Mass spectrometry-based quantification of steroids for the diagnostic workup of adrenal tumors

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Massenspektrometrische Quantifizierung von Steroiden zur Diagnostik von Nebennierentumoren

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submitted by

Nora Johanna Vogg

from Bayreuth

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Submitted on: ……

Office stamp

Members of the Thesis Committee

Chairperson: Prof. Dr. Peter Heuschmann

Primary Supervisor: Prof. Dr. Martin Fassnacht

Second Supervisor: Prof. Dr. Dr. Matthias Kroiss

Third Supervisor: Prof. Dr. Oliver Scherf-Clavel

Fourth Supervisor: Prof. Dr. Petra Högger

Date of Public Defence: …….

Date of Receipt of Certificates: ……

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Summary

Tumors of the adrenal gland belong to the most frequent neoplasms in humans with a prevalence of 3–10 % in adults > 50 years. Often, the lesion is detected incidentally during imaging for other reasons. The aim of the diagnostic workup is the identification of potentially hormone-secreting and / or malignant tumors, because most of these tumors will require surgical resection. Hormonally functional tumors can be benign or malignant and are characterized by autonomous secretion of adrenal steroid hormones. Malignant adrenocortical carcinomas (ACC) are very rare and associated with a poor prognosis in advanced stages. As therapeutic options for ACC are limited, an early and accurate diagnosis is crucial. Within this thesis, two liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the quantification of steroids in different biomaterials were developed to improve the diagnostic workup of adrenal tumors.

The 1 mg dexamethasone suppression test (DST) is the standard screening test for the assessment of autonomous cortisol secretion. Thereby, the suppressibility of cortisol secretion is tested after oral administration of dexamethasone. An LC-MS/MS method for the simultaneous quantification of cortisol and dexamethasone in DST serum samples was developed, validated, and applied to 400 clinical samples. Newly established method-specific threshold concentrations for cortisol and dexamethasone increased specificity from 67.5 % to 92.4 % while preserving 100.0 % sensitivity.

The second part of this thesis aimed at improving the differentiation between ACC and adrenocortical adenomas (ACA). An LC-MS/MS method for the quantification of eleven urinary steroids was developed and validated. A classification model requiring only two steroids was trained and tested based on 24-h urine samples from 268 patients with adrenal tumor. Both the analytical method and the classification model were optimized to comply with the demands for clinical routine implementation, namely robustness, time efficiency, and a simple and comprehensible data evaluation and interpretation. Malignancy was excluded with a negative predictive value of 100 % in an independent validation cohort of 84 samples of 24-h urine. A newly proposed simplified diagnostic workflow with urinary steroid profiling as first tier test could obviate additional adrenal-specific imaging in 42 of 64 patients with ACA. Moreover, spot urine samples were tested as surrogate matrix for 24-h urine in the cohort of 84 patients. While analysis of spot urine resulted in a positive predictive value of 86.7 % compared to 87.5 % in 24-h urine samples, spot urine was likewise able to exclude ACC with a negative predictive value of 100.0 %.

Both the simultaneous measurement of cortisol and dexamethasone as well as the 24-h urinary steroid profiling are ready for clinical application, whereas the transfer of the urine method to the considerably simplified spot urine analysis will require prospective validation in a larger cohort. Indeed, the new DST method is already in clinical use at the University Hospital Würzburg since September 2021.

Zusammenfassung

Nebennierentumoren gehören zu den häufigsten Neoplasmen beim Menschen und treten mit einer Prävalenz von 3–10 % bei Erwachsenen über 50 Jahren auf. Häufig wird die Raumforderung zufällig im Rahmen einer bildgebenden Untersuchung erkannt. Die meisten dieser sogenannten Inzidentalome sind gutartige und hormoninaktive Nebennierenrindenadenome (ACA), die keine therapeutische Intervention erfordern. Das Ziel der Nebennierentumor-Diagnostik ist die Abklärung potentieller Hormonaktivität und Malignität, denn diese Tumoren müssen zum Großteil operativ entfernt werden. Hormonaktive Tumoren können benigne oder maligne sein und sind durch die autonome Sekretion von Steroidhormonen charakterisiert. Maligne Nebennierenrindenkarzinome (ACC) sind sehr selten, aber aggressiv und mit einer schlechten Prognose im fortgeschrittenen Tumorstadium assoziiert. Da die therapeutischen Möglichkeiten für das ACC limitiert sind, ist eine schnelle und sichere Diagnostik erforderlich. Im Rahmen dieser Doktorarbeit wurden zwei Flüssigchromatographie-Tandemmassenspektrometrie (LC-MS/MS) Methoden zur Quantifizierung von Steroiden in Biomaterialien entwickelt, um damit die Diagnostik von Nebennierentumoren zu verbessern.

Der 1 mg-Dexamethason-Hemmtest (DST) ist ein häufig durchgeführter Screening-Test zur Untersuchung auf autonome Cortisolsekretion. Dabei wird die Supprimierbarkeit der Cortisolsekretion durch die orale Einnahme von Dexamethason überprüft. Eine LC-MS/MS Methode zur simultanen Quantifizierung von Cortisol und Dexamethason im Serum wurde entwickelt, validiert und zur Messung von 400 DST-Patientenproben genutzt. Durch methodenspezifische Schwellenwertkonzentrationen für Cortisol und Dexamethason konnte die klinische Testspezifität von 67.5 % auf 92.4 % bei unveränderter Sensitivität von 100.0 % erhöht werden.

Der zweite Teil dieser Arbeit befasst sich mit der Verbesserung der Unterscheidung von ACC und ACA. Dafür wurde eine LC-MS/MS Methode zur Quantifizierung von elf Steroiden im Urin entwickelt und validiert. Über die Messung von 24-h Sammelurinproben von 268 Nebennierenrindentumor-Patienten wurde ein Klassifikationsmodell, das auf nur zwei Steroiden basiert, trainiert und getestet. Sowohl die analytische Methode als auch das Klassifikationsmodell wurden hinsichtlich Robustheit und Zeiteffizienz optimiert, um sich möglichst gut in die klinische Routine implementieren zu lassen. Außerdem lag der Fokus auf einer einfachen, nachvollziehbaren und direkten Datenauswertung und -interpretation. Als ein Hauptergebnis konnte Malignität in einer unabhängigen Validierungskohorte von 84 Patienten mit einem negativen prädiktiven Wert von 100 % ausgeschlossen werden. Nach einem vereinfachten diagnostischen Schema mit der Urin-Steroid-Analytik als erstem Screening-Test könnte bei 42 von 64 Patienten mit ACA auf eine

zusätzliche nebennierenspezifische Bildgebung verzichtet werden. Des Weiteren wurden erstmals Spontanurinproben als Surrogatmatrix für 24-h Sammelurin in der Validierungskohorte getestet. Dabei unterschied sich der positive prädiktive Wert der Spontanurine mit 86.7 % kaum von den 87.5 % der 24-h Sammelurine, während auch mit Spontanurin ein negativer prädiktiver Wert von 100 % erzielt werden konnte, was einen wichtigen Schritt in die Richtung einer vereinfachten Probensammlung darstellt.

Sowohl die simultane Quantifizierung von Cortisol und Dexamethason als auch die 24-h Urin-Steroid-Methode haben ihre Eignung für die klinische Routineanwendung bewiesen. Der Transfer der Urinmethode auf den deutlich einfacheren Spontanurin erfordert jedoch eine prospektive Validierung in einer größeren Patientenkohorte. Die neue DST-Methode wurde bereits im September 2021 in die klinische Routine am Universitätsklinikum Würzburg eingeführt.

1 Introduction

1.1 The adrenal glands

Adrenal glands (lat. *Glandulae suprarenalis*) are small, triangular-shaped paired endocrine glands with vital functions for humans that are located retroperitoneal on top of both kidneys. A fibrous capsule surrounds the adrenal gland, which is composed of the outer adrenal cortex and the inner adrenal medulla. Both parts have different embryological origins. While the cortex is derived from the mesoderm, the adrenal medulla is derived from the ectoderm and is developed from migrating cells of the neural crest (Avisse et al. 2000). The adrenal cortex is moreover subdivided into the three histologically and functionally distinct zones zona glomerulosa, zona fasciculata, and zona reticularis (Rosol et al. 2001), as depicted in Figure 1.1.

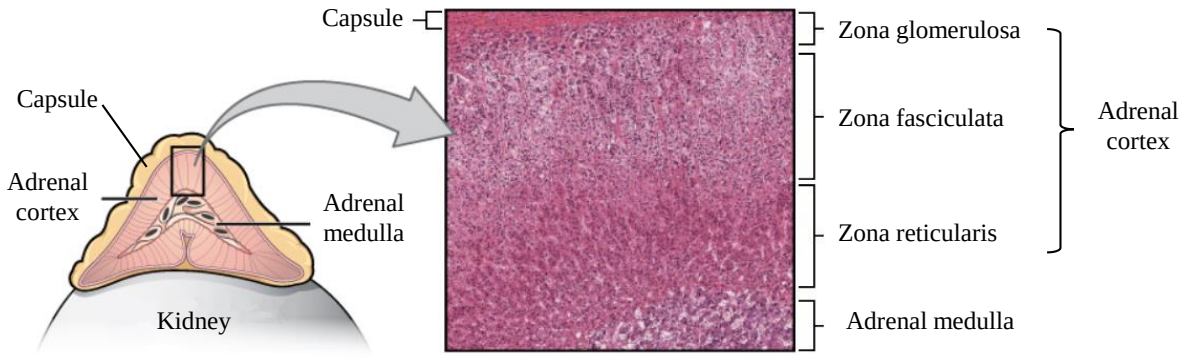

Figure 1.1. Schematic cross-sectional and microscopic structure of the adrenal gland. Modified from (Betts et al. 2013).

Besides morphology, the two parts of the adrenal differ in functionality. Both produce and secrete various hormones into the bloodstream. Chromaffin cells of the medulla release the catecholamines epinephrine and norepinephrine, regulated by the sympathetic nervous system. The neurotransmitters are derivates of the amino acid tyrosine and act as acute stress hormones initiating the “fight-or-flight” response (Carmichael and Winkler 1985). On the other hand, the adrenal cortex secretes steroid hormones whereby each layer produces specific types of steroids due to their specific enzyme expression. The outermost zona glomerulosa is the synthesis site of mineralocorticoids. In the zona fasciculata, the thickest zone of the adrenal cortex, glucocorticoids are produced. The innermost zona reticularis secretes mainly androgens and androgen precursors (Schiffer et al. 2019).

1.2 Physiological action and classification of steroid hormones

Steroid hormones are biologically active organic compounds that can be synthesized in specialized organs (e.g. adrenal glands, testes, ovaries). In very low concentrations they are capable of affecting metabolic processes and regulate the interplay between cells and organs in a wide range of physiological functions. Chemically, steroids are classified as small molecules with a molecular weight around 300 Da (O'Malley and Schrader 1976). The cyclopentaphenanthrene skeleton with three cyclohexane rings (A–C) and one cyclopentane ring (D) as well as lipophilic properties are characteristic for steroid hormones (Figure 1.2A).

Steroid hormones are transported via the blood stream to reach their target cells. Because of their lipophilic character, they can diffuse directly through the cell membrane. Intracellularly, they bind to specific steroid receptors and thereby activate the receptor to a nuclear transcription factor. The hormone receptor complex moves into the nucleus where it binds to the respective hormone response element, a short sequence of DNA within the promoter of a gene. This affects the transcription of the corresponding gene positively, also referred to as transactivation, or negatively, also known as transrepression (Beato and Klug 2000).

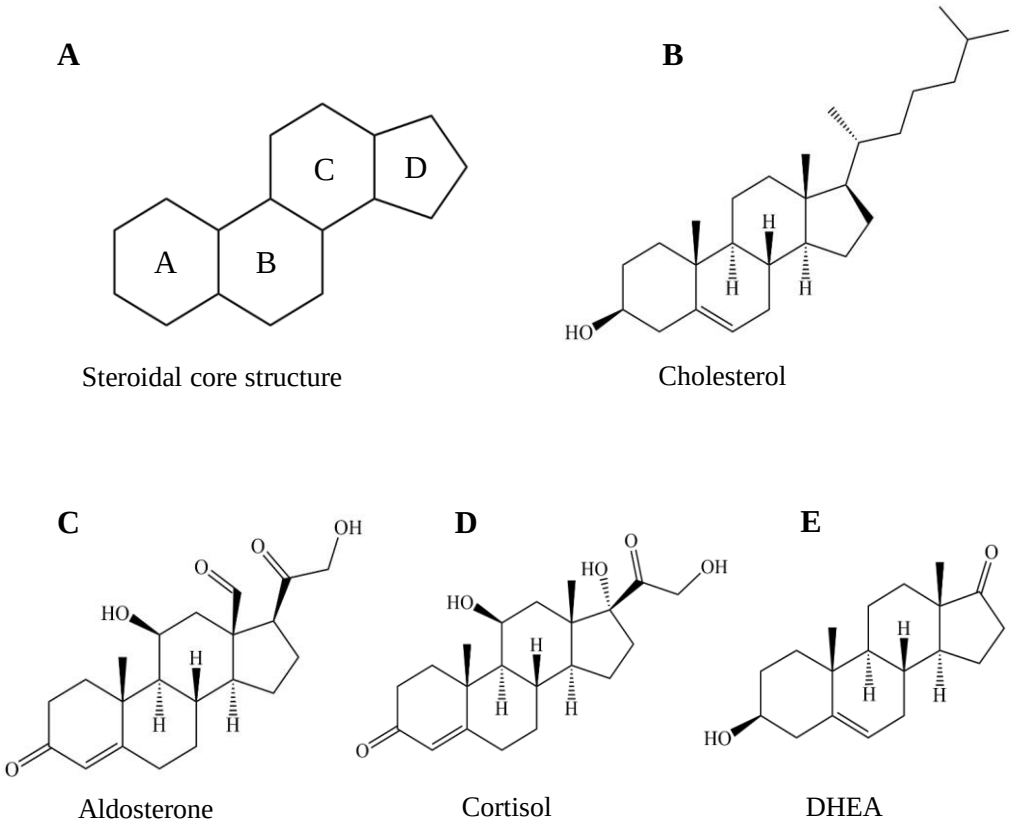

Figure 1.2. Structural formulae of steroid hormones, A: Tetracyclic steroidal core structure with rings A–D, B: Cholesterol as precursor in steroidogenesis, C: Aldosterone as mineralocorticoid, D: Cortisol as glucocorticoid, E: DHEA as androgen precursor.

Five major classes of steroids can be classified according to their binding to a specific receptor triggering specific effects, namely mineralocorticoids, glucocorticoids, androgens, estrogens, and progesterone. Thereof, mineralocorticoids, glucocorticoids, and androgen precursors and androgens are biosynthesized in the adrenal cortex, whereas most androgens, estrogens, and progesterone are of gonadal origin (Giguere et al. 1988, Schiffer et al. 2019).

The steroid hormones synthesized in each of the three adrenocortical zones have specific physiological functions. Mineralocorticoids regulate the electrolyte and fluid homeostasis and thereby the blood pressure. Their secretion is controlled by the renin-angiotensin-aldosterone system. The peptide hormone angiotensin II and potassium stimulate the synthesis of the mineralocorticoid aldosterone (Figure 1.2C). Main effects of aldosterone are increased sodium and water resorption in combination with an increased renal excretion of potassium (Booth et al. 2002).

Glucocorticoids regulate a variety of homeostatic, cardiovascular, metabolic, and immunologic functions. Cortisol (Figure 1.2D) as the main endogenous glucocorticoid is released in a circadian rhythm with a peak blood concentration in the early morning (Mohd Azmi et al. 2021). As a steroid hormone secreting organ, the adrenal cortex is part of the hypothalamic-pituitary-adrenal (HPA) axis, a physiological stress response system that connects the central nervous system to the endocrine system. Secretion of glucocorticoids is regulated by the HPA axis to maintain physiological homeostasis. The complex regulatory and control system consists of hierarchic neuroendocrine pathways and feedback mechanisms involving the hypothalamus, the anterior pituitary gland, and the adrenal cortex. Briefly, the hypothalamus produces corticotropin releasing hormone (CRH) that stimulates the pituitary gland to release adrenocorticotropin (ACTH) which further triggers the adrenal cortex to release glucocorticoids. The negative feedback of an increased glucocorticoid concentration on hypothalamus and pituitary gland inhibits further stimulation of the adrenal cortex resulting in decreased glucocorticoid secretion (Papadimitriou and Priftis 2009).

Androgens belong to the class of sex hormones and promote reproductive and anabolic functions after binding to the androgen receptor (Roy et al. 1999). The virilizing function of androgens are relevant for the metabolism of both males and females. The major adrenocortical-derived androgens dehydroepiandrosterone (DHEA, Figure 1.2E) and androstenedione show low androgenic effects but act as precursor hormones for the conversion to testosterone and dihydrotestosterone (Mo et al. 2006).

1.3 Adrenocortical steroid biosynthesis and metabolism

All adrenocortical steroids derive from the precursor cholesterol (Figure 1.2B), a C_{27} steroid, which is obtained from multiple sources. This includes *de novo* biosynthesis from acetate in the endoplasmatic reticulum, hydrolysis of cholesteryl esters stored in lipid droplets, exogenous lipoprotein-derived esters, and free cholesterol from the plasma membrane (Schiffer et al. 2019). The two major functional enzyme classes cytochrome P450 (CYP) and hydroxysteroid dehydrogenases (HSD) are involved in the biosynthesis of steroid hormones which occurs in two organelles. Synthesis begins in the mitochondria, continues in the smooth endoplasmatic reticulum, and is completed in the mitochondria (Rosol et al. 2001, Payne and Hales 2004).

As initial and rate-limiting step of steroid biosynthesis, the *steroidogenic acute regulatory protein* (StAR) transports cholesterol to the inner mitochondrial membrane (Miller 2013). Next, the side chain cleavage enzyme CYP11A1 catalyzes the conversion of cholesterol to pregnenolone (Turcu and Auchus 2015). At the stage of pregnenolone, the synthesis pathways split for mineralocorticoids, glucocorticoids, and androgens, as depicted in Figure 1.3.

In the zona glomerulosa of the adrenal cortex, pregnenolone is converted to progesterone by the enzyme HSD3B2 which is further processed to 11-deoxycorticosterone catalyzed by CYP21A2. Two isoforms of CYP11B catalyze the 11β-hydroxylation of 11-deoxycorticosterone yielding corticosterone. CYP11B2, also known as aldosterone synthase, converts corticosterone to aldosterone via 18-hydroxycorticosterone as intermediate (Schiffer et al. 2019).

For the conversion of pregnenolone to cortisol, three hydroxylation steps catalyzed by CYP monooxygenases are required that occur in the zona fasciculata. The first hydroxylation by CYP17A1 leads to 17α-hydroxyprogesterone. Subsequently, CYP21A2 catalyzes the hydroxylation to 11-deoxycortisol. The final CYP11B1-catalyzed conversion to cortisol occurs in the mitochondria of zona fasciculata cells (Schiffer et al. 2019).

Androgen precursors are produced in the adrenocortical zona reticularis and are mostly activated to the active androgens in peripheral tissues. After conversion of pregnenolone to 17α-hydroxypregnenolone, the 17,20-lyase activity of CYP17A1 yields DHEA. Some of the resulting DHEA is converted to androstenedione which serves as substrate for the aldo-keto reductase AKR1C2/4, yielding testosterone (Schiffer et al. 2019).

