldosteronism (93.5 to 97.2%), or primary hypertension (70.1 to 92.6%). This data represents the diagnostic agreement of 23 patients in the PROSALDO study (Fig 4a to 4c below).

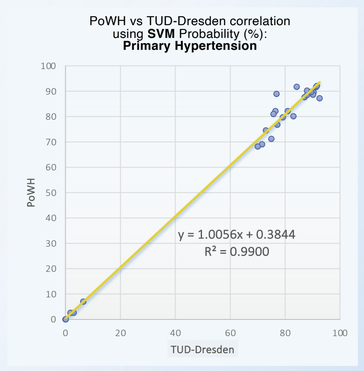


Figure 4a. Primary Hypertension probability correlation

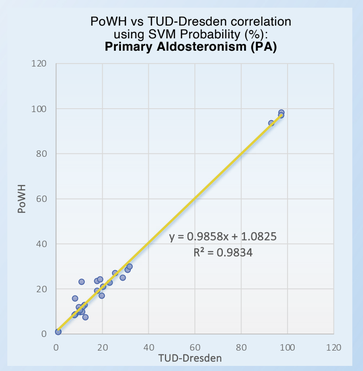


Figure 4b. Primary Aldosteronism probability correlation

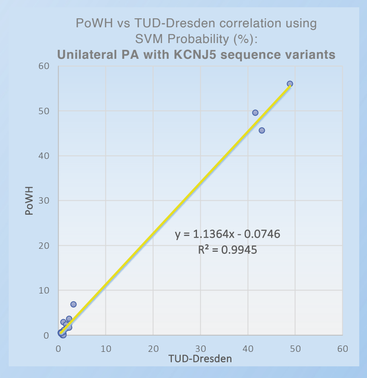


Figure 4c. Unilateral PA with KCNJ5 mutation probability correlation

Table 3. ML probability analysis from a representative patient showing concordance between POWH vs TUD-Dresden for Primary hypertension, Bilateral and Unilateral PA, and Unilateral PA with KCNJ5 mutation probability. This data is from a **patient confirmed to having a right adrenal adenoma by CT scan**. A right adrenalectomy procedure, and histology results are pending.

Probability in %	Model 1 (LDA)		Model 2 (SVM)		Model 3 (RF)	
	POWH	DRESDEN	POWH	DRESDEN	POWH	DRESDEN
Primary hypertension	1.0	0.4	5.8	4.45	0.6	0.4
Bilateral	1.5	0.6	11.1	4.05	23.4	5.4
Unilateral	8.4	5	31.4	30.41	23.4	18.4
Unilateral w/ KCNJ5	89.0	94.1	51.6	61.09	52.6	75.8
PA	99.0	99.7	94.2	95.6	99.4	99.6
			POW	DRESDEN		
Mean PA probability			97.5	98.3		

Discussion

Conclusion: Our multi-steroid LC-MS/MS assay panel has acceptable analytical performance and good agreement with an independent method at TUD-Dresden. It offers comparable clinical performance when used in an established machine learning algorithm for the differential diagnosis of various forms of hypertension. Future adoption of machine learning using steroid profile data will facilitate more non-invasive means of diagnosing primary hyperaldosteronism.

Acknowledgements

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