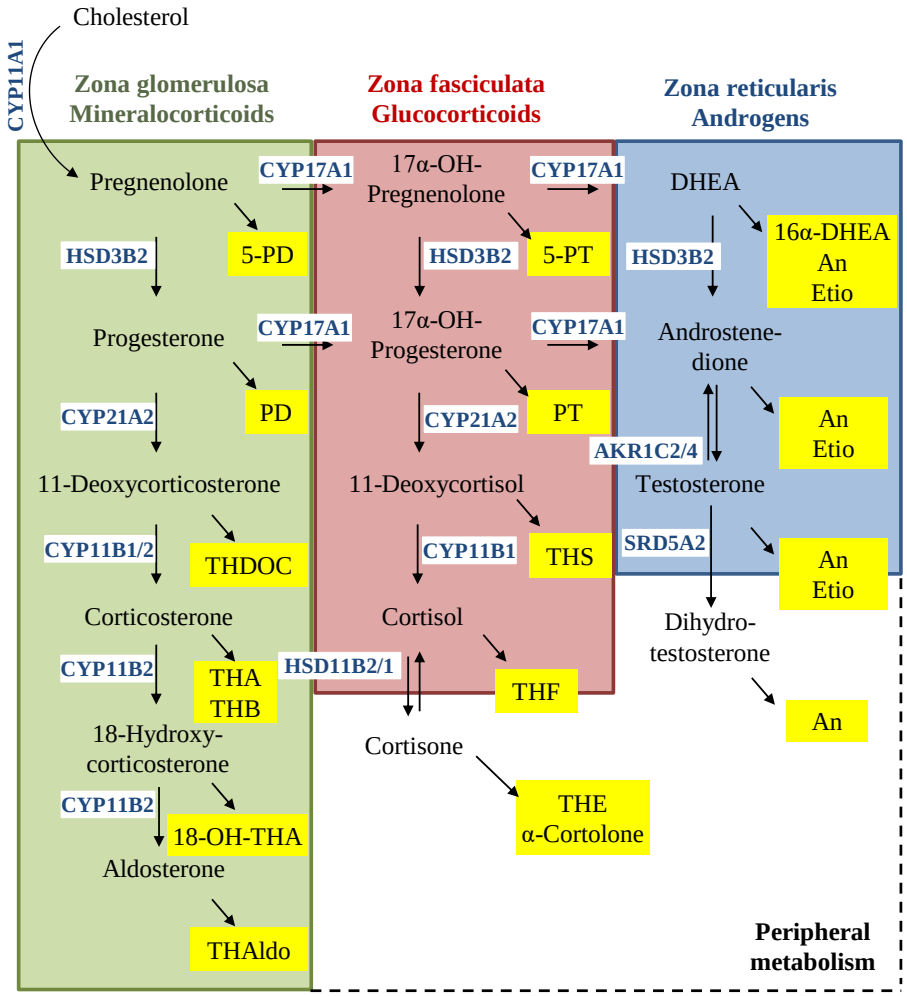

Figure 1.3. Adrenocortical steroid biosynthesis pathway with urinary metabolites pictured in yellow. An, androsterone; Etio, etiocholanolone; DHEA, dehydroepiandrosterone; PD, pregnanediol; 5-PD, 5-pregnenediol; PT, pregnanetriol; 5-PT, 5-pregnenetriol; THA, tetrahydro-11-dehydrocorticosterone; THB, tetrahydrocorticosterone; THAldo, tetrahydro-aldosterone; THDOC, tetrahydro-11-deoxycorticosterone; THE, tetrahydrocortisone; THF, tetrahydrocortisol; THS, tetrahydro-11-deoxycortisol; CYP, cytochrome P450; HSD, hydroxysteroiddehydrogenase; AKR, aldo-keto reductase; SRD, Steroid-5α-reductase. Modified from (Turcu and Auchus 2015, Storbeck et al. 2019).

Metabolism plays an important role in steroid action, inactivation, and excretion and is divided into phase I and phase II reactions. During phase I biotransformation, steroids undergo hydroxylation, reduction, or oxidation reactions. Therefore, urinary steroid metabolites are often characterized by several hydroxyl-groups and reduced double-bonds compared to their precursors. Phase I biotransformation alters the biological activity and provides functional groups as sites for conjugation during phase II metabolism. The classic phase II conjugation reactions are sulfation and glucuronidation, increasing water solubility and polarity for subsequent urinary or bile excretion (Schiffer et al. 2019).

1.4 Adrenal tumors and adrenal hormone excess

Tumors of the adrenal gland exist frequently in approximately 3–10 % of the population. Prevalence increases with age and reaches up to 10 % in people older than 70 years (Mansmann et al. 2004, Ebbehoj et al. 2020). A large proportion thereof is discovered incidentally on cross-sectional abdominal imaging for other reasons as so called incidentaloma (Young 2007). Adrenal tumors can be distinguished in terms of hormone activity, dignity, and histological origin.

1.4.1 Adrenocortical adenoma

Benign adrenocortical adenomas (ACA) represent around 85 % of all adrenal tumors (Ebbehoj et al. 2020). Tumor size is < 4 cm in 95–98 % of ACA and approximately 15 % of patients with ACA present with bilateral lesions. Most ACA are hormonally non-functional and harmless. However, a considerable proportion show biochemical evidence of adrenal hormone excess, like (possible) autonomous cortisol secretion (ACS, see also chapter 1.4.4) of variable clinical significance and severity in 40–50 % (Bancos and Prete 2021). ACA with ACS are associated with an increased risk for cardiometabolic comorbidities and mortality, and predominantly affects women younger than 65 years (Reimondo et al. 2020, Deutschbein et al. 2022, Prete et al. 2022). Aldosterone excess is diagnosed in 5–10 % of ACA and very rarely, ACA produce androgens or estrogens which results in virilization or feminization (Sherlock et al. 2020, Bancos and Prete 2021).

For unilateral adrenal tumors with clinically relevant ACTH-independent hormone excess, adrenalectomy is recommended as the standard of care according to international clinical practice guidelines. Patients with asymptomatic, non-functional ACA do not require surgery. For patients who do not fall in these categories, e.g. with ACS, an individualized approach considering age, degree of cortisol excess, comorbidities, and patient's preference is suggested. Patients with an adrenal tumor with ACS who undergo surgery should be substituted with glucocorticoids perioperatively. If the tumor is not surgically removed, annual clinical reassessments for cortisol excess and comorbidities potentially related to cortisol excess are suggested for patients with (possible) ACS (Fassnacht et al. 2016).

1.4.2 Adrenocortical carcinoma

Adrenocortical carcinoma (ACC) is a very rare but aggressive malignancy with an annual incidence of 0.5–2/1.000.000 habitants. The distribution of age at diagnosis shows a peak in the fourth and fifth decade of life. Women are affected more frequently with the female-to-male ratio being 1.2:1 (Kerkhofs et al. 2013). Approximately 60 % of patients with ACC present with evidence of adrenal steroid hormone excess, most frequently a Cushing's syndrome (CS) with or without virilization. The rapid progression of ACC is associated with a poor, but heterogeneous prognosis with a stage-dependent 5-year survival rate ranging from 13–82 % (Fassnacht et al. 2009, Else et al. 2014).

To classify the progress of a tumor and to evaluate prognosis, the European Network for the Study of Adrenal Tumors (ENSAT) established a staging system. ENSAT stages range from I to IV with increasing tumor progress (Table 1.1). Stage I includes non-infiltrating tumors ≤ 5 cm and stage IV characterizes large infiltrating tumors with distant metastases (Fassnacht et al. 2009, Lughezzani et al. 2010, Libe 2015).

Table 1.1. ENSAT classification (Fassnacht et al. 2009)

ENSAT stage	TNM-classification
I	T1, N0, M0
II	T2, N0, M0
III	T1–2, N1, M0 T3–4, N0–1, M0
IV	T1–4, N0–1, M1

T1, tumor ≤ 5 cm; T2, tumor > 5cm;
T3, tumor infiltration into surrounding tissue;
T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein;

N0, negative lymph nodes;
N1, positive lymph node(s);

M0, no distant metastases;
M1, presence of distant metastases.

The proliferation marker Ki67 as well as the mitotic count, assessed during histopathological investigation of the operated tumor, are powerful prognostic markers and important for treatment decisions (Beuschlein et al. 2015, Libe 2015). Recently, a point-based score (S-GRAS) calculated from ENSAT tumor stage, Ki67 index, resection status, age, and symptoms, was established as prognostic tool superior to tumor stage and Ki67 individually (Elhassan et al. 2021).

Medical treatment options are limited for ACC. Generally, a complete operative tumor resection (R0) is the most important curative approach and the primary therapy for nonmetastatic ACC (Fassnacht et al. 2013). However, even after tumor resection, the risk of recurrence remains high. Mitotane is the only drug approved for systemic treatment of ACC and is used both in an adjuvant setting and for advanced disease (Megerle et al. 2018, Paragliola et al. 2018). Mitotane is associated with severe adverse effects and a narrow therapeutic window while response rates are only approximately 30 % (Alyateem and Nilubol 2021). For patients with a localized and resectable tumor, adjuvant therapy depends on prognostic factors regarding the risk of recurrence. For patients with R0-resected ACC and a low risk (ENSAT stage I-II and Ki67 ≤ 10 %), active surveillance with close follow-up investigations is indicated. In contrast, patients with high-risk features (ENSAT stage III-IV or Ki67 > 10 % or recurrence) should be treated adjuvant with mitotane (Fassnacht et al. 2020, Alyateem and Nilubol 2021, Terzolo and Fassnacht 2022). The management of patients with metastatic ACC is challenging. First-line therapy for patients with irresectable ACC in advanced stages is a platinum-based chemotherapy with the combination of etoposide, doxorubicine, and cisplatine plus mitotane (EDP-M) which has proven significantly higher response rates compared to mitotane plus streptozocin (23.2 % vs. 9.2 %). Progression-free survival was longer for patients under EDP-M therapy (5.0 months vs. 2.1 months), however, there was no significant difference in overall survival between the EDP-M group and the streptozocin-mitotane group (Fassnacht et al. 2012). Several clinical trials investigating potential alternative therapies have failed to improve outcomes of patients with advanced ACC. Until now, no therapy has been shown to be similarly effective or even better than EDP-M (Alyateem and Nilubol 2021, Kiesewetter et al. 2021, Lagana et al. 2022). This makes an early and accurate diagnosis particularly important and the timepoint of initial diagnosis might be decisive for prognosis. The complete surgical R0 resection of the tumor provides the only chance of cure, however, feasibility is limited to well differentiated and nonmetastatic tumors.

1.4.3 Other adrenal tumors

Besides ACA and ACC, other benign lesions, other malignant tumors, and pheochromocytomas exist. Other benign tumors include myelolipomas, which are non-functional lesions composed of mature adipose tissue and myeloid tissue. Following ACA, myelolipoma is second most common in adrenal incidentalomas representing 6–16 %. Although benign and non-functioning, myelolipomas can present with diameters >10 cm and therefore require resection

due to mass effect symptoms (Decmann et al. 2018). Further benign noncortical tumors are rare and include ganglioneuromas, cysts, hemangiomas, lymphangiomas, and schwannomas (Bancos and Prete 2021).

Most other malignant lesions are adrenal metastases of other malignancies representing 7.5 % of all adrenal tumors. Lymphomas, sarcomas, and neuroblastomas are very rare other malignant tumors (Ebbehoj et al. 2020).

Pheochromocytomas are rare catecholamine-secreting tumors derived from chromaffin tissue of the adrenal medulla with an annual incidence of 0.6 cases/100.000 habitants and typical symptoms of headaches, palpitations, and profuse sweating. They can be benign or malignant. Rapid diagnostic workup is vital as surgical resection is the cornerstone of therapy and undiagnosed pheochromocytomas can be lethal (Neumann et al. 2019, Sherlock et al. 2020).

1.4.4 Cushing's syndrome and autonomous cortisol secretion

Cushing's syndrome (CS) is a rare disease, which results from a persistently excessive level of cortisol in the human body. Typical symptoms are central obesity with thin extremities, facial plethora, hirsutism, proximal muscle weakness, purple striae, and easy bruising (Nieman et al. 2008). Relevant comorbidities include hypertension, osteoporosis, diabetes, psychiatric disorders, and high risk for infections and cardiovascular diseases (Arnaldi et al. 2012).

Endogenous hypercortisolism can be caused by ACTH-independent cortisol secretion from adrenal tumors or bilateral adrenal hyperplasia, which makes up approximately 20 % of endogenous CS. The majority of endogenous CS is ACTH-dependent with the most common cause being ACTH-secreting pituitary tumors (Cushing's disease, ~70 %) or ectopic ACTH secretion (~10%) (Braun et al. 2019).

In patients with adrenal incidentaloma, who are biochemically evaluated, a frequent situation is biochemical evidence of hypercortisolism without clinical symptoms of CS. This constellation is nowadays called (possible) autonomous cortisol secretion (ACS). Its diagnosis is based on the result of the 1 mg dexamethasone suppression test (DST) that will be further addressed in chapter 1.5.2. Serum cortisol suppression after DST below 1.8 µg/dL excludes ACS with high sensitivity (Wood et al. 1997). Insufficient cortisol suppression with DST results between 1.8 µg/dL and 5 µg/dL are defined as "possible ACS" and cortisol above 5 µg/dL is called ACS (Fassnacht et al. 2016).

1.5 Current diagnostic workup of adrenal tumors

The term incidentaloma indicates a preliminary diagnosis of the unexpected finding of the adrenal mass and requires careful differential diagnostics. Assessment of autonomous hormone secretion and potential malignancy are the main goals of the diagnostic workup of adrenal tumors (Kerkhofs et al. 2015b). Although the vast majority are benign and non-functional ACA and require no therapeutic intervention in this case, about 20% of patients suffer from clinically relevant hormone excess and / or malignant tumors requiring active therapy. Especially the rare ACC is aggressive and associated with a high mortality in advanced stages. Therefore, the diagnostic workup is extremely important as it enables an early and potentially curative resection of ACC. On the other hand, unnecessary surgeries should be minimized to avoid potential postoperative complications or even deaths (Murphy et al. 2010), but also for economic reasons.

The differential diagnosis of adrenal tumors can be challenging. Preoperative diagnostic workup is based on morphologic evaluation by imaging and functional evaluation by hormone measurements, followed by post-operative histopathology if applicable (Libe 2015, Fassnacht et al. 2016).

1.5.1 Imaging modalities

The appearance on imaging is an important criterion for the evaluation of adrenal tumors. The most commonly used imaging techniques for characterization of adrenal pathologies are abdominal unenhanced computed tomography (CT), often followed by contrast CT, chemical shift magnetic resonance imaging (MRI), or ^{18}fluoro-deoxyglucose positron emission tomography (FDG-PET/CT) (Lockhart et al. 2002).

In general, the tumor diameter is considered for the assessment of potential malignancy, as the risk of ACC increases with tumor size. A tumor diameter larger than 4 cm diagnoses ACC with high sensitivity of 93 % at relatively poor specificity of 42 % in a retrospective multicenter study, whereas a cutoff at 6 cm improves specificity at the expense of sensitivity (Mantero et al. 2000). Tumor size remains an important predictor of malignancy, however there is a clear overlap of benign and malign tumors, as small ACC in early stages and large benign adrenal tumors are found occasionally. Therefore, the diagnostic accuracy is insufficient when solely considering tumor size (Barnett et al. 2000). Follow-up investigations to monitor potential tumor growth are required.

Besides tumor size and growth within a period of time, morphology is considered for the evaluation of adrenal tumors. Three imaging techniques are mainly used for differential diagnostics: CT, MRI, and positron emission tomography (PET) with radiotracer-labelled fluorodeoxyglucose (^{18}F-FDG). CT and MRI are suitable tools for the identification of benign lesions and the exclusion of ACC, whereas FDG-PET is superior for the detection of malignant disease (Fassnacht et al. 2018).

Tumor tissue attenuation in Hounsfield units (HU) on unenhanced CT scan indicates the density and lipid content of the tumor. Approximately 70 % of ACA contain significant intracellular lipid appearing with low HU, whereas almost all ACC appear with high HU due to low intracellular lipid content (Blake et al. 2010). The threshold < 10 HU is frequently applied for the diagnosis of ACA with 71 % sensitivity and 98 % specificity (Young 2011). Recent findings suggest better diagnostic accuracy for the differentiation of benign tumors and ACC using 20 HU as cutoff (Bancos et al. 2020). For lesions with HU > 10, a specific contrast washout procedure should be performed. Rapid relative wash-out > 40 % or absolute wash-out > 60 % after 15 minutes are indicative of ACA (Kerkhofs et al. 2015b). However, the established thresholds are associated with limited diagnostic accuracy and a relative percentage wash-out cutoff at 58 % was recently proposed for excellent ACC identification although the negative predictive value (NPV) was moderate (Schloetelburg et al. 2021). Drawbacks of CT imaging are the fact that still a substantial amount of ACA is false-positively considered as suspicious for ACC. Moreover, the ionizing radiation exposure is associated with risk of inducing malignancies (Cawood et al. 2009).

MRI can be an alternative if CT-iodinated contrast is contraindicated, exposure to radiation should be avoided (e.g. during pregnancy), or if findings on CT are inconclusive (Young 2011). Especially the development of chemical shift analysis has improved the diagnostic accuracy of MRI as it allows for detection of intracellular lipid in adrenal masses. ACA with a high content of intracellular lipid are characterized by a signal loss on out-of-phase images compared to in-phase images while malignant lesions remain unchanged (Fassnacht et al. 2016).

Functional nuclear medical imaging modalities like PET and the combination with CT as PET/CT have shown valuable results for differentiating benign from malignant adrenal masses (Boland et al. 2009, Groussin et al. 2009, Deandreis et al. 2011). Adrenal uptake of ^{18}F-FDG higher than hepatic uptake is considered to be of malignant origin (Blake et al. 2010). FDG-PET is suitable for detection of metastases and thereby for staging and follow-up investigations. However, solely FDG-PET cannot differentiate between ACC, adrenal metastases, or malignant

pheochromocytoma (Deandreis et al. 2011) and recent data indicate that even few ACC present with low FDG-uptake (He et al. 2021). As relatively new tracer, [^{123}I] iodometomidate has shown suitability for proving adrenocortical origin as it specifically binds to adrenocortical CYP11B enzymes but is not suitable for the differentiation between benign and malignant lesions (Hahner et al. 2008, Hahner et al. 2013).

Even though imaging modalities generally provide good performances in the diagnostics of adrenal tumors, current evidence on cut-offs for ACC diagnosis is heterogenous and disappointingly poor (Dinnes et al. 2016). Many patients with adrenal incidentaloma have to undergo multimodal imaging and even unnecessary surgeries, therefore the second pillar of preoperative diagnostics, the hormonal workup, is of great importance.

1.5.2 Hormonal workup

Endocrine workup begins with a physical examination for overt symptoms of hormone excess. These include signs of CS like central obesity with thin extremities, a round face, abdominal stretch marks, and easy bruising. Moreover, signs of androgen excess like virilization, hirsutism, or acne and aldosterone excess (hypertension, hypokalemia) are assessed (Stifelman and Fenig 2005). Overall, it is the aim to prove or (mostly) to exclude a hormonally functional adrenal tumor. The following aspects are important to consider during the hormonal workup.

Exclusion of pheochromocytoma

An important part of the endocrine workup is the determination of metanephrines, the metabolites of epinephrine and norepinephrine, in plasma or 24-h urine to test for pheochromocytoma. Elevated metanephrine concentrations indicate catecholamine-secreting tumors of the adrenal medulla. However, certain medication may generate false positive results, e.g. tricyclic antidepressants, serotonin reuptake inhibitors, and sympathomimetics (Neary et al. 2011).

Identification or exclusion of autonomous cortisol secretion

As indicated above, patients with adrenal tumors frequently present with some kind of autonomous cortisol secretion. Various biochemical tests for the assessment of potential autonomous cortisol secretion (ACS) can be applied. The low-dose overnight DST is the most

frequently performed initial screening test (Terzolo et al. 2011). Therefore, the patient takes 1 mg dexamethasone (Dex) as a single oral dose at night (11 pm). In the next morning between 8–9 am, blood is withdrawn and the serum cortisol concentration is determined. In patients without ACS, the exogenous glucocorticoid Dex causes a suppression of the endogenous cortisol biosynthesis due to the negative feedback mechanism via the HPA axis, as depicted in Figure 1.4. A suppression of serum cortisol below 1.8 µg/dL excludes autonomous cortisol secretion with high sensitivity (Wood et al. 1997).

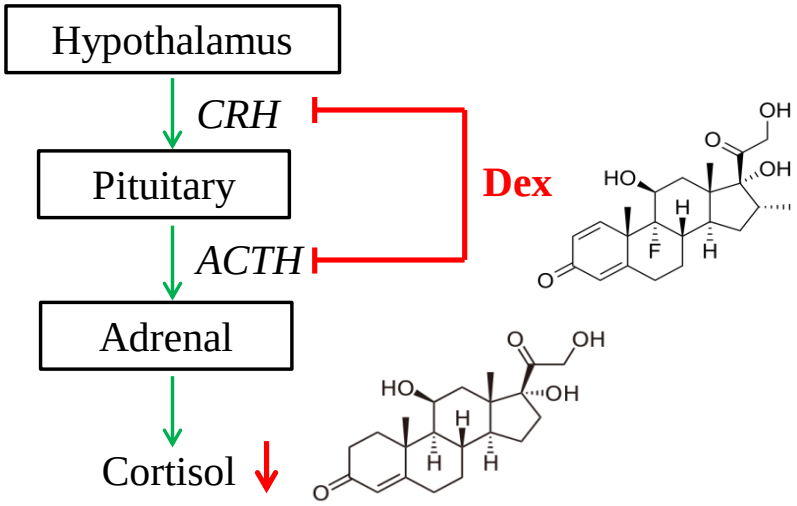

Figure 1.4. Principle of the dexamethasone suppression test. By exogenous dexamethasone, in patients without autonomous cortisol secretion, endogenous cortisol production is suppressed due to negative feedback on corticotropin releasing hormone (CRH) and adrenocorticotropin (ACTH) in the HPA axis.

Besides the 1 mg DST, determination of cortisol in saliva or urine are alternatives as initial screening tests with high diagnostic accuracy. Because of the physiological circadian rhythm of cortisol secretion, salivary samples should be collected between 23:00–24:00 h at night and a 24-h urine collection should be performed. Patients with abnormal results are recommended to undergo a second test, either one of the above, determination of serum midnight cortisol, or a 2 mg-48h-DST. If two tests are positive, the next step is to look for the cause auf hypercortisolism, which is commonly performed by determination of plasma ACTH. Suppressed plasma ACTH is highly suggestive of ACTH-independent adrenal CS (Nieman et al. 2008, Debono and Newell-Price 2016).

Exclusion of primary aldosteronism

In patients with hypertension or hypokalemia, potential autonomous aldosterone excess should be investigated. The diagnostic workup of primary aldosteronism is a multistep process consisting of screening, confirmatory testing, and subtype differentiation. The commonly performed screening test is measurement of the plasma aldosterone-to-renin ratio, however, interfering medication is often causing false-positive test results. Therefore, a confirmatory test like the saline load test or the captopril challenge test is necessary to confirm or exclude primary aldosteronism (Reincke et al. 2021).

Steroid hormone profiling

The term steroid hormone profiling which describes a multicomponent chromatographic analysis has been used since the mid-1960s (Shackleton 1986). The simultaneous detection of several steroids is time-efficient and attractive for diagnosis and monitoring of adrenal tumors. For the evaluation of preoperative adrenocortical hormone excess, various glucocorticoids, androgens, and mineralocorticoids are determined in body fluids. In particular, concentrations of the sex steroids and precursors dehydroepiandrosterone sulfate (DHEAS), 17-hydroxyprogesterone, androstenedione, 11-deoxycortisol, testosterone, and estradiol should be measured in blood plasma of patients with suspected ACC (Fassnacht et al. 2020).

Analytical methods used for hormone assessment

Hormone measurements in various biomatrices are commonly performed with immunoassays in clinical routine as these assays are quick and comparably cheap. Examples are radioimmunoassays, enzyme-linked immunosorbent assays, chemiluminescent immunoassays, and electrochemiluminescent immunoassays (Karashima and Osaka 2022). A severe drawback of these assays are cross-reactions of similar metabolites with antibodies of the assay, which lead to interferences and consequently the determination of excessive concentrations. As an alternative to immunoassays, mass spectrometry-based quantification methods can detect steroid hormones with high specificity and sensitivity and significantly reduce interfering cross-reactions. Especially the coupling of chromatographic separation to mass spectrometry, for example gas chromatography mass spectrometry (GC-MS) or liquid chromatography mass spectrometry (LC-MS), results in excellent specificity (Soldin and Soldin 2009).

The preoperative diagnostic workup based on morphologic and functional evaluation is summarized in Table 1.2.

Table 1.2. Preoperative diagnostic workup of adrenal incidentalomas

Morphologic evaluation	**Functional evaluation**
• Abdominal CT with and without contrast or MRI with chemical shift → Determination of size, heterogeneity, lipid content, and contrast washout • CT thorax → Screening for metastases • Possibly FDG-PET scan	Biochemical workup for • Pheochromocytoma o Plasma metanephrines o 24h-urinary metanephrines • Cushing syndrome o 1 mg DST o 24h-urinary free cortisol o Midnight salivary cortisol • Hyperaldosteronism o Potassium in serum o Aldosterone-renin ratio in plasma • Hyperandrogenemia (all in serum) o DHEAS o 17-hydroxyprogesterone o Androstenedione o Testosterone

1.5.3 Histopathology

A fine-needle biopsy is not recommended for primary tumors suspicious of ACC as diagnostic accuracy is low and violation of the tumor capsule might induce tumor cell spread and metastasis (Williams et al. 2014, Fassnacht et al. 2018). Adrenal biopsy should only be performed in the diagnosis of adrenal metastasis in patients with a history of extra-adrenal malignancy and if the expected findings are likely to alter the management of the individual patient after exclusion of catecholamine-producing tumors (Bancos et al. 2016).

Histopathological examination is performed after surgical tumor resection for final diagnosis. It is currently considered as the gold standard for diagnosing an ACC. However, the pathology reports also provide the basis for prognosis and decisions on adjuvant therapy. Adrenocortical origin of the tumor must be confirmed, e.g. by determination of steroidogenic factor 1, a transcription factor characteristic of steroidogenic tissue, which is moreover of prognostic

relevance (Sbiera et al. 2010, Duregon et al. 2013). Multiple macroscopic and microscopic parameters can be used to discriminate benign from malignant tumors. Macroscopically, ACC are often large and heterogenous with a brown to yellow surface and areas of necrosis. Microscopically, the Weiss system is the best validated score for the differentiation of ACC and ACA. Nine characteristics regarding tumor morphology, cytology, and invasion are evaluated, and the sum of positive items makes up the Weiss score (Table 1.3). Less than three applicable features indicate a benign tumor, whereas a Weiss score ≥ 3 indicates the diagnosis of ACC (Weiss 1984, Aubert et al. 2002, Volante et al. 2008, Papotti et al. 2011).

Table 1.3. Weiss scoring system

Characteristic
High nuclear grade (III or IV) using the criteria of (Fuhrman et al. 1982)
Mitotic rate > 5 per 50 high-power fields
Abnormal mitoses
Clear cells ≤ 25 % of the tumor volume
Diffuse architecture in > 1/3 % of the tumor
Necrosis
Venous invasion
Sinusoidal invasion
Capsular invasion
Total Weiss Score = Sum of applicable characteristics

1.6 Analytical principles of the applied LC-MS/MS technique

LC-MS/MS is an emerging tool for steroid quantification in biomaterials and is increasingly implemented in endocrinological laboratories (Vogeser and Parhofer 2007, Kushnir et al. 2010) due to its high sensitivity and specificity, which is crucial for the analysis of hormones. In the early 1970s liquid chromatography was first coupled to mass spectrometry and revolutionized by the development of the electrospray ion source in the 1980s (Pitt 2009, Busetti and Swann 2013). As shown in Figure 1.5, the combination of a chromatographic separation via high performance liquid chromatography (HPLC) and determination of ion mass-to-charge ratios (m/z) by mass spectrometry enables a highly selective and quantitative analysis of complex mixtures. Target compounds in complex matrices are quantified by signal responses in relation to an internal calibration of standards with known concentrations. Considering the peak area ratio of analytes to constant amounts of stable isotopically labelled internal standards as signal response helps to control variability due to potential sample loss and matrix effects in a quantitative assay (Sargent 2013).

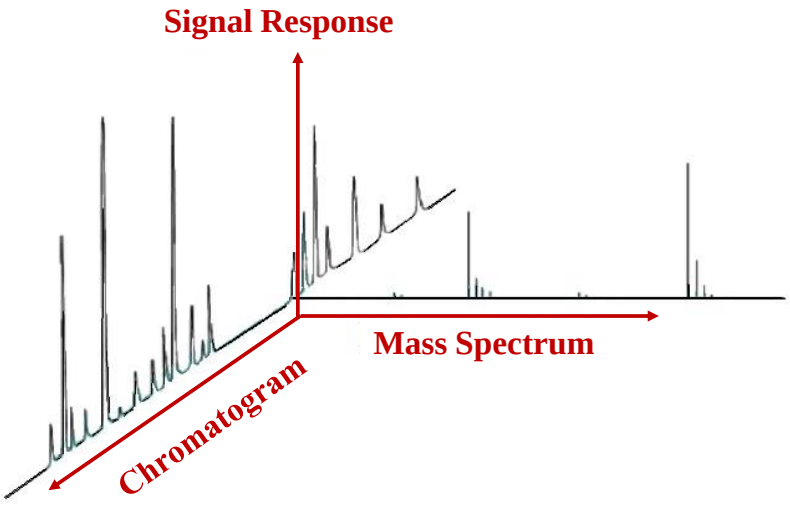

Figure 1.5. Three dimensions of LC-MS: The components of a complex mixture are chromatographically separated, identified, and quantified. Modified from (Sargent 2013).

In the HPLC system, analytes are separated from matrix residuals and other molecules chromatographically according to their physical interactions between a stationary and a mobile phase. The mobile phase elutes the analytes from the analytical column (stationary phase) within a specific retention time depending on the analytes' polarity. Reversed phase (RP) chromatography is a frequently used mode of action with a non-polar stationary phase and a polar elution solvent. In this case, non-polar compounds are retained stronger by the column

and polar compounds elute first. To change the polarity of the mobile phase, a solvent mixture can be used for elution. Isocratic elution with constant eluent composition and step gradients or gradient elution where the eluent composition changes in steps or continuously can be used for an effective separation of components (Belanger et al. 1997).

Following chromatography, the analytes reach the mass spectrometer. In general, a mass spectrometer consists of an ion source, a mass analyzer, and a detector. In the ion source analytes are ionized and evaporated into gas phase. Electromagnetic forces guide the charged particles in the mass analyzer and filter specific mass-to-charge ratios, which are captured in the detector. In both methods of this thesis, analytes are ionized by electrospray ionization (ESI), a soft ionization technique with generally minimal molecule fragmentation upon ionization (Banerjee and Mazumdar 2012). In the next step, analytes are transferred from liquid to gas phase by ESI via the following processes (Figure 1.6): the analyte solution enters the ionization region via a high-voltage capillary tip at atmospheric pressure. Charged droplets are formed as the electric field causes an excess of equally charged ions on the capillary tip that repel each other and leave the capillary as a fine aerosol. The droplet size decreases with solvent evaporation supported by heat and inert gas. Finally, ions pass an aperture in the counterpole to reach the mass analyzer (Banerjee and Mazumdar 2012, Lottspeich and Engels 2012).

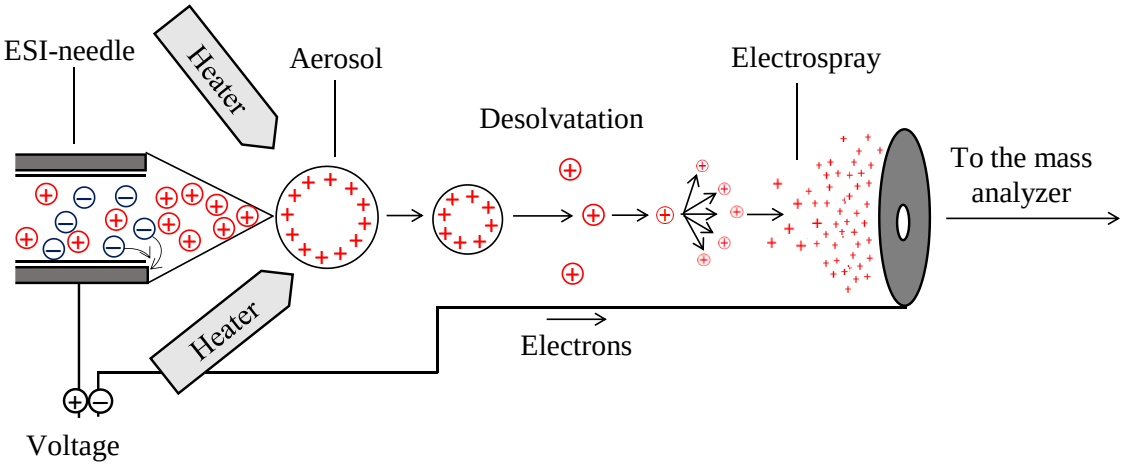

Figure 1.6. Process of electrospray ionization. Modified from (Lottspeich and Engels 2012).

A quadrupole mass analyzer indicates four rod magnets that cause an alternating electric field. By the application of a specific voltage and frequency of polarity-switching, ions are forced on a spiral track and only ions of a certain mass-to-charge ratio (*m/z*) are accelerated through the quadrupole as they fly on the stable trajectory. Other ions are deflected and neutralized (Wong 2020).

Tandem mass spectrometry (MS/MS) is an analytical technique which subjects ions to two or more sequential stages of mass analysis according to their mass-to-charge ratio (m/z) (Banerjee and Mazumdar 2012). A triple-quadrupole mass spectrometer is an example for MS/MS with increased selectivity due to the coupling of two mass analyzers and is the mass analysis technique used within this thesis. Three quadrupoles are connected in series (Figure 1.7). The first quadrupole (Q1) acts as a filter for a precursor ion m/z which receives a compound-specific collision energy in the collision cell (Q2) where the precursor ions dissociate to form product ions. Only a specific product ion m/z is selected from the fragments by the third quadrupole (Q3) and reaches a detector, typically an electron multiplier. Within one chromatographic run, many mass transitions can be recorded simultaneously in so-called multiple reaction monitoring (MRM) mode which is highly specific because of the two selection steps and the typical fragmentation patterns of most analytes (Vogeser and Parhofer 2007).

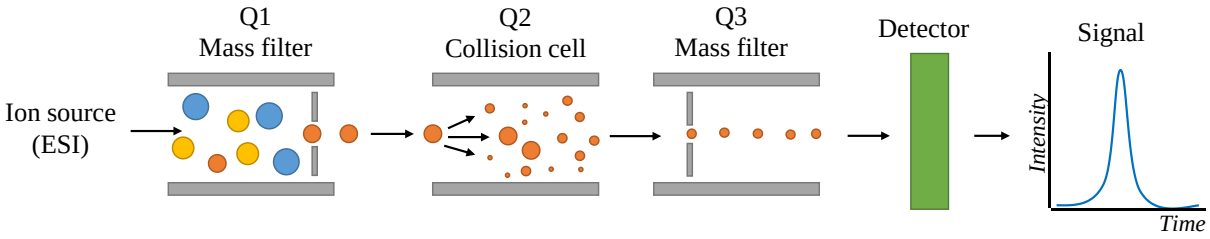

Figure 1.7. Triple-quadrupole mass spectrometer in MRM-mode. Modified from (Schmidt et al. 2008).

1.7 Mass spectrometry-based methods in the endocrine workup of adrenal tumors

Steroid profiling by mass spectrometry-based methods has been reported to have promising potential in the endocrine workup of adrenal tumors. A 13-steroid serum panel based on LC-MS/MS was applied to 10 ACC patients and 38 non-ACC patients and significantly increased concentrations particularly for the cortisol precursor 11-deoxycortisol were found in patients with ACC (Taylor et al. 2017). Schweitzer et al. quantified 15 steroids in plasma samples of 66 patients with ACA and 42 patients with ACC and discovered sex-specific diagnostic signatures composed of six steroids after logistic regression modeling (Schweitzer et al. 2019). Berke et al. retrospectively evaluated plasma steroid profiles of 19 steroids in a cohort of 577 patients with adrenal incidentaloma and showed valuable benefits for the identification of ACC, primary aldosteronism, and pheochromocytoma (Berke et al. 2022).

Several groups have performed steroid metabolite profiling in 24-h urine for the differentiation of ACC and ACA using GC-MS (Arlt et al. 2011, Kerkhofs et al. 2015a, Velikanova et al. 2016). More recently, two LC-MS-based methods were developed for urinary steroid quantification. Hines et al. determined 26 steroids in 24-h urine samples of 71 patients with adrenal diseases and found statistically significant differences in the steroid excretions of ACC vs. ACA and adrenal vs. pituitary CS (Hines et al. 2017). In the prospective multicenter study EURINE-ACT, Bancos et al. measured 15 steroids by LC-MS/MS in 24-h urine and tested a machine-learning algorithm to differentiate ACC from other adrenal tumors. In combination with the imaging characteristics tumor diameter and CT attenuation in unenhanced CT scans, a positive predictive value (PPV) of 76.4 % and NPV of 99.7 % were achieved (Bancos et al. 2020).

1.8 Objectives of this thesis

The diagnostic workup of adrenal tumors is based on imaging and hormone assessment with the aim to assess potential functionality and malignancy. Currently, the initial evaluation of adrenal tumors includes at least the unenhanced CT attenuation and the 1 mg DST (Corssmit and Dekkers 2019). The overall aim of this thesis is to improve adrenal tumor diagnostics by the development, validation, and clinical application of LC-MS/MS methods for the quantification of steroid hormones in biological matrices. Therefore, the focus was set on the optimization of the DST and the application of urinary steroid profiling in the diagnostic workup of adrenal tumors.

The DST, a frequently performed screening test in the hormonal workup of adrenal incidentalomas, exhibits two weaknesses in interpretation when only the serum cortisol concentration is quantified. First, the inter-individual pharmacokinetics of Dex are neglected that result in a variability of Dex exposure between individuals and thereby substantially affect the cortisol suppression (Meikle 1982). Second, the threshold to rule out autonomous cortisol secretion in the low-dose DST is set very low at 1.8 µg/dL (50 nmol/L) cortisol, yielding excellent clinical sensitivity at moderate specificity. This threshold was derived from the early era of immunoassays (Wood et al. 1997) but is commonly applied to any laboratory technique for cortisol quantification. The first part of this work included the optimization and validation of an LC-MS/MS method for the simultaneous quantification of cortisol and Dex in DST serum samples (chapter 2).

The following questions are aimed to be answered:

- What is the extent of inter-individual variation in Dex exposure after the 1 mg DST?
- Which demographic or clinical factors affect the serum Dex concentration?
- Can method-specific cutoff concentrations for Dex and cortisol increase clinical test specificity?

The second part of this thesis addresses the differential diagnosis of frequently occurring benign adrenocortical adenomas (ACA) and rare, malignant adrenocortical carcinoma (ACC) which can be challenging with the currently applied methods in clinical routine. However, a fast and assured diagnosis with high clinical sensitivity is extremely important, as operative tumor resection is the only treatment to potentially cure ACC (Fassnacht et al. 2013). On the other hand, redundant surgeries of hormone-inactive and harmless ACA should be avoided. In contrast to the DST, the quantification of steroid metabolites in 24-h urine is not yet part of the current guidelines for the diagnostic workup of adrenal tumors (Fassnacht et al. 2016, Fassnacht et al. 2020). In recent years, promising results were published using urinary steroid profiling by LC-MS/MS for the differentiation of ACC and ACA (Hines et al. 2017, Bancos et al. 2020). Due to the increasing application of LC-MS/MS in clinical laboratories, urinary steroid profiling might be a suitable complementation to the existing biochemical analyses in adrenal tumor investigation. However, existing methods include large multi-steroid panels or are combined with machine learning algorithms for classification. The complex and time consuming data evaluation and interpretation has hampered the transfer to routine application in clinical practice. Within this thesis, an LC-MS/MS method was developed and validated for the simultaneous quantification of eleven steroid metabolites in urine (chapter 3). Furthermore, the method was applied to urine samples of patients with adrenal tumor with the aim of establishing a simple and comprehensible classification model suitable for clinical routine application (chapter 4).

The following questions are aimed to be answered:

- Which urinary steroids are most suitable for the classification of ACC and ACA?
- How good is the diagnostic performance of urinary steroid profiling for the differentiation of ACC from ACA?
- Is 24-h urine replaceable by spot urine?

2 Simultaneous quantification of cortisol and dexamethasone in DST serum samples

The content of this chapter has been published in Clinical Chemistry 2021, 67(7), 998–1007, https://doi.org/10.1093/clinchem/hvab056 (Vogg et al. 2021). LC-MS/MS method development was started by Benedict Gräsl as part of his Bachelor thesis (Graesl 2019). Within this PhD thesis, the method was optimized, validated, and applied to clinical samples of patients who underwent DST.

For individual author contributions, see Table A5 and Table A8. Permissions for reprint were obtained from all co-authors and from the publisher under a Creative Commons license (CC BY-NC-ND 4.0).

Method-Specific Cortisol and Dexamethasone Thresholds Increase Clinical Specificity of the Dexamethasone Suppression Test for Cushing Syndrome

Nora Vogg[1,2], Max Kurlbaum[1,2], Timo Deutschbein[1,3], Benedict Gräsl[1], Martin Fassnacht[1,2], Matthias Kroiss[1,2,4]

[1]Department of Internal Medicine I, Division of Endocrinology and Diabetes, University Hospital Würzburg, Germany

[2]Central Laboratory, Core Unit Clinical Mass Spectrometry, University Hospital Würzburg, Germany

[3]Medicover Oldenburg MVZ, Oldenburg, Germany

[4]Department of Internal Medicine IV, LMU University Hospital Munich, University of Munich, Munich, Germany

Abstract

Background: The dexamethasone suppression test (DST) is the recommended first-tier test for suspected Cushing syndrome (CS). Missed dexamethasone intake or insufficient dexamethasone serum exposure may yield false positive results. Quantification of serum dexamethasone in DST samples may therefore improve test performance.

Methods: Simultaneous quantification of dexamethasone and cortisol by liquid chromatography-tandem mass spectrometry in 400 DST serum samples (100 overt CS, 200 excluded CS, 100 adrenal incidentalomas with (possible) autonomous cortisol secretion, AI-ACS) randomly selected within the indication groups. The 2.5th percentile of dexamethasone in patients with excluded CS was considered the lower limit of normal (LLN).

Results: Serum dexamethasone varied from undetectable to 20.2 ng/mL with a median of 4.8 ng/mL (95 % CI 4.5-5.1 ng/mL). Dexamethasone was undetectable in only 16 patients (4 %), suggesting non-compliance. The dexamethasone LLN was 1.8 ng/mL (4.6 nmol/L). Decreased glomerular filtration rate and diabetes mellitus were associated with higher serum dexamethasone concentration, while body mass index, sex, age, nicotine, and oral contraceptives had no significant effect. By excluding the 27 samples with dexamethasone <LLN and applying the method-specific cortisol cutoff of 2.4 µg/dL (66 nmol/L) to samples with suspected CS, the clinical specificity for CS increased from 67.5 % to 92.4 % while preserving 100 % clinical sensitivity. Among 100 AI-ACS samples (defined by immunoassay), 4 samples had dexamethasone <1.8 ng/mL and 14 samples had cortisol <2.4 µg/dL, which excluded autonomous cortisol secretion.

Conclusions: Quantification of dexamethasone and method-specific cortisol cutoffs in DST samples may reduce the false positive rate and lower the proportion of patients requiring further workup.

Introduction

Cushing syndrome (CS) is a rare disease characterized by hypercortisolism and associated with relevant morbidity and impaired overall survival *(1-4)*. The low-dose overnight dexamethasone (Dex) suppression test (DST) is the most widely used laboratory test for the diagnosis of CS and recommended first-tier test if CS is suspected *(5-9)*. Following oral administration of 1 mg Dex at 11:00 PM, serum cortisol concentration is determined in a blood sample collected the next morning between 8.00 AM and 9:00 AM. Serum cortisol suppression to 1.8 µg/dL (50 nmol/L) or lower excludes autonomous cortisol secretion with high clinical sensitivity *(10)*. In addition, the test has been recommended to stratify further workup of adrenal incidentaloma patients in current guidelines *(11)*. For patients without clinical signs of CS but with insufficient cortisol suppression after Dex, the term possible autonomous cortisol secretion has been suggested when cortisol is in the range of 1.8–5.0 µg/dL. Autonomous cortisol secretion (ACS) is suggested for a cortisol concentration >5.0 µg/dL *(11)*. However, there is an ongoing debate about the best test strategy in this setting *(12)*.

Given the high clinical sensitivity of DST as a first-tier test, the low cutoff value chosen to exclude hypercortisolism leads to a relatively low clinical specificity *(13)*. Positive DST results require further diagnostic workup by using late night salivary cortisol or 24-h urinary free cortisol measurement *(5)*. Various reasons may lead to insufficient Dex exposure and thereby also to false-positive tests. Among those, missed Dex ingestion by the patient is frequently suspected but can rarely be ascertained. Variable absorption, distribution, metabolism, and elimination of Dex may confound test results *(14, 15)*. Known examples are food or drug interactions through enzyme induction (e.g., phenytoin, rifampicin).

The quantification of Dex in serum in addition to cortisol may provide information about possibly insufficient Dex exposure and may help to identify false positive tests. Dex in DST samples has historically been measured by radioimmunoassay *(16-19)* which is associated with lack of analytical specificity and cross-reactivity with structurally similar compounds *(20, 21)*. More analytically specific and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods with the ability to measure several analytes simultaneously have recently been developed *(22-26)*. The impact of Dex measurement by LC-MS/MS on DST performance was evaluated in two studies with a total case number of 502 patients, but including only 27 patients with overt CS and 27 patients with ACS *(23, 26)*. Even if earlier studies suggested cutoff values between 1.3 ng/mL (3.3 nmol/L) and 1.8 ng/mL (4.6 nmol/L) to verify sufficient Dex exposure allowing for adequately suppressed serum cortisol *(23-26)*,

the value of concomitant Dex quantification and the threshold to apply are still under discussion.

We here report the development and validation of an LC-MS/MS method for the simultaneous determination of Dex and cortisol in the same DST serum sample. We applied this method to 400 DST samples of patients in whom CS was suspected. The aim of our study was to evaluate whether Dex quantification reduced the proportion of false positive test results in a large number of pathological DST samples, to establish method-specific Dex and cortisol cutoff concentrations, and to investigate factors that could possibly influence serum Dex exposure during DST.

Materials and Methods

Patients and samples

The retrospective study was approved by the ethics committee of the university of Würzburg (correspondence 20200930 01) and individual patient informed consent waived. DST blood samples of patients in whom endogenous CS was suspected or who were diagnosed with an adrenal incidentaloma at the University Hospital Würzburg between February 2008 and November 2019 were collected in an S-Monovette (Serum-Gel, Sarstedt AG & Co. KG). After 30 min resting at room temperature, samples were centrifuged for 5 min at 4,000 x g and stored at -20°C.

Patients with cortisol concentrations >1.8 mg/dL post Dex underwent further workup according to current guidelines *(5, 11)*.

400 samples were included in the study: 100 from patients with overt endogenous CS, 200 samples from patients in whom CS was excluded, and 100 samples from patients with adrenal incidentalomas with (possible) autonomous cortisol secretion (AI-ACS) (Supplemental Figure 2.1). The sample size for the control cohort used for Dex threshold development as lower limit of the reference range was determined by the availability of biomaterial and considering applicable guidelines *(27, 28)*. The sample size for the method-specific cortisol threshold adaption can be justified by the precision of estimates of diagnostic accuracy. Analyses were performed in an unblinded fashion.

Clinical and further biochemical data were obtained from patients' records. Renal function was evaluated by the estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease formula with eGFR>90 mL/min/1.73m^2 considered normal and chronic

kidney disease stages mild (60–89 mL/min/1.73m^2), moderate (30–59 mL/min/1.73m^2), and severe (<30 mL/min/1.73m^2).

Measurement of serum cortisol

Routine measurements of cortisol were performed immediately after blood sampling with a standard immunoassay (Immulite® 2000 XPi, Siemens Healthcare GmbH) following the manufacturer's instructions. Limit of detection of the immunoassay was 0.2 µg/dL with a quantification limit of 1.0 µg/dL and a precision <9.4 %. The LC-MS/MS method for cortisol measurement is described below.

Standards and reagents

Cortisol, cortisol-d4, dexamethasone, acetic acid, and ammonium acetate were purchased from Sigma-Aldrich Chemie GmbH. Dexamethasone-d5 was purchased from Toronto Research Chemicals Inc. MS-grade water and methanol were from VWR International GmbH and acetonitrile from Merck KGaA.

Sample preparation

200 µL sample (calibrator, QC or unknown patient sample) were mixed for 30 s with 200 µL precipitation reagent [(methanol:acetonitrile (1:1) containing deuterated internal standards at 30 ng/mL dexamethasone-d5 and 50 ng/mL cortisol-d4, stored at -20°C]. After centrifugation at 21,382 x g for 10 min, 200 µL supernatant were diluted with 100 µL mobile phase A (see below) and centrifuged again. 150 µL supernatant were transferred into an HPLC vial for further analysis.

LC-MS/MS conditions

An Agilent 1290 Infinity HPLC system (Agilent Technologies Germany GmbH & Co. KG) was used for chromatography. Mobile phases consisted of LC-MS-grade water with 2 mM ammonium acetate and 0.04 % (V/V) acetic acid adjusted to pH=3.8 (mobile phase A) and LC-MS-grade methanol with 2 mM ammonium acetate and 0.04 % (V/V) acetic acid (mobile phase B). 25 µL prepared sample were injected onto an Oasis HLB 15 µm 2.1x20 mm online solid phase extraction column (Waters GmbH) with valve position to waste and after one-minute run time switching to the analytical column. Analytes were separated chromatographically on an XBridge BEH C18, 2.5 µm, 3.0x75 mm analytical column during a total run time of 5.35 min

and additional column auto-equilibration of one minute before each injection. Retention times were at 2.92 min for cortisol and 2.98 min for Dex.

For LC-MS/MS, a QTRAP 4500 MD (AB Sciex Germany GmbH) was used in electrospray ionization positive mode. Measurements were performed in the multiple-reaction monitoring mode with the following mass transitions (*m/z*) for cortisol (quantifier: 363.1→120.9, qualifier: 363.1 → 97.1), dexamethasone (393.1→355.1), cortisol-d4 (367.1→120.9), and dexamethasone-d5 (398.1→360.1). Methodological details are provided in the Supplemental Tables (Chromatography: Supplemental Table 2.1; Mass spectrometry: Supplemental Table 2.2) and a representative chromatogram in Supplemental Figure 2.2.

Linearity of quantification was assessed for Dex from 1.0 to 60.0 ng/mL and for cortisol from 1.0 to 60.0 µg/dL in water as surrogate matrix. Quality controls (QC) were prepared by spiking standard solution into plasma with low QC (QC1) containing 2.0 ng/mL Dex and 9.4 µg/dL cortisol and high QC (QC2) containing 10.0 ng/mL Dex and 12.9 µg/dL cortisol.

Concentrations were calculated with Analyst Software (1.6.3, Sciex) via 6-point calibration and 1/x weighting. Correctness of quantification was verified for cortisol by measurement of commercial in vitro diagnostics quality controls (Mass*Chrom*® Steroids in Serum/Plasma, Chromsystems Instruments & Chemicals GmbH) and certified ring trial samples from the German Reference Institute for Bioanalytics (RfB).

Method validation

Method validation was oriented to the recommendations of the Center for Drug Evaluation and Research, May 2018 *(29)*. Calibration curves of 9 independent runs were utilized to evaluate linearity. The coefficients of determination for Dex were >0.988 in all runs and >0.998 for cortisol in every calibration curve. Detailed linearity data are supplied in Supplemental Table 2.3. The limit of detection, defined by a signal-to-noise ratio >3, was 0.5 ng/mL for Dex and 0.5 µg/dL for cortisol and the lower limit of quantitation was the lowest calibration level at 1 ng/mL for Dex and 1 µg/dL for cortisol, both with a signal-to-noise ratio >10.

Intra-assay precision (percent coefficient of variation) and accuracy (percent relative error) were calculated by analyzing 10 QC samples of each level in one run. Inter-assay precision was determined by measuring concentrations of both QC levels in triplicate in 8 independent runs (n=24 for each QC level). Intra-assay precisions were <8.8 % and inter-assay precisions <13.8 % for both analytes. Details for precision and accuracy are provided in Supplemental Table 2.4.

Matrix effects were evaluated by comparing slopes of a matrix calibration curve with a calibration curve in water. Ion enhancement of 112 % for Dex and 110 % for cortisol were detected. Recovery was found to be 101 % for Dex and 83 % for cortisol.

Statistical analysis

Statistical analyses were performed using SPSS version 26 (IBM Corp.) and OriginPro 2020b (OriginLab Corp.). Subject characteristics are given as median (range) for continuous data. Normal distribution of data was evaluated by Shapiro-Wilk test. Groups were compared by Mann-Whitney U test or Kruskal-Wallis test, with a p-value <0.05 considered statistically significant. Pearson coefficient was used to test for linear correlation. Test performance was evaluated by receiver operating characteristics analyses. From a clinical perspective, we considered excellent clinical sensitivity more important than an optimized compromise between clinical sensitivity and specificity (Youden's index) and therefore aimed to maintain a 100 % clinical sensitivity. We applied three different cortisol thresholds: 1.8 µg/dL as the commonly used threshold in the literature, but also the threshold with the highest clinical specificity while maintaining 100 % clinical sensitivity and the threshold with the highest Youden's index. Positive predictive value was calculated as the ratio of true positives to total positives and negative predictive value results in the true negatives divided by the total negatives. Influences of clinical variables were assessed by multiple linear regression modelling with eGFR, body mass index (BMI), sex, age, nicotine consumption, use of oral contraceptives, and the diagnosis of diabetes mellitus as covariates.

Results

Clinical characteristics

Dex and cortisol were quantified by LC-MS/MS in 400 DST samples tested for clinical suspicion of CS or during the endocrine workup of an adrenal incidentaloma. Demographic and clinical characteristics are listed in Table 2.1.

Table 2.1 Demographic and clinical characteristics of DST samples.

	Overt CS	CS excluded	AI-ACS
Patient samples, n	100	200	100
Females, n	81	130	62
Age in years, median, (range)	52 (20–77)	52 (17–85)	65 (26–83)
BMI [kg/m^2], median, (range)	27.5 (18.9–57.4)	32.0 (11.6–62.5)	27.8 (18.9–50.4)
Diabetes mellitus, n	32	39	36
Smokers, n /	21 /	31 /	32 /
Ex-smokers, n	20	33	18
eGFR (MDRD) [mL/min/1.73m^2], median, (range)	91 (13–263)	86 (27–157)	76 (7–145)
Oral contraceptives, n	7	10	1
Serum cortisol concentration after Dex [µg/dL] (median, range)	12.1 (2.5–59.8)	1.5 (0.6*–43.0)	3.9 (1.7–17.4)
Serum dexamethasone in ng/mL, (median, range)	5.0 (0.0*–20.2)	4.6 (0.0*–11.4)	5.4 (1.0–14.9)
Serum dexamethasone concentration <1.80 ng/mL, n	11	16	4

* below LOD; BMI, body mass index; MDRD, Modification of Diet in Renal Disease; CS, Cushing syndrome; AI-ACS, Adrenal incidentaloma with (possible) autonomous cortisol secretion.

Serum Dex concentrations in DST samples

Serum Dex concentrations of the 400 study samples were highly variable and ranged from undetectable to 20.2 ng/mL with a median concentration of 4.8 ng/mL Dex. Overall, Dex was undetectable in only 16 of 400 samples (4 %), indicating missed Dex administration. CS could be excluded in 10 of these patients while the diagnosis of CS in the remaining 6 patients was supported by further diagnostic testing.

Comparison of serum Dex concentration in DST samples from patients with CS (median = 5.1 ng/mL), patients with excluded CS (median = 4.7 ng/mL), and AI-ACS-samples (median = 5.4 ng/mL) missed the prespecified significance level of 0.05 (p=0.059, Kruskal-Wallis; Figure 2.1). Samples with undetectable Dex were excluded prior to this analysis.

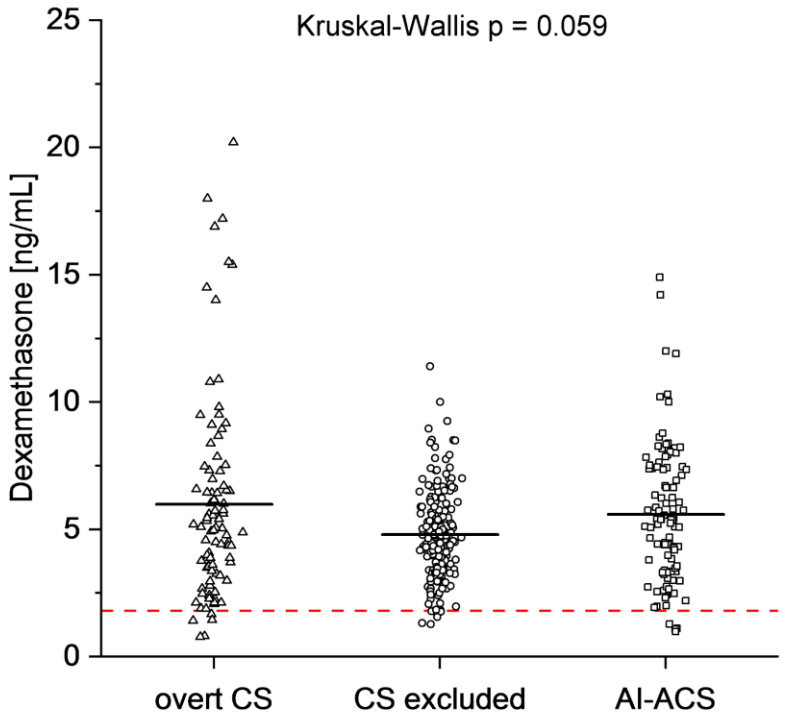

Figure 2.1. Serum dexamethasone concentrations after dexamethasone suppression test in 94 patients with Cushing syndrome (CS) compared to 190 patients in whom CS was excluded, and 100 samples of patients with adrenal incidentaloma with (possible) autonomous cortisol secretion (AI-ACS) missed the prespecified significance level of 0.05 (p=0.059). 16 samples without detectable dexamethasone were excluded. Black horizontal lines indicate the mean serum dexamethasone concentrations in each group. The broken red line represents the threshold of 1.8 ng/mL Dex.

Dexamethasone threshold development

To determine reference values for Dex after DST, the Dex concentrations in all 137 DST samples with a negative DST result (defined by serum cortisol <1.8 µg/dL during routine testing) were analyzed resulting in a median serum Dex concentration of 4.8 ng/mL and a serum Dex range from 1.3 ng/mL to 11.4 ng/mL. The 2.5th percentile at 1.8 ng/mL was considered as lower limit of the reference range and hence set as the minimal Dex concentration leading to an adequate serum cortisol suppression. By applying the Dex cutoff and excluding 27 samples

with Dex below 1.8 ng/mL among 100 samples with overt CS and 200 samples with excluded cortisol excess, test specificity increased from 67.5 % to 71.7 %.

Impact of clinical characteristics on serum Dex concentration

To investigate influences on serum Dex concentration, the following factors were investigated: eGFR, BMI, sex, age, nicotine consumption, use of oral contraceptives, and diagnosis of diabetes mellitus. DST samples with no detectable Dex (n=16) that may therefore be false positive (e.g. due to non-compliance) were excluded.

Median Dex concentration increased from 4.5 ng/mL (range 0.8–16.9 ng/mL) in 165 patients with a normal renal function to 4.9 ng/mL (range 1.1–17.2 ng/mL) in 175 patients with a mild chronic kidney disease and 7.2 ng/mL (range 2.3–20.2 ng/mL) in 38 patients with a moderate chronic kidney disease. Highest median Dex was measured in 6 patients with severe chronic kidney disease at 9.5 ng/mL (range 4.6–18.0 ng/mL) (Figure 2.2 A). A moderate but significant correlation was found between eGFR and Dex concentration with a Pearson correlation coefficient $r=-0.25$ ($p=2.2*10^{-5}$).

A significantly higher Dex concentration (0.8 ng/mL difference) was found in 104 patients with diabetes mellitus with a median of 5.5 ng/mL compared to 280 samples from patients without diabetes mellitus with a median of 4.7 ng/mL ($p=0.009$) (Figure 2.2 B).

BMI did not show any effect on the serum Dex concentration, neither did sex, age, nicotine consumption, or use of oral contraceptives. eGFR and diabetes mellitus retained statistically significant association with Dex concentration after multiple linear regression (Supplemental Table 2.5).

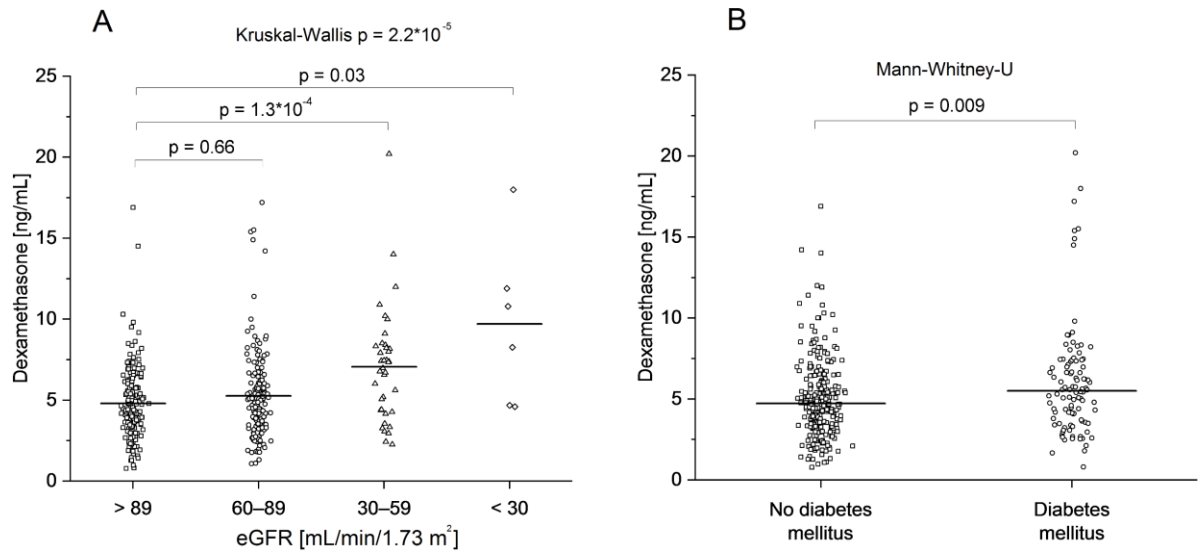

Figure 2.2. Effects of eGFR and diabetes diagnosis on serum dexamethasone A) A lower eGFR [mL/min/1.73 m^2] leads to a higher serum dexamethasone concentration. B) Patients with diabetes mellitus showed a significantly higher serum dexamethasone concentration than patients without diabetes mellitus.

Method-specific cortisol threshold

As recommended by the Endocrine Society Clinical Practice Guideline for the diagnosis of Cushing syndrome *(5)*, a method-specific threshold for our LC-MS/MS assay was established. For this, only samples with Dex concentrations above 1.8 ng/mL were considered (i.e., 89 samples from patients with confirmed CS and 184 samples from patients with excluded CS) to exclude bias from insufficient Dex exposure. Positive predictive value and negative predictive value with 95 % confidence intervals are listed in Table 2.2.

Table 2.2. DST performance at different cortisol cutoff concentrations. 2x2 tables and calculated positive predicted value (PPV) and negative predictive value (NPV) for the cortisol cutoff concentrations 1.8 µg/dL, 2.4 µg/dL, and 3.1 µg/dL

Cortisol cutoff [µg/dL]		CS	CS excluded	Total	PPV [%] (95% CI)	NPV [%] (95% CI)
1.8	Test positive	89	52	141	63.1 (54.6–71.0)	100 (96.5–100)
	Test negative	0	132	132		
	Total	89	184	**273**		
2.4	Test positive	89	14	103	86.4 (77.9–92.1)	100 (97.3–100)
	Test negative	0	170	170		
	Total	89	184	**273**		
3.1	Test positive	86	4	90	95.6 (88.4–98.6)	98.4 (94.9–99.6)
	Test negative	3	180	183		
	Total	89	184	**273**		

CS, Cushing syndrome.

Receiver operating characteristics analysis was performed using the clinical diagnosis based on routine endocrine workup for classification. Clinical sensitivity and specificity with 95 % confidence intervals were calculated with adjusted cortisol cutoff values. Specificity increased from 71.7 % at 1.8 µg/dL (Figure 2.3 A) over 92.4 % at 2.4 µg/dL (Figure 2.3 B) to 97.8 % at 3.1 µg/dL (Figure 2.3 C). Even though the threshold at 3.1 µg/dL cortisol resulted in the best sum of clinical sensitivity and specificity (Youden index), we defined 2.4 µg/dL as the method-specific cortisol cutoff concentration, since it maintained 100 % clinical sensitivity. The adaption of the cortisol threshold relies on consideration of diagnostic accuracy at three cortisol thresholds after receiver operating characteristics analysis of the 273 samples with i) ascertained diagnosis and ii) Dex >1.8 ng/mL (89 CS/184 CS excluded).

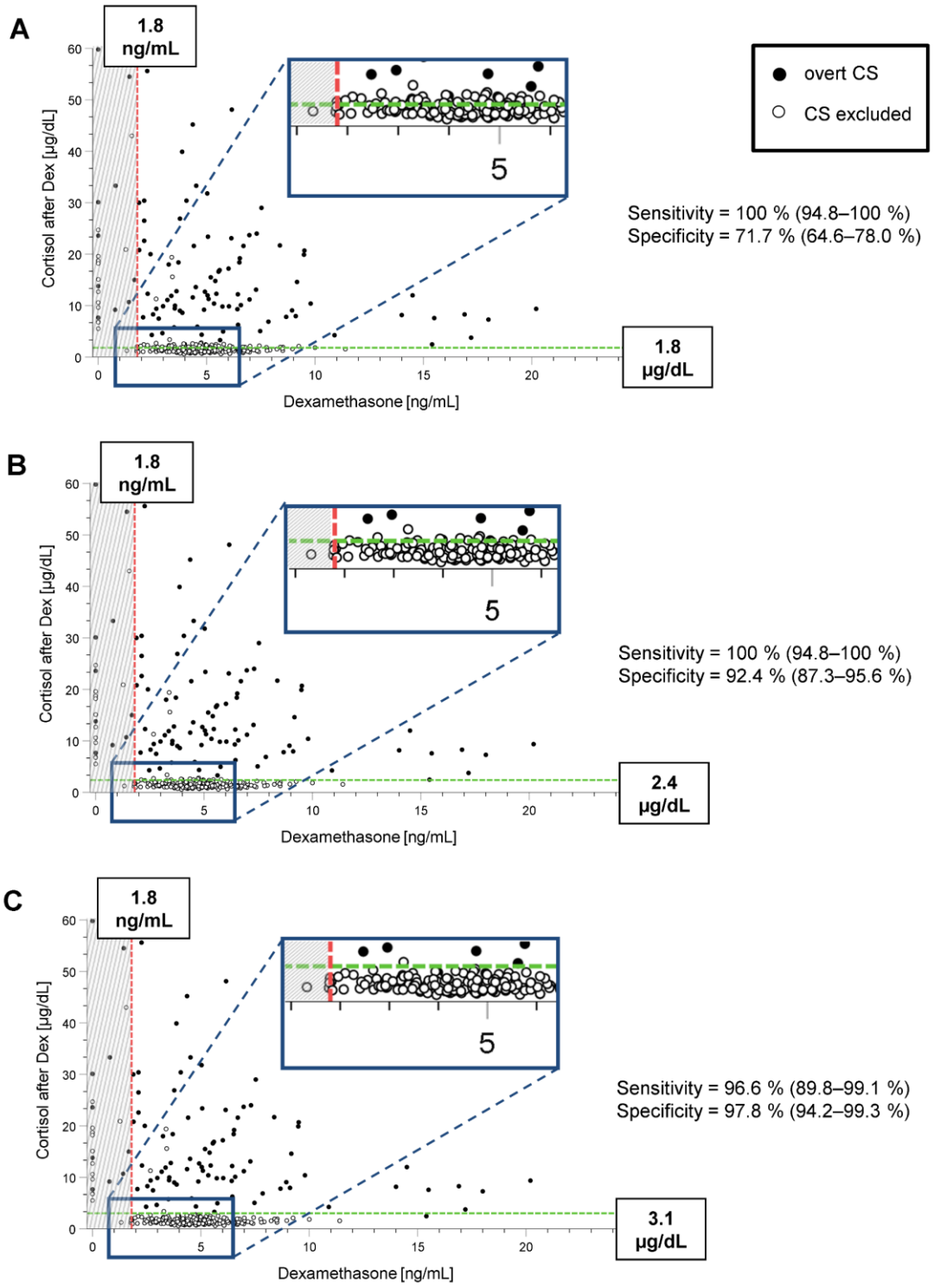

Figure 2.3. Diagnostic specificity of the dexamethasone suppression test improves by adjusting the serum cortisol cutoff from 1.8 µg/dL (A) to 2.4 µg/dL (B) and 3.1 µg/dL (C, broken green line). To maintain clinical sensitivity, the method-specific cortisol cutoff of 2.4 µg/dL (B) was chosen. Samples in the grey-shaded area are excluded from receiver operating characteristics analysis due to insufficient dexamethasone (Dex) exposure (broken red line).

The split between the groups does not represent prevalence in general population or a broader population with suspected CS but approximates proportions in a specialized center with many suspected cases. Under the assumption of a CS proportion of 33 % in the available sample collection, with the reported sample size clinical specificity of 71.7 % (cortisol cutoff at 1.8 µg/dL) can be estimated with a precision of 13.4 %. Clinical specificity of 92.4 % (cortisol cutoff at 2.4 µg/dL) can even be estimated with a precision of 8.3 % and clinical sensitivity of 100 % can be estimated with a precision of 5.2 % (=width of the 95% CI according to the method of Score (Wilson)). Therefore, the sample size can be considered sufficient for a precise estimate of clinical sensitivity and specificity.

Clinical relevance of Dex measurement in DST samples from patients with adrenal incidentalomas with autonomous cortisol secretion (AI-ACS)

After establishing the new thresholds, Dex and cortisol were quantified in 100 DST samples from patients with AI-ACS. The former diagnosis was re-evaluated applying the newly established cutoff values for Dex and cortisol (Figure 2.4).

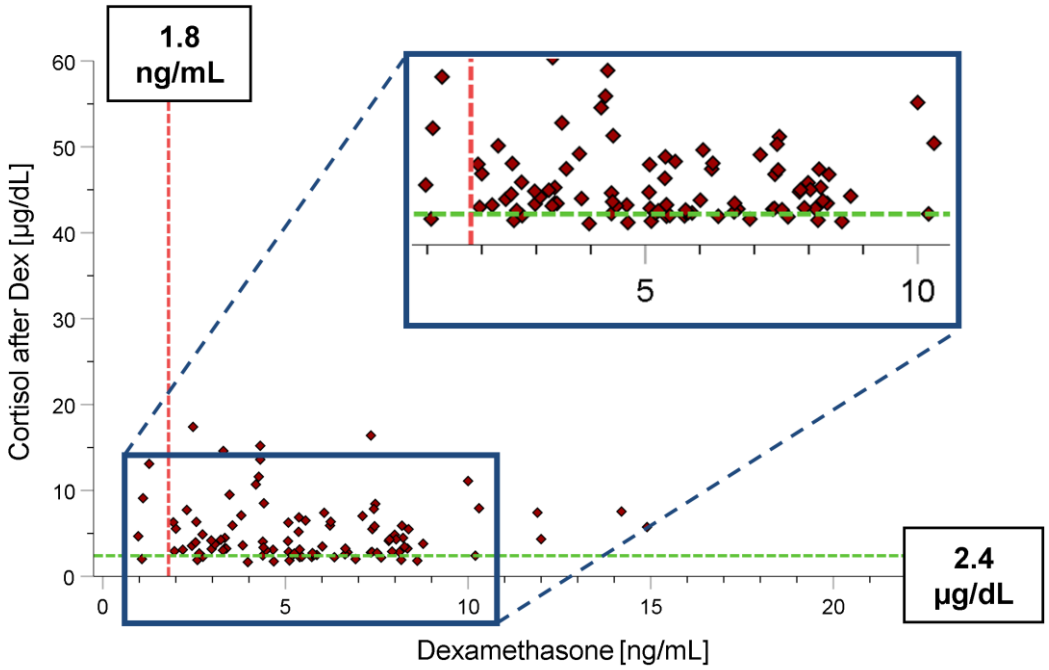

Figure 2.4. Application of the cortisol and Dex thresholds to the AI-ACS validation cohort. In 100 samples from patients with adrenal incidentaloma with (possible) autonomous cortisol secretion, 4 samples had dexamethasone (Dex) concentrations below the Dex threshold of 1.8 ng/mL (broken red line) and 14 samples showed suppression below the method-specific cortisol threshold of 2.4 µg/dL (broken green line).

Appropriate test execution can be questioned in four samples for which the threshold for Dex of 1.8 ng/mL was not reached (thereby indicating possibly inadequate Dex exposure). In 14 samples, serum cortisol was below the adapted cortisol cutoff of 2.4 µg/dL. For these patients, the exclusion of autonomous cortisol secretion could be considered.

Discussion

Here we developed and applied an LC-MS/MS method for the simultaneous quantification of cortisol and Dex to a large population of patients, thereby demonstrating its diagnostic value for an improved interpretation of DST results. False positive tests due to non-compliance or insufficient Dex exposure (<1.8 ng/mL) can now be clearly identified and – by applying the method-specific cutoff for cortisol (2.4 µg/dL) – clinical specificity improved in a clinically relevant manner.

First, we found an extremely broad range of Dex concentrations after administration of 1 mg at 11:00 PM the previous day; this finding is similar to earlier reports *(10, 16)*. This stresses the relevance of inter-individual variations in absorption, distribution, metabolism, and elimination. There was no association of Dex exposure with disease state. High variability of Dex exposure was also reported, using therapeutic Dex doses *(30)*. Blood sampling in the morning after Dex administration (8 AM – 9 AM) may not accurately assess real exposure given that the peak Dex in a pharmacokinetics study was observed already after ~1 h *(31)*. In addition, the rather low dose of only 1 mg Dex may contribute to the variable exposure. An unusually high Dex exposure could cause false negative test results, even though this case might be extremely rare. Nevertheless, Dex concentrations have to be interpreted individually and cortisol may be suppressed even below the Dex threshold due to the different sensitivity of hypothalamic CRH neurons. This is illustrated by the fact that 5 patients with Dex <1.8 ng/mL in our cohort still had suppressed cortisol.

Ueland et al. proposed a Dex cutoff value of 1.3 ng/mL (3.3 nmol/L) to verify a minimal concentration required for an adequate cortisol suppression *(23)*. This cutoff value was later confirmed by Hawley et al. *(24)*. In contrast, Ceccato et al. calculated a Dex threshold of 1.8 ng/mL (4.5 nmol/L) *(26)*. The latter cutoff complies very well with our currently calculated threshold of 1.8 ng/mL.

Importantly, variabilities of Dex exposure are also relevant for other tests such as the 8 mg overnight Dex suppression test that is used for the differential diagnosis of corticotropin-dependent CS *(32)*.

No impact on the serum Dex concentration was detected by age, sex, BMI, or nicotine consumption, confirming results from previously published studies *(23, 26, 33)*. We could observe a negative correlation with kidney function, which was also described by Ueland et al. *(23)*, whereas Ceccato et al. only detected this effect in their small CS cohort (n=16) and not in the control group *(26)*. Additionally, we found a significant effect by diabetes mellitus also after multivariable adjustment.

The cutoff for cortisol after 1 mg DST of 1.8 µg/dL recommended by current guidelines has the aim of maximizing clinical sensitivity. This value has been proposed in the early era of immunoassays *(10)*. Although it is important to adapt cutoffs to the specific method applied, the limited number of CS patients severely hampers definition of own cutoffs by each center. An advantage of our study is that we were able to compare 100 patients with proven CS to 200 patients in whom CS was ruled out. The high clinical sensitivity of DST was not decreased when we adapted the cortisol threshold from 1.8 µg/dL to 2.4 µg/dL for our LC-MS/MS method, thereby increasing test specificity from 71.7 % to 92.4 %. This outcome is similar to the specificity of the more inconvenient-to-perform 2-day low dose DST which is not recognized as a first-tier test by most centers anymore. While for many tests the aim is to find an appropriate compromise between clinical sensitivity and specificity that is reflected in the Youden index, the DST is used as a screening test and aims at maximizing sensitivity, which is why we rather accepted false positive results than false negative results.

The high frequency of cross-sectional imaging leads to an increasing number of incidentally discovered adrenal tumors *(11)*. While imaging criteria in combination with steroid mass spectrometry of 24-h urine samples have recently confirmed to enable reliable detection of malignancy *(34, 35)*, the endocrine workup for clearly benign adrenal incidentalomas still poses a relevant clinical challenge. The identification of patients with subclinical or only mild CS has remained a matter of controversy and ongoing research. Clinically, the risk of unnecessary surgery needs to be balanced against the potentially deleterious effects of chronic tissue exposure to long-term glucocorticoid excess *(11)*. Using our new cutoffs for Dex and cortisol, among the 100 DST samples of patients with AI-ACS, autonomous cortisol secretion was excluded in 14 patients. This is a clinically relevant proportion in whom the current practice of repeated testing (with its potential psychological disturbance) may be omitted.

Our study has potential limitations. First, the sample size is still limited due to the rarity of patients with overt CS. However, the number of patients in our study considerably exceeds that of previously published studies and appears sufficient for reliable statistical analyses. In

addition, the proportion of patients with CS compared to those in whom CS was excluded may reflect the situation in a referral center and not that in general population or primary endocrine care. Moreover, since no external quality controls were available for Dex, a certified reference standard was used to prepare quality controls in our laboratory, ensuring the best possible level of analytical quality. Further, samples were collected for several years resulting in a comparably long period of time between cortisol quantification by immunoassay and the LC-MS/MS analysis. However, cortisol degradation was considered marginal in view of the good comparability of cortisol concentrations between methods. Even though we developed thresholds based on a relatively large study given the rarity of CS, the absolute number is still limited and the study is retrospective in nature. Consequently, independent validation is necessary.

In conclusion, the developed Dex threshold turns out to be a valuable tool to evaluate sufficient Dex exposure during DST. The patients with unsuppressed cortisol and insufficient Dex exposure should either perform a repeat DST in case of non-compliance or undergo another diagnostic testing procedure like late-night salivary cortisol or 24-h urinary free cortisol measurement. Applying method-specific cutoffs for Dex and cortisol significantly improved the specificity of DST from 67.5 % to 92.4 %, while preserving 100 % sensitivity. Thus, our data clearly highlight the necessity to establish method-specific cutoffs, which is often neglected in clinical practice.

Funding

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Disclosures

The authors have no conflicts of interest to disclose.

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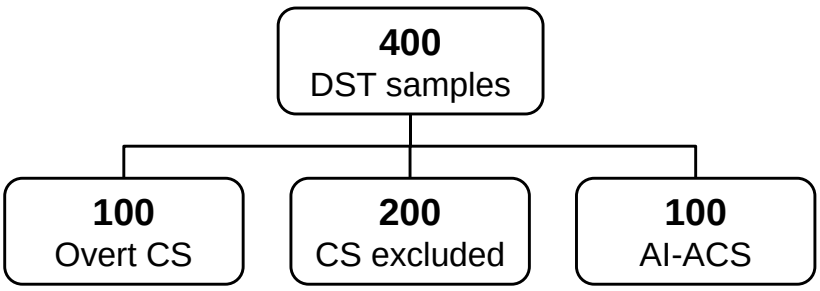
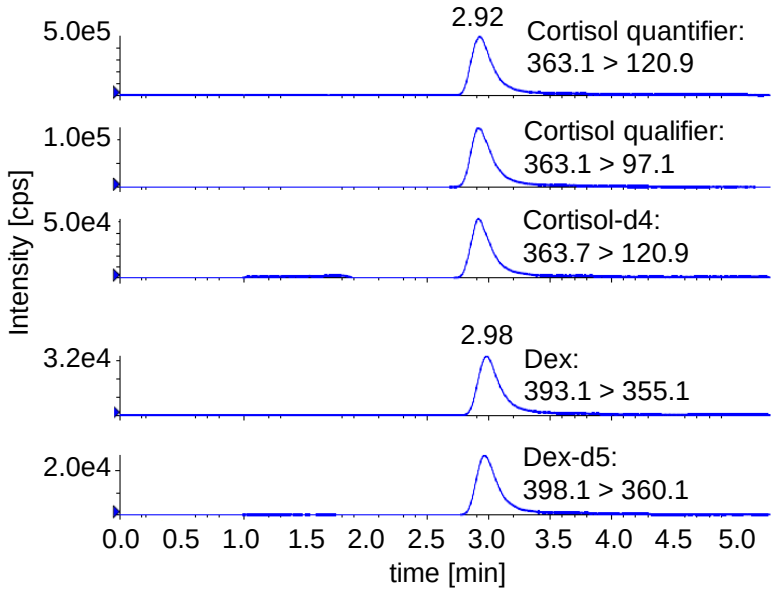
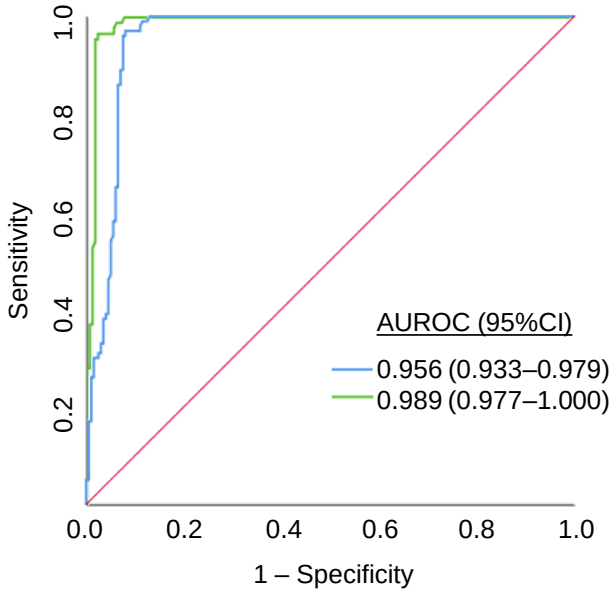
Supplemental Figures



Supplemental Figure 2.1. Diagnoses of the 400 DST serum samples.



Supplemental Figure 2.2. Chromatogram of the m/z transitions for cortisol and cortisol-d4 at a retention time of 2.92 minutes and of Dex and Dex-d5 at 2.98 minutes.



Supplemental Figure 2.3. Comparison of ROC-curves including 100 samples of CS patients and 200 samples with excluded CS (blue curve) versus 89 CS patients and 184 excluded CS patients with serum Dex > 1.8 ng/mL (green curve).

Supplemental Tables

Supplemental Table 2.1. Flow rates and mobile phase composition during chromatography.

Total time [min]	Flow rate [µL/min]	Mobile phase B [%]
0.00	500	5
1.00	500	5
1.01	350	85
5.00	350	95
5.01	500	85
5.30	500	85
5.31	350	5
5.35	350	5

Supplemental Table 2.2. Mass transitions and MS parameters for dexamethasone (Dex), cortisol and their internal standards.

Analyte	Precursor ion (*m/z*)	Fragment ion (*m/z*)	DP [V]	EP [V]	CE [V]	CXP [V]
Dexamethasone	393.1	355.1	85	10	19	10
Cortisol	363.1	120.9	95	10	30	7
		97.1	125	10	55	8.5
Dexamethasone-d5	398.1	360.1	85	10	19	10
Cortisol-d4	367.1	120.9	95	10	30	7

CE, Collision energy; CXP, Collision cell exit potential; DP, Declustering potential; EP, Entrance potential

Supplemental Table 2.3. Method linearity.

Analyte	Intercept, N=9, mean (SD)	Slope, N=9, mean (SD)	Residuals, N=9, mean (SD)
Dexamethasone	0.0112 (0.0042)	0.0112 (0.0042)	-0.0055 (0.0263)
Cortisol	-0.0069 (0.0101)	0.0208 (0.0013)	0.0002 (0.0198)

Supplemental Table 2.4. Precision and accuracy of the method.

Analyte		Intra-assay precision, CV [%]	Inter-assay precision, CV [%]	Intra-assay accuracy [%]	Inter-assay accuracy [%]
Dexamethasone	QC1	8.8	13.8	104.3	112.6
	QC2	4.6	9.3	98.9	119.5
Cortisol	QC1	7.6	6.4	104.0	96.2
	QC2	8.2	6.3	107.7	90.3

Supplemental Table 2.5. Multiple linear regression of the independent variables sex, age, body mass index, diabetes mellitus, nicotine consumption, estimated glomerular filtration rate (eGFR), oral contraceptives on the dependent variable serum dexamethasone concentration.

Factor	Standardized coefficient Beta	p-value
Sex	-0.073	0.167
Age	0.059	0.369
Body mass index	-0.013	0.807
Diabetes mellitus	0.123	**0.022**
Nicotine consumption	0.020	0.703
eGFR (MDRD)	-0.231	**1.04×10^{-4}**
Oral contraceptives	0.068	0.223

MDRD, Modification of Diet in Renal Disease.

3 Urinary steroid profiling: LC-MS/MS method development and validation

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Targeted metabolic profiling of urinary steroids with a focus on analytical accuracy and sample stability

Nora Vogg[1,2], Tobias Müller[3], Andreas Floren[3], Thomas Dandekar[3], Oliver Scherf-Clavel[4], Martin Fassnacht[1], Matthias Kroiss[1,5], Max Kurlbaum[1,2]

[1]Department of Internal Medicine I, Division of Endocrinology and Diabetes, University Hospital Würzburg, Germany

[2]Central Laboratory, Core Unit Clinical Mass Spectrometry, University Hospital Würzburg, Germany

[3]Department of Bioinformatics, Biocenter, Am Hubland, University of Würzburg, Germany

[4]Institute for Pharmacy and Food Chemistry, Am Hubland, University of Würzburg, Germany

[5]Department of Internal Medicine IV, University Hospital Munich, Ludwig-Maximilians-Universität München, Munich, Germany

Abstract

Introduction: Preoperative diagnostic workup of adrenal tumors is based on imaging and hormone analyses, but charged with uncertainties. Steroid profiling by liquid chromatography tandem mass spectrometry (LC-MS/MS) in 24-h urine has shown potential to discriminate benign and malignant adrenal tumors. Our aim was to develop and validate a specific and accurate LC-MS/MS method for the quantification of deconjugated urinary marker steroids, to evaluate their pre-analytical stability and to apply the method to clinical samples of patients with adrenal tumors.

Methods: A method for the quantification of 11 deconjugated steroids (5-pregnenetriol, dehydroepiandrosterone, cortisone, cortisol, α-cortolone, tetrahydro-11-deoxycortisol, etiocholanolone, pregnenolone, pregnanetriol, pregnanediol, and 5-pregnenediol) in human urine was developed and validated based on international guidelines. Steroids were enzymatically deconjugated and extracted by solid phase extraction before LC-MS/MS quantification in positive electrospray ionization mode.

Results: Excellent linearity with R^2>0.99 and intra- and inter-day precisions of <10.1% were found. Relative matrix effects were between 96.4% and 101.6% and relative recovery was between 98.2% and 115.0%. Sufficient pre-freeze stability for all steroids in urine was found at 20–25 °C for seven days and at 4–6 °C for up to 28 days. Samples were stable during long-term storage at -20 °C and -80 °C for 6 months.

Conclusions: A sensitive and robust LC-MS/MS method for the quantification of 11 urinary steroids was developed and validated according to international guidelines. Pre-analytical matrix stability was evaluated and the suitability of the method for the analysis of clinical samples and prospective validation studies was shown.

Introduction

Adrenal tumors are among the most common neoplasms in humans. With the increasingly frequent use of cross-sectional imaging, incidental adrenal masses are observed in 3–5 % [1-4]. While most of these are benign adrenocortical adenomas (ACA) that are more frequent with increased age [5-7], rare but aggressive adrenocortical carcinomas (ACC) have an annual incidence of only 0.7–2.0 cases per million population and a peak incidence between 40 and 50 years of age [8]. Early diagnosis of ACC is crucial because complete surgical removal is the only chance of cure [9]. Malignancy assessment is based on both imaging techniques and hormonal workup [9-11], which is often tedious and charged with uncertainties. Quantification of cortisol in serum and urine with or without dynamic testing helps to determine the presence of autonomous cortisol secretion and Cushing's syndrome [12, 13]. Profiling of a broader set of steroid hormones by liquid chromatography tandem mass spectrometry (LC-MS/MS) in serum or plasma has been proposed to accelerate the diagnosis of ACC [14, 15]. The circadian rhythm underlying most steroids increases the variability of results rendering standardized sampling conditions essential. Urine collection over 24 hours is a non-invasive procedure and overcomes the problem of circadian variability as net steroid output over a day can be assessed, and both diurnal fluctuations and dilution effects can be considered.

Steroid quantification by LC-MS/MS has increasingly replaced immunoassays over the last decades due to its improved analytical specificity and the possibility to quantify several biomarkers in a single run by multiple reaction monitoring (MRM) [16, 17]. Bancos, Taylor et al. recently published a prospective validation study using urine steroid metabolomics in conjunction with imaging features for the differential diagnosis of adrenal incidentalomas [18], which resulted in a positive predictive value of 76.4 % for ACC detection.

Our aim was to develop an LC-MS/MS method with higher clinical diagnostic value through optimal analytical accuracy suitable for clinical routine application. We selected a marker set of 11 deconjugated urinary steroids to meet this demand.

The selected marker panel included urinary steroid metabolites with the highest clinical diagnostic value for ACC diagnosis according to published reports [18-20]. Arlt et al. found nine steroid markers as most relevant for differentiation between ACC and ACA, including tetrahydro-11-deoxycortisol (THS), 5-pregnen-3β,17,20α-triol (5-PT), 5-pregnen-3β,20α-diol (5-PD), 5β-pregnan-3α,17,20α-triol (PT), etiocholanolone (Etio), and 5β-pregnan-3α,20α-diol (PD) [19]. This finding was largely confirmed by Hines et al., who found Etio, dehydroepiandrosterone (DHEA), 5-PT, 5-PD, PD, 17-hydroxypregnanolone, PT, and THS to

be the strongest indicators of ACC [20]. Bancos et al. investigated a panel of 15 urinary steroids that included the markers mentioned above, as well as androsterone, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone, cortisol, cortisone, tetrahydrocortisone, and β-cortolone [18]. By excluding strongly intercorrelated steroids, we were able to narrow down the marker set further. Pregnenolone was included additionally as a representative precursor in steroid biosynthesis, which we found useful in preliminary experiments using various biomaterials and cell culture experiments (data not shown).

Figure 3.1 depicts the simplified pathway of steroid biosynthesis. In Supplemental Figure 3.1 the chemical structures of the 11 marker substances are shown with their molecular masses and corresponding quantifier transitions in positive MRM mode.

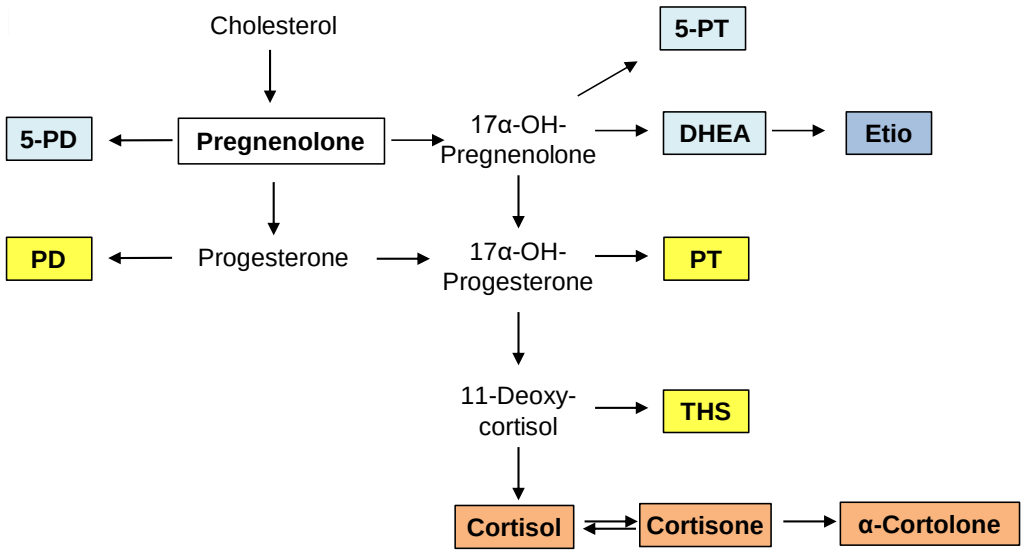

Figure 3.1. Simplified pathway of steroid biosynthesis and metabolism with boxed diagnostic analytes included in the quantification method. Colors indicate their role in steroid metabolism. White: early steroid hormone precursor, light blue: androgen precursor, dark blue: androgen, yellow: glucocorticoid precursor, orange: glucocorticoid.

Moreover, the focus was on validation of the pre-analytical stability of the urinary steroids, which is relevant for sample storage and handling in clinical practice. In an outpatient setting, most patients collect 24-h urine at home and send the sample to the laboratory by mail with unknown consequences on stability. Steroid degradation or intramolecular rearrangements might falsify the determined concentration and possibly lead to a misclassification of the

adrenal tumor differential diagnosis. To our knowledge, for most analytical methods pre-analytical stability is usually validated for up to 24 h at room temperature and maximally 48h at refrigerator temperature, e.g. in a stability study for urinary estrogens [21]. The necessity of pre-analytical stability assessment of urine samples in metabolomic analysis is frequently referred to, however only general recommendations for cooled or even frozen sample storage and transport are given without addressing steroid hormone metabolites specifically [22-26].

Herein we describe a validated LC-MS/MS method for the quantification of a panel of 11 urinary steroids. We investigated the pre-analytical stability of urinary steroid metabolites at room temperature and refrigerator temperature for up to 28 days and the method was successfully applied to 24-h urine samples collected from adrenal tumor patients.

Materials and methods

Instrumentation and materials

An Agilent 1290 HPLC (G4226A autosampler, infinityBinPump, G1316C column oven, G1330B thermostat) coupled to a QTRAP 6500+ MS-system (SCIEX, Framingham, USA) was used for LC-MS/MS measurements. LC-MS/MS data acquisition and quantification was performed with Analyst 1.6.3 (Sciex). The analytical column was an Acquity UPLC Premier HSS T3 1.8µm 2.1x50mm (Waters GmbH, Eschborn, Germany) and offline solid phase extraction (SPE) was performed on SepPak tC18 100mg 96-well Plates (Waters GmbH, Eschborn, Germany). Cortisol, pregnenolone, DHEA-d6, β-glucuronidase/arylsulfatase from *Helix pomatia*, and Sigmatrix urine diluent were purchased from Sigma-Aldrich (Taufkirchen, Germany). Etio, 5-PD, PD, PT, cortisone, α-cortolone, 5-PT, and DHEA were purchased from Steraloids (Newport, RI, USA) and THS from Cayman Chemical (Ann Arbor, MI, USA). THS-d5, Etio-d5, and PT-d5 were obtained from IsoSciences (Ambler, PA, USA) and PD-d5 and pregnenolone-d4 from Toronto Research Chemicals Inc. (Toronto, Canada). MS-grade methanol and water were purchased from VWR International GmbH (Darmstadt, Germany). Formic acid, ammonium acetate, and acetic acid were from Thermo Fisher Scientific Inc. (Schwerte, Germany).

Standard preparation

Stock solutions of all compounds were prepared in methanol at a concentration of 1.0 mg/mL (5-PT, DHEA, cortisone, cortisol, α-cortolone, Etio, pregnenolone, PT) or 0.5 mg/mL (THS, PD, 5-PD). From these, two methanolic working solutions were prepared (working solution 1:

pregnenolone, PD, α-cortolone, Etio, 5-PD, cortisone, cortisol; and working solution 2: 5-PT, PT, THS, DHEA). Deuterated internal standards (IS) were dissolved in methanol (1 mg/mL), combined, and diluted with water/methanol (1:1) to an IS mix with the following concentrations: 5000 ng/mL (THS-d5), 2500 ng/mL (PD-d5), 1000 ng/mL (Etio-d5, PT-d5, DHEA-d6), and 500 ng/mL (cortisol-d4, pregnenolone-d4).

Two approaches for preparation of calibration standards and quality controls (QC) were performed and compared regarding linearity of calibration curves. First, standards were prepared by spiking the two working solutions into steroid-free urine matrix, resulting in six calibration levels. Second, working solutions were spiked into a mixture of steroid-free urine matrix and methanol (1:1). Four QC levels – lower limit of quantitation (LLOQ), low, medium, and high – were prepared analogously to the calibration standards from separate stock solutions. Table 3.1 lists calibration range and QC concentrations for each analyte.

Table 3.1. Calibration range and QC concentrations of the steroid standards

Analyte	**Calibration range [ng/mL]**	**QC LLOQ [ng/mL]**	**QC low [ng/mL]**	**QC medium [ng/mL]**	**QC high [ng/mL]**
5-PT	20–5000	20	100	1000	2500
DHEA	20–5000	20	100	1000	2500
Cortisone	10–1000	10	20	200	500
Cortisol	10–1000	10	20	200	500
α-cortolone	50–5000	50	100	1000	2500
THS	20–5000	20	100	1000	2500
Etio	50–5000	50	100	1000	2500
Pregnenolone	5–500	5	10	100	250
PT	20–5000	20	100	1000	2500
PD	50–5000	50	100	1000	2500
5-PD	50–5000	50	100	1000	2500

QC quality control, *LLOQ* lower limit of quantitation

Sample preparation

150 µL of sample (calibration standard, QC, or urine sample) were gently mixed with 300 µL of deconjugation buffer consisting of 30 µL of β-glucuronidase/arylsulfatase (glucuronidase activity: 6.9 U/mL at 25 °C with 4-nitrophenylglucuronide, arylsulfatase activity: 19 U/mL at 25 °C with 5-nitrophenylsulfate) and 270 µL of ammonium acetate buffer (pH 4.9, 0.2M). Next, samples underwent an incubation for 3h at 55 °C for enzymatic deconjugation of sulfate and glucuronide moieties. Final incubation conditions were established by systematic variations of enzyme amount and incubation time. Increasing enzyme concentration or deconjugation time did not lead to an additional increase of 9 out of 11 deconjugated metabolites, indicating quantitative deconjugation. Deconjugated steroid concentrations using various amounts of arylsulfatase/glucuronidase mix are shown in Supplemental Figure 3.2. 30 µL of IS mix were added to the incubated samples followed by addition of 180 µL of methanol to urine samples and 180 µL of urine matrix/methanol (1:1) to calibration standards and QC samples to ensure equal solvent composition in all samples before SPE. The last step was left out when calibration standards and QC samples were prepared in pure steroid-free urine matrix without methanol. For offline-SPE, the SepPak tC18 100mg 96-well plate was pre-conditioned sequentially with 1 mL of methanol and 1 mL of water per well. Incubated samples were loaded, followed by two washing steps with 700 µL of water. Extracted steroids were eluted into a collection plate using 2 x 300 µL of methanol in two consecutive steps. Following complete solvent evaporation at 50 °C under a gentle flow of nitrogen, samples were reconstituted in 150 µL of methanol and diluted with 150 µL of water. 10 µL of the extracted sample were injected into the HPLC system. Samples with concentrations above the upper limit of quantification (ULOQ) were incubated again and diluted with a mixture of steroid-free urine matrix and ammonium acetate buffer, pH 4.9, 0.2M (1:2) according to the calibration range.

LC-MS/MS conditions

The column oven temperature was set to 45 °C. Source and gas parameters were set as follows: curtain gas: 40 psi, collision gas: medium, ion spray voltage: 4500 V, temperature: 500 °C, ion source gas 1: 50 psi, and ion source gas 2: 30 psi. Mobile phases consisted of MS-grade water with 0.1 % (V/V) formic acid (mobile phase A) and MS-grade methanol with 0.1 % (V/V) formic acid (mobile phase B). The flow rate was set to 500 µL/min with a gradient as follows: 0.0–1.0 min: 45% B; 1.0–8.5 min: from 45% to 80% B; 8.5–9.0 min: from 80% to 98%B; 9.0–10.0 min: 98% B; 10.0–10.5: from 98% to 45% B; 10.5–12.0 min: 45% B. After 9.0 minutes of run time, the valve position switched to waste. To increase the number of data points per peak,

MRM transitions were measured in three periods. The first period lasted from 0 to 4.7 min (detection of cortisone, cortisol, and α-cortolone), the second period from 4.7 to 5.7 min (detection of 5-PT and DHEA), and the third period from 5.7 to 9.0 min (detection of THS, Etio, 5-PD, pregnenolone, PT, and PD). One minute of automatic re-equilibration time preceded each analytical run. For every analyte, a quantifier and a qualifier MRM transition were identified and optimized to maximum intensity. Compound-specific MS-parameters for analytes and IS are listed in Supplemental Table S1. Chromatographic separation in a urine sample of an ACC patient and analyte retention times are shown in Figure 3.2. Isobaric compounds are baseline separated from the analyte peaks.

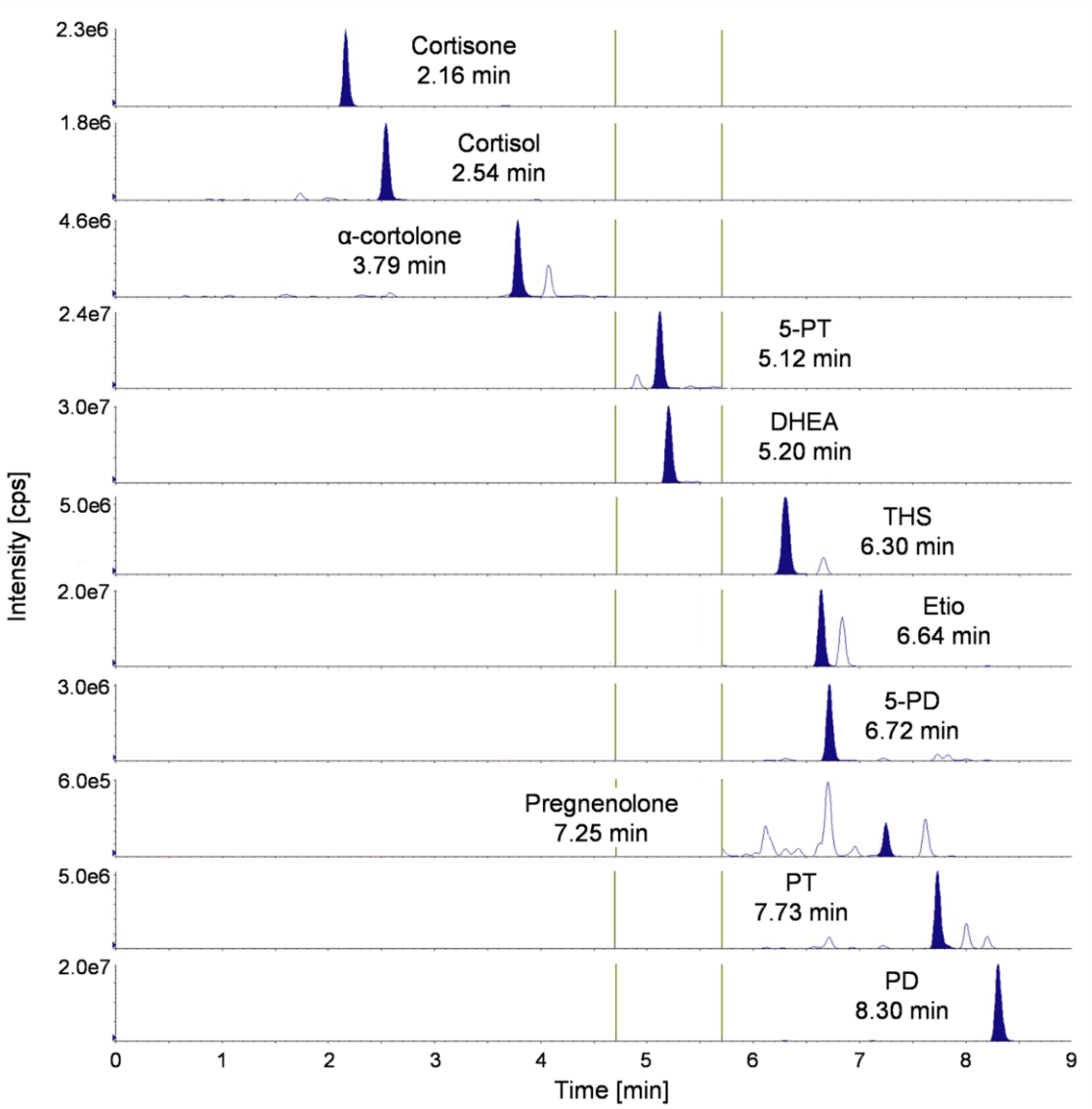

Figure 3.2. Extracted ion chromatograms of the 11 quantifier MRM transitions with corresponding retention times in a urine sample of an ACC patient. Vertical lines represent borders of the three periods.

Method validation

The LC-MS/MS method was validated based on current guidelines for bioanalytical method validation by the European Medicines Agency (2011) [27] and the Food and Drug Administration (2018) [28].

Selectivity

Six different lots of human urine were evaluated to test whether endogenous compounds were interfering with the seven deuterated IS. To this end, urine samples were measured with and without IS, respectively, taking into consideration the ratio between the two as the blank IS response percentage.

Sensitivity and carry-over

The limit of detection (LOD) was defined as the concentration with a signal-to-noise ratio (s/n) > 3. Carry-over was determined by a solvent injection after injection of the highest calibrator. Acceptance criteria were fulfilled by an analyte peak area measured in the blank of less than 20 % of the analyte peak area at the LLOQ.

Accuracy, precision, and reinjection reproducibility

Precision was defined as the percent coefficient of variation (%CV) and accuracy as the ratio of calculated concentration to nominal concentration. Inter-day accuracy and precision were determined in three independent runs with four QC levels (LLOQ, low, medium, and high), each measured in four replicates. Intra-day accuracy and precision were calculated from six replicates of the four QC levels within one validation run. Reinjection reproducibility was determined by the %CV of five injections from the same processed sample.

Matrix effect and recovery

Matrix effects were evaluated by comparing the responses (analyte peak area for absolute matrix effect and the ratio of analyte peak area to IS peak area for relative matrix effect) of matrix QC samples versus QC samples prepared in water (Eq. 1).

$$Matrix\ effect\ (\%) = \frac{\text{response (presence of matrix)}}{\text{response (absence of matrix)}} \times 100 \quad (1)$$

Recovery was calculated by comparing analytes' responses (analyte peak area for absolute recovery and the ratio of analyte peak area to IS peak area for relative recovery) in processed QC samples via SPE versus post-extract spiked samples (Eq. 2).

$$Recovery\ (\%) = \frac{\text{response (extracted sample)}}{\text{response (post-extracted spiked sample)}} \times 100 \quad (2)$$

QC samples were measured in triplicate at three concentration levels (low, medium, and high) for matrix effect and recovery.

Dilution integrity

To cover the case of patient samples with steroid concentrations above the calibration range, dilution integrity was tested with QC samples prepared in a concentration of twofold the ULOQ. After enzymatic hydrolysis, samples were diluted with a mixture of steroid-free urine matrix and ammonium acetate buffer, pH 4.9, 0.2M (1:2) in a fourfold and a tenfold dilution to concentration levels within the calibration range. Each dilution was prepared in six replicates.

Stability

Steroid stability in urine before freezing and processing was determined at room temperature (20–25 °C) and in the refrigerator (4–6 °C) for 28 days (pre-freeze stability). At each time point, a triplicate of a the patient urine pool was transferred from the evaluation temperature to -80°C and, once all of the time points were passed, all of the samples were measured together in a single run. Stability of temperature conditions during enzymatic hydrolysis was tested by comparison of pooled urine samples after pre-heating (3h, 55°C) against unheated samples. To exclude an effect of the 55 °C heating phase during incubation, enzymatic hydrolysis was performed for 6h at 30 °C.

Stock solution stability was measured after six months by comparing freshly prepared stock solutions with the original stock solutions at two concentration level (diluted to 100 ng/ml and 500 ng/ml). Long-term stability of frozen samples was determined for up to six months with QC sample storage at -20 °C and -80 °C. Three QC levels (low, medium, and high) were measured in triplicate. Autosampler stability of the processed sample was investigated over 24 hours. Freeze-thaw stability was determined with three cycles of a triplicate of QC standards at three concentration levels.

Application to clinical samples

The method was applied to 24-h urine samples of 19 patients with an adrenal tumor diameter ≥ 2 cm, composed of 4 ACC patients and 15 ACA patients. The four ACC cases were all of a classical type. Three of these ACC cases were functional and one case was non-functional. 24-h Urine samples were collected consecutively between January and May 2019 as part of the European Network for the study of adrenal tumors (ENSAT) registry study, which has been approved by the local ethics committee of the University of Würzburg (#88/11). All patients provided written informed consent. Total collection volume was documented and a urine

aliquot was stored at -20°C in a urine Monovette® (Sarstedt, Nümbrecht, Germany). Diagnosis was made following standard workup (imaging, hormone measurements in serum, and histology after adrenalectomy, if available) [9, 10]. After measurement of steroid concentrations in ng/mL, the steroid excretion in µg/24 h was normalized via the individual total collection volume. The tumor diameter and Hounsfield units (HU) in unenhanced computed tomography (CT) were documented from the patients' imaging records.

Statistics

Statistical analyses were performed using IBM SPSS version 26. Urinary steroid excretion data were found not to be normally distributed by applying the Shapiro-Wilk test. Groups were compared using the Mann Whitney U test with p-values <0.05 considered statistically significant. Correlations between steroid excretions and tumor diameter were tested by determination of the Pearson correlation coefficient (Pearson r).

Results

Sample preparation

Preparation of calibration standards and QC samples was initially based on spiking methanolic working solutions into pure steroid-free urine matrix. However, early experiments illustrated the need for optimization for the standard and calibration preparation due to insufficient linearity of some steroids; for example PD with R^2=0.9448 (Supplemental Figure 3.3 A). Improved linearity (R^2=0.9994 for PD) was found after spiking a mixture of methanol and steroid-free urine matrix (1:1) with methanolic working solutions (Supplemental Figure 3.3 B). Method validation and measurements of patient urine samples were thus conducted with a calibration and QC samples prepared in methanol and steroid-free urine matrix (1:1). To ensure equal solvent composition and extraction properties for calibration standards and clinical urine samples during SPE, 180 µL of methanol were added to each urine sample after enzymatic hydrolysis. Accordingly, 180 µL of a 1:1 mixture of methanol and steroid-free urine matrix were added to calibration standards and QC samples.

Method validation

Calibration curves were plotted with peak area ratios (analyte/IS) against the nominal concentration of each analyte. Cortisone, cortisol, DHEA, Etio, α-cortolone, PD, and PT showed the best results with a linear curve fit with 1/x-weighting and a quadratic curve fit with

$1/x^2$-weighting was used for 5-PT, THS, pregnenolone, and 5-PD. All calibration curves showed coefficients of determination (R^2) > 0.99. R^2 of calibration curves of five validation runs are listed as mean (SD) in Table 2.

For selectivity, the blank IS response percentage was <1.0 % for cortisol-d4, Etio-d4, THS-d4, PD-d4, PT-d4, and pregnenolone-d4, and 1.3 % for DHEA-d4. All steroids were baseline separated from co-eluting isobaric substances. Steroid identification was verified by monitoring of the quantifier-to-qualifier ion ratio and comparison of quantifier and qualifier retention times with the retention time of the corresponding analytical standard.

The LOD of each analyte is listed in Table 2. No relevant carry-over was found in any analyte or IS.

Inter- and intra-day accuracy and precision was acceptable for all analytes both at the LLOQ and as the mean of QC levels low, medium, and high. Highest imprecisions were calculated for THS with an inter-day (im)precision at the LLOQ of 10.1 %. The highest %CV after five reinjections of the same processed sample was also detected for THS with 5.8 %. All results for accuracy, precision, and reinjection reproducibility are listed in Table 2.

Low matrix effects were detected for the artificial steroid-free urine matrix. Absolute matrix effects were between 96.4 % (Etio, 5-PD) and 102.0 % (THS) and relative matrix effects between 96.4 % (pregnenolone) and 101.6 % (PD). Absolute recovery was found to be between 106.0 % (PD) and 121.4 % (cortisone). However, relative recovery was closer to 100 % for all analytes, as suitable internal standards normalize the positive recovery effect detected for the analyte peak areas. Results for matrix effects and recovery are listed in Table 2.

Dilution of QC samples with concentrations above the ULOQ did not affect accuracy and precision. The mean accuracy of both dilution levels was considered as dilution integrity and is listed in Table 3.2 for all analytes.

Table 3.2. Validation results

	5-PT	DHEA	Cortisone	Cortisol	α-corto-lone	THS	Etio	Preg-nenolone	PT	PD	5-PD
Absolute matrix effect (%)	97.3	97.9	99.0	98.3	97.2	102.0	96.4	101.1	98.5	100.1	96.4
Relative matrix effect (%)	99.5	97.9	100.8	100.2	98.8	99.7	101.5	96.4	98.9	101.6	98.3
Absolute recovery (%)	116.5	117.1	121.4	120.1	120.1	120.7	116.1	108.9	111.8	106.0	113.6
Relative recovery (%)	99.2	99.8	101.3	101.7	115.0	98.3	101.7	101.6	98.2	101.2	107.4
Inter-day accuracy (%)	97.8	100.6	100.3	99.6	101.7	99.8	101.9	99.3	101.2	100.2	98.9
Inter-day accuracy LLOQ (%)	105.1	100.7	102.0	102.3	98.7	99.6	102.4	112.4	100.3	100.5	109.4
Intra-day accuracy (%)	100.3	99.1	100.0	99.3	95.1	97.2	99.4	102.3	98.9	100.6	102.8
Intra-day accuracy LLOQ (%)	109.0	107.1	100.2	94.6	91.4	101.1	107.3	106.7	98.7	105.2	111.4
Inter-day precision (%CV)	2.3	2.6	2.0	1.9	2.4	8.3	2.9	3.4	2.5	2.4	2.7
Inter-day precision LLOQ (%CV)	3.3	2.7	2.1	4.3	2.8	10.1	3.3	2.4	3.0	2.4	2.8
Intra-day precision (%CV)	3.3	2.8	2.2	2.1	3.0	8.8	3.3	3.6	2.9	3.2	3.3
Intra-day precision LLOQ (%CV)	3.8	2.5	2.1	1.6	2.0	7.1	1.4	2.1	5.6	2.8	3.9
LOD (ng/mL)	2.0	2.0	1.0	1.0	5.0	4.0	5.0	2.5	2.0	5.0	10.0
Dilution Integrity (%)	103.7	104.8	108.7	109.4	104.6	105.2	108.0	107.1	107.9	111.3	115.2
Reinjection reproducibility (%CV)	0.9	1.2	0.9	1.0	1.6	5.8	2.0	3.4	2.2	1.6	2.7
Linearity, R^2, n=5 (mean, SD)	0.9963 (0.0022)	0.9993 (0.0005)	0.9998 (0.0002)	0.9995 (0.0003)	0.9992 (0.0009)	0.9947 (0.0067)	0.9994 (0.0004)	0.9963 (0.0023)	0.9996 (0.0003)	0.9994 (0.0002)	0.9940 (0.0015)

Evaluation of pre-freeze stability of the urinary steroids at 20–25 °C and at 4–6 °C showed variable stability (Figure 3.3). While all 11 steroids were stable for the period of 28 days at 4–6 °C (+/- 10 % of the initial concentration at day 0), changes from baseline of less than +/- 10 % were found for seven days at 20–25 °C. Cortisone, cortisol, Etio, α-cortolone, and PD did not show any alteration over the period of 28 days for both conditions. However, significant degradation at room temperature was present for 5-PT, DHEA, pregnenolone, and 5-PD, whereas PT concentration significantly increased over time at room temperature.

Stock solutions were stable for up to at least six months with concentration changes below +/– 15%. Results of stock solution stability and pre-freeze stability are listed in Supplemental Table S2. Concentration changes below +/– 15% were observed in spiked QC samples for up to 6 months at -20°C and -80°C, as well as for 3 freeze-thaw cycles and for 24 h of the processed sample in the autosampler at 4 °C. Results of the long-term stability, autosampler stability, and freeze-thaw stability are listed in Supplemental Table S3.

Steroids showed sufficient stability for 3h at 55°C with change in concentration ≤5 % (Supplemental Table S4).

Application to clinical urine samples

Steroid concentrations were measured in 24-h urine samples of adrenal tumor patients (n=19) and normalized to µg/24 h via total collection volume. Steroid excretions of ACC vs. ACA samples were compared by Mann-Whitney U test (Table 3.3).

Steroid excretions of 5-PT, cortisone, cortisol, PT, and 5-PD were positively correlated with the tumor diameter, with cortisone and cortisol showing a highly significant correlation ($p<0.004$), whereas 5-PT, PT, and 5-PD were slightly below the level of significance (Supplemental Table S5). The four patients with ACC had an attenuation of >10 HU in unenhanced CT. Nine of the patients with ACA had an attenuation ≤10 HU, while four ACA had >10 HU and two cases had no available unenhanced CT images.

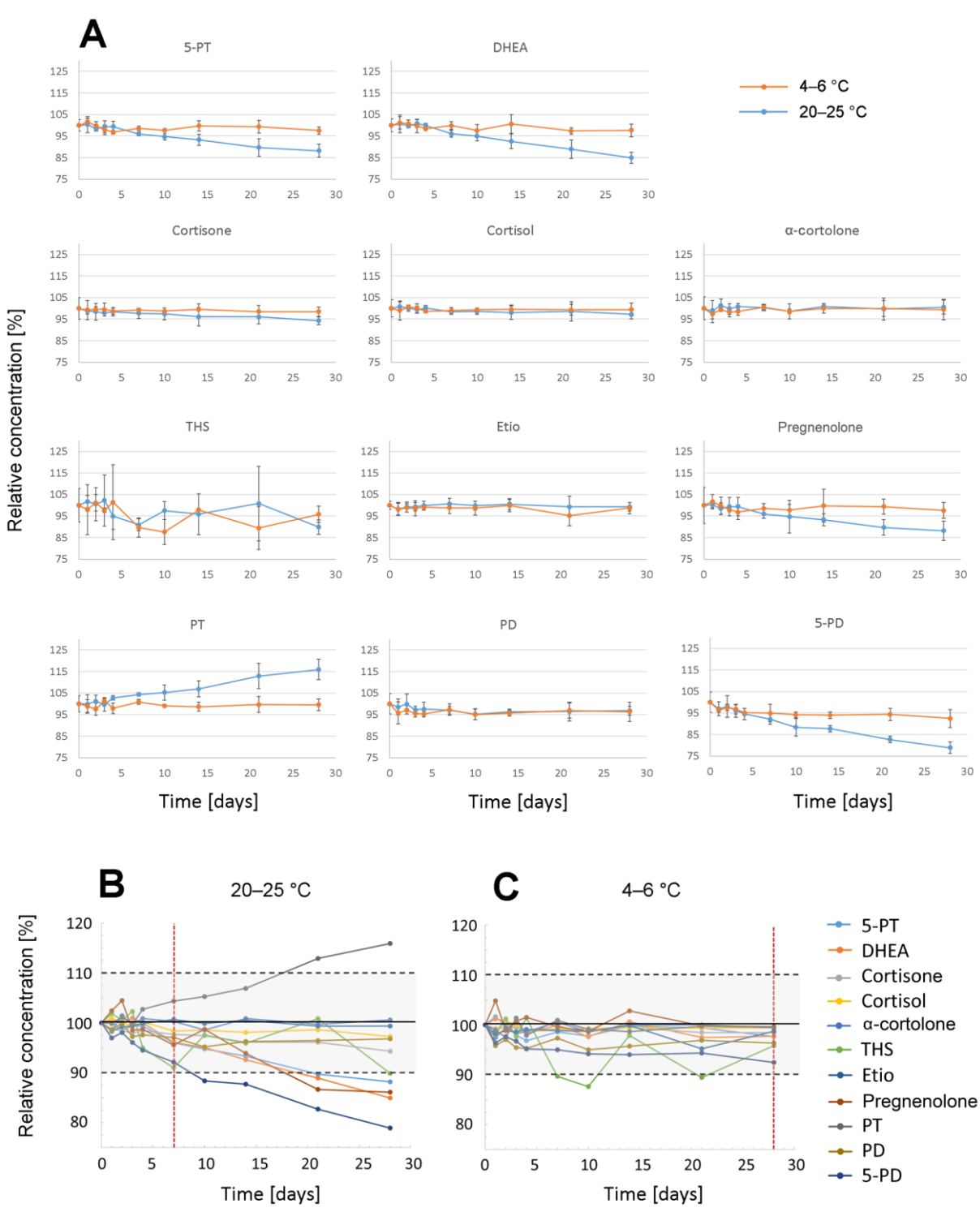

Figure 3.3. (A) Pre-freeze stability of urinary steroids at 20–25 °C (blue line) and at 4–6 °C (orange line) shown as mean of three independent measurements. Steroids were stable at 20–25 °C for seven days (B) and at 4–6 °C for 28 days (C) within the prespecified limits of 90–110 % of the initial concentration.

Table 3.3. Comparison of steroids in ACC vs. ACA urine samples in µg/24 h by Mann-Whitney U test

	ACC (n=4)	**ACA (n=15)**		**p-Value**
Analytes	**Mean (SD), range [µg/24 h]**	**Mean (SD), range [µg/24 h]**	**Samples below LOD [n]**	
5-PT	1190 (899), 695–2534	81.2 (94.7), 14.3–347	-	0.001
DHEA	2695 (4518), 235–9468	133 (435), 1.4–1702	2	0.004
Cortisone	231 (164), 56.2–383	124 (56.1), 38.5–241	-	0.357
Cortisol	406 (472), 80.6–1087	109 (64.4), 26.1–224	-	0.221
α-cortolone	1858 (189), 1609–2046	1741 (938), 371–3753	-	0.221
THS	1656 (2102), 326–4788	198 (164), 33.5–556	-	0.002
Etio	6712 (6201), 1305–15300	856 (665), 82.6–2192	-	0.004
Pregnenolone	10.3 (8.9), 0.0–20.9	0.9 (2.3), 0.0–7.1	14	0.027
PT	2513 (1535), 1261–4680	596 (503), 80.6–2000	-	0.002
PD	1154 (1369), 279–3188	184 (218), 32.6–928	-	0.004
5-PD	237 (88.3), 118–331	101 (33.2), 50.4–168	-	0.004
Tumor diameter [cm]	7.0 (2.2), 4.4–9.7	3.6 (1.4), 2.2–7.0		0.006
Tumor HU in unenhanced CT, n				
≤10	0	9		
>10	4	4		
n/a	0	2		

LOD limit of detection

Discussion

We have developed and validated an LC-MS/MS method for the quantification of deconjugated urinary steroids and applied it to a set of 24-h urine samples of adrenal tumor patients. Steroids are excreted mainly as urinary sulfate or glucuronide conjugates and may even be measured directly as intact conjugates by LC-MS/MS [29-32]. However, due to a lack of commercially available steroid conjugate standards for most diagnostically relevant steroid precursor metabolites and the large number of possible metabolites, most published quantitative methods include a deconjugation step [18, 20, 33-36]. To capture the total urinary steroids including sulfates and glucuronides as well as the free steroid fraction, we performed a hydrolysis step and quantified deconjugated steroids. Measuring deconjugated urinary steroids for the hormonal workup of adrenal tumors has been performed by others, but most previously published methods were based on gas chromatography mass spectrometry (GC-MS) [19, 37-41]. GC-MS provides an excellent resolution, but sample pre-treatment is laborious and time consuming as derivatization steps are necessary. Two previously published works describe LC-MS/MS methods for the quantification of deconjugated steroids in urine for the application in the diagnostic workup of adrenal tumors [18, 20]. Hines et al. isolated the steroids from urine by liquid-liquid extraction [20], which has the drawbacks of a time-intensive and difficult to standardize extraction process [16]. Bancos, Taylor et al. used offline-SPE for steroid extraction [18], which was, likewise, our preferred extraction method due to its excellent recoveries and reproducibility, and lower organic solvent usage; we consider these major advantages of SPE in comparison to liquid-liquid extraction.

In contrast with other published methods, we focused on a panel of 11 urinary steroids to will facilitate clinical implementation and reduce cost. An improved analytical accuracy was obtained by the usage of seven stable isotope labelled IS, which allows more accurate measurements to be achieved over the existing methods that use fewer IS [18, 20] due to the reduction of potential matrix interferences. Moreover, we have overcome linearity issues of steroids in synthetic steroid-free urine matrix by modifying sample preparation. As linearity improved for critical analytes after addition of methanol to the urine matrix, we hypothesize that insufficient standard solubility may lead to inhomogeneous distribution within the samples during sample preparation in the absence of methanol. It is possible that precipitated steroids in real urine samples are dissolved by the addition of methanol after incubation and before SPE. This step also ensures an equal solvent composition in calibration standards, quality control samples and real urine samples.

As our method focused on deconjugated steroids, sample pre-treatment included a deconjugation step with Arylsulfatase/Glucuronidase. The combined enzymatic activity in the digestive juice of *Helix pomatia* is suitable for cleavage of sulfate and glucuronide esters for both (i) steroids excreted mainly as glucuronides like etiocholanolone, and (ii) mainly sulfated steroids like DHEA [32]. Enzymatic hydrolysis is complex and requires the optimization of enzyme type and concentration, incubation time, and temperature [42]. Both Hines et al. and Bancos, Taylor et al. use a mixture of glucuronidase/arylsulfatase and incubation conditions of 2h at 50 °C and 3h at 60°C, respectively [18, 20]. In our sample preparation procedure, a 3h-incubation at 55 °C with 30 µL of the liquid digestive juice of *Helix pomatia* resulted in most reproducible quantitative results.

We analyzed pre-freeze urinary steroid stability, which is highly relevant for clinical practice. There is a paucity of data pertaining to the acceptable storage duration and steroid stability in urine samples at room temperature, or at refrigerator temperature before long-term storage in a freezer, as most previously published methods have focused on clinical studies rather than routine clinical implementation. We were able to determine sufficient stability of 90–110% of all steroids for at least seven days at 20–25 °C and for 28 days at 4–6 °C. This information gives confidence for the frequently performed postal dispatch of 24-h urine samples.

Finally, we showed the successful application of the method to 19 urine samples of adrenal tumor patients. Specific quantification was achieved by chromatographic baseline separation of analytes from isobaric compounds. After normalization of steroid concentrations to steroid excretion in µg/24 h, significant differences between the urine samples of ACC and benign tumor patients were found in 8 of the 11 analyzed steroids. Even with our small sample size, the method showed potential value for broad application to clinical samples. The method showed excellent sensitivity for the detected urinary steroids, as only two patients with ACA had urinary DHEA below the LOD and 14 samples contained no measurable pregnenolone, which is generally excreted in very low concentrations. The steroid excretion ranges in µg/24 h are comparable to previously published data of absolute values, even though they were measured by GC-MS/MS [19, 20, 39]. The overall increase of steroid excretion in patients with ACC compared to ACA is also in accordance with other studies. Most groups identified THS to be the most discriminative steroid to classify the tumors [19, 38, 39], whereas our results show the lowest p-values for 5-PT, followed by THS and PT.

In conclusion, a robust and specific LC-MS/MS method with optimized analytical accuracy was developed, validated, and applied to a modest set of clinical samples. Significant clinical

diagnostic performance may be achieved by combining targeted metabolic profiling of urinary steroids via LC-MS/MS with bioinformatic algorithms of characteristic steroid patterns to improve the differentiation between ACC and benign tumors in clinical routine.

Declaration of Competing interests

The authors declare they have no known competing financial interests or personal relationships that could affect the work described in this article.

Ethical approval

This study was part of the European Network for the study of adrenal tumors (ENSAT) registry, which has been approved by the local ethics committee of the University of Würzburg (#88/11).

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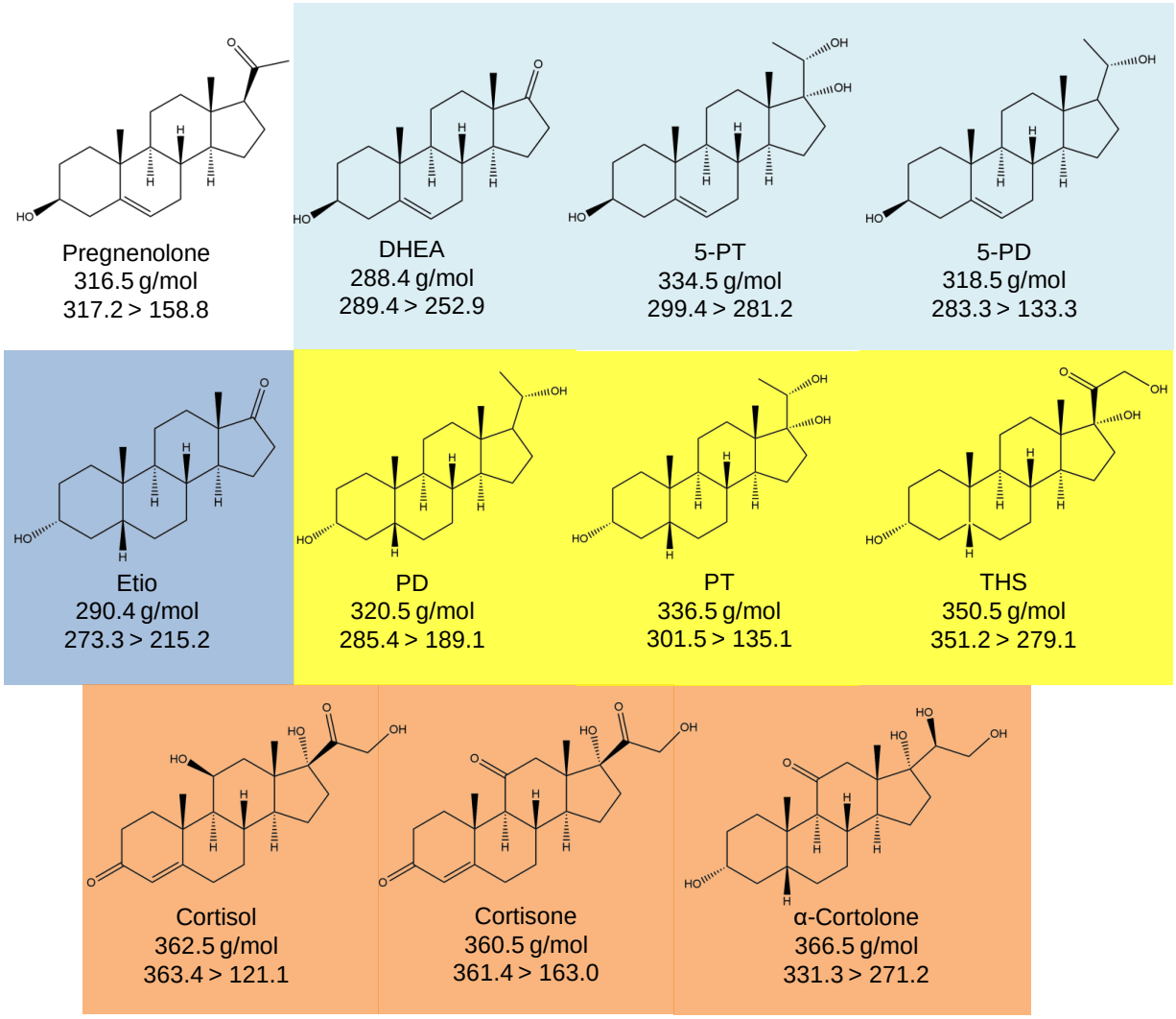
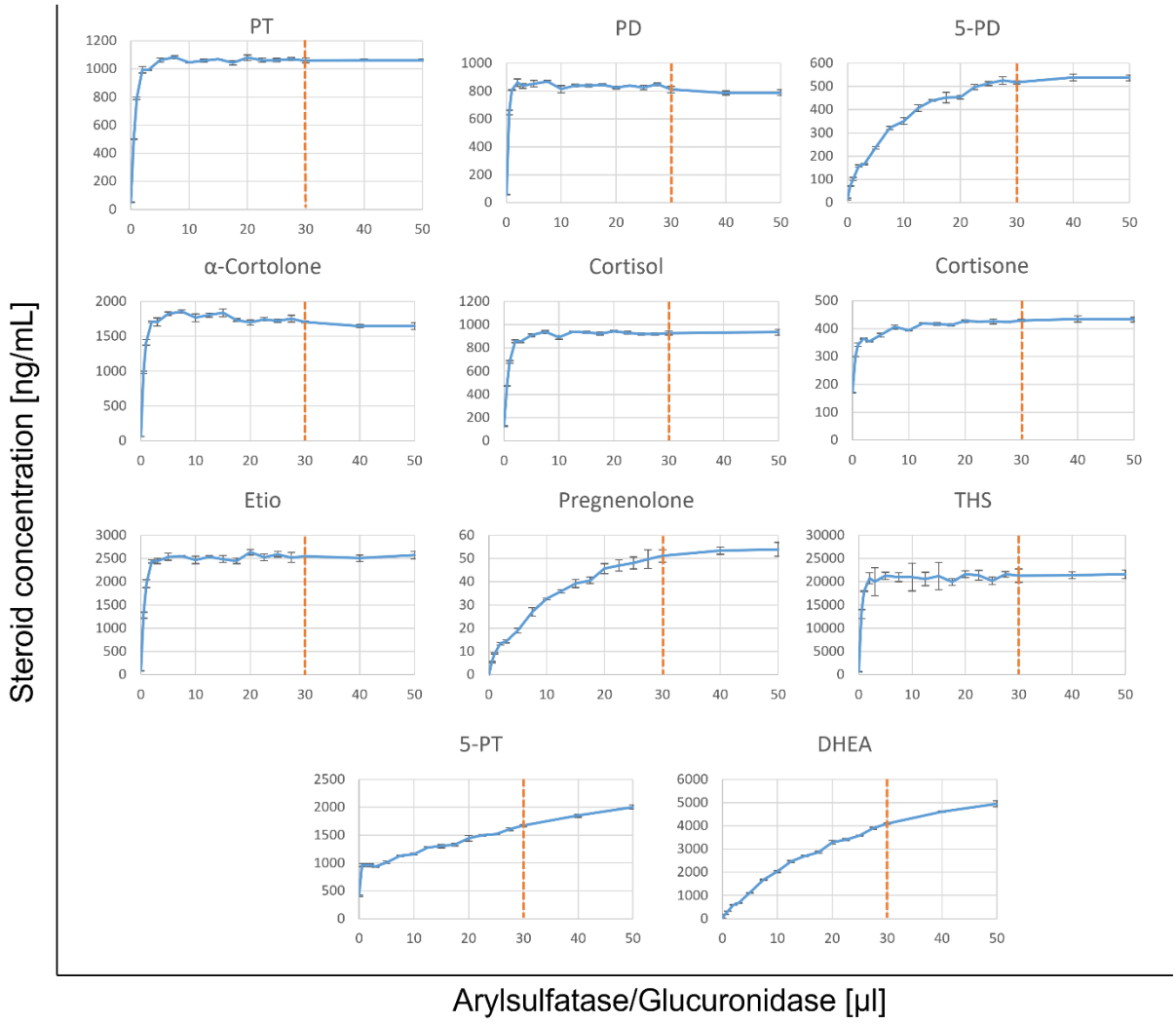
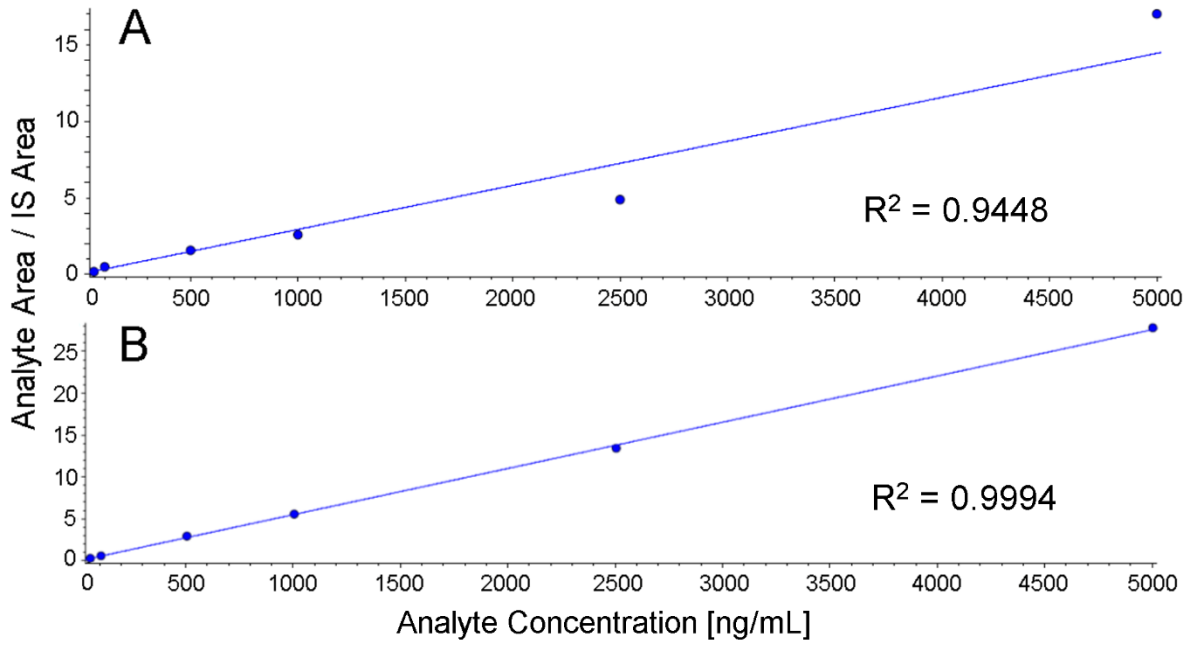
Supplemental Figures



Supplemental Figure 3.1. Chemical structures with molecular masses and quantifier MRM transitions of the marker substances. Colors indicate the steroids' role in steroid metabolism. White: general precursor, light blue: androgen precursor, dark blue: androgen, yellow: glucocorticoid precursor, orange: glucocorticoid.



Supplemental Figure 3.2. Deconjugated steroid concentrations during incubation for 3h at 55°C using various amount of arylsulfatase/glucuronidase. The vertical orange line represents 30 µL enzyme mix (with 270 µL ammonium acetate buffer) which results in a concentration plateau for 9/11 steroids and excellent reproducibility for all analytes. Measurements are shown as the mean of a triplicate.



Supplemental Figure 3.3. (A) Calibration curves of PD spiked into steroid-free urine matrix and (B) into a mixture of methanol and steroid-free urine matrix (1:1) with improved linearity. Calibration curves were fitted linear with 1/x weighting.

Supplemental Table 3.1. Quantifier and qualifier m/z transitions and compound specific MS-parameters of the 11 steroids and the 7 internal standards

Analyte	Internal standard	Retention time [min], (Period)	Dwell time [ms]	Precursor ion (m/z)	Product ion (m/z)	Declustering potential [V]	Entrance potential [V]	Collision energy [V]	Collision cell exit potential [V]
5-PT	DHEA-d6	5.12, (2)	100	299.4	281.2*	120	6	15	20
					135.2	120	5	31	13
DHEA	DHEA-d6	5.20, (2=	100	289.4	252.9*	30	9	14	24
					271.2	45	9	12	19
Cortisone	Cortisol-d4	2.16, (1)	50	361.4	163.0*	90	7	31	15
					121.0	90	7	36	15
Cortisol	Cortisol-d4	2.54, (1)	50	363.4	121.1*	90	10	30	13
					327.2	90	10	22	21
α-cortolone	PD-d5	3.79, (1)	20	331.3	271.1*	140	6	24	18
					313.1	140	6	31	18
THS	THS-d5	6.30, (3)	20	351.2	279.1*	45	7	23	16
					297.4	45	7	18	18
Etio	Etio-d4	6.64, (3)	20	273.3	215.2*	85	9	21	12
					147.2	85	9	32	18
Pregnenolone	Pregnenolone-d4	7.25, (3)	20	317.2	158.8*	80	7	26	15
					299.4	80	7	13	20
PT	PT-d5	7.73, (3)	20	301.5	135.1*	130	8	24	12
					189.3	130	8	28	11
PD	PD-d5	8.30, (3)	20	285.4	189.1*	90	10	21	14
					175.2	90	10	23	16
5-PD	PD-d5	6.72, (3)	20	283.3	133.3*	55	6	18	12
					189.1	55	6	26	12
DHEA-d6	-	5.17, (3)	100	295.4	258.9	30	9	14	24
Cortisol-d4	-	2.53, (1)	50	367.4	121.1	90	10	30	13

THS-d5	-	6.28, (3)	20	356.1	283.3	45	7	23	16
Etio-d5	-	6.62, (3)	20	278.4	220.3	85	9	21	14
Pregnenolone-d4	-	7.22, (3)	20	321.2	159.2	80	7	26	15
PT-d5	-	7.72, (3)	20	306.5	140.2	120	8	24	12
PD-d5	-	8.29, (3)	20	290.4	189.1	90	10	21	14

* used as quantifiers

Supplemental Table 3.2. Stock solution stability and pre-freeze stability

Analyte	**Stock solution stability**		**Pre-freeze stability**	
		6 months at -80°C (n=3 per dilution)	**7 days at 20–25°C** (n=3)	**28 days at 4-6 °C** (n=3)
5-PT	Dilution 1	102.3 (0.7)	96.0 (0.9)	97.6 (1.7)
	Dilution 2	104.0 (1.7)		
DHEA	Dilution 1	101.7 (0.6)	96.1 (1.7)	97.7 (2.9)
	Dilution 2	102.1 (0.9)		
Cortisone	Dilution 1	103.5 (1.0)	97.7 (2.4)	98.4 (2.2)
	Dilution 2	103.8 (1.3)		
Cortisol	Dilution 1	95.3 (1.4)	98.3 (1.2)	99.4 (3.0)
	Dilution 2	95.5 (1.0)		
α-cortolone	Dilution 1	95.5 (3.7)	100.3 (1.3)	99.4 (4.7)
	Dilution 2	102.0 (7.5)		
THS	Dilution 1	98.7 (1.7)	91.0 (2.7)	95.8 (3.8)
	Dilution 2	100.0 (1.7)		
Etio	Dilution 1	104.0 (1.5)	100.6 (2.5)	98.7 (2.7)
	Dilution 2	104.4 (1.1)		
Pregnenolone	Dilution 1	106.4 (4.0)	95.5 (2.1)	99.6 (3.6)
	Dilution 2	105.6 (3.8)		
PT	Dilution 1	100.7 (1.2)	104.3 (0.8)	99.6 (2.7)
	Dilution 2	99.5 (4.0)		
PD	Dilution 1	102.4 (3.9)	97.1 (1.8)	96.3 (4.4)
	Dilution 2	100.5 (2.6)		
5-PD	Dilution 1	97.0 (1.8)	92.1 (2.4)	92.5 (4.2)
	Dilution 2	105.3 (4.8)		

All values presented as relative concentration (% of day 0), mean (%CV)

Supplemental Table 3.3. Long-term stability, autosampler stability, and freeze-thaw stability

Analyte	QC Level (n=3 per level)	Long-term stability		Autosampler stability	Freeze-thaw stability
		6 months at -20°C	6 months at -80°C	24 h at 4-6°C, processed	3 cycles
5-PT	Low	96.6 (2.1)	101.9 (1.3)	101.2 (1.7)	101.9 (2.9)
	medium	97.7 (2.9)	100.3 (4.5)	101.1 (0.8)	100.4 (1.6)
	high	99.8 (1.7)	100.5 (3.2)	101.2 (3.7)	105.0 (3.2)
DHEA	Low	104.7 (2.4)	106.7 (2.4)	99.9 (1.9)	99.3 (2.7)
	medium	103.5 (3.3)	104.8 (2.9)	99.6 (1.5)	97.1 (1.1)
	high	101.7 (2.0)	102.5 (3.8)	99.6 (5.3)	101.0 (3.0)
Cortisone	Low	89.5 (1.7)	91.5 (1.4)	100.1 (2.1)	99.7 (1.8)
	medium	96.2 (1.5)	95.8 (1.3)	99.4 (1.2)	97.7 (0.5)
	high	96.4 (0.8)	95.1 (1.5)	99.4 (3.1)	100.5 (2.4)
Cortisol	Low	90.9 (1.5)	94.5 (0.5)	99.4 (1.9)	101.8 (3.1)
	medium	94.5 (2.0)	93.7 (1.1)	99.4 (0.7)	97.4 (1.5)
	high	94.6 (1.1)	94.0 (1.7)	99.7 (2.6)	101.0 (2.0)
a-Cortolone	Low	94.7 (2.9)	95.3 (0.2)	99.9 (5.9)	101.6 (2.5)
	medium	103.0 (1.5)	105.9 (2.8)	103.4 (1.2)	97.6 (1.9)
	high	98.7 (1.2)	101.6 (2.2)	107.1 (3.1)	100.3 (3.5)
THS	Low	98.6 (13.3)	103.4 (4.1)	103.8 (8.5)	110.5 (15.3)
	medium	102.3 (2.2)	90.2 (4.6)	98.9 (4.3)	113.8 (2.3)
	high	97.5 (3.6)	106.0 (4.1)	98.2 (7.8)	101.9 (5.6)

Etio	Low	90.9 (5.7)	89.9 (1.7)	94.9 (2.5)	93.4 (3.0)
	medium	99.6 (3.6)	95.5 (4.9)	98.7 (1.8)	94.7 (2.5)
	high	97.0 (0.9)	97.9 (4.1)	97.8 (3.2)	93.2 (2.3)
Pregnenolone	Low	102.0 (3.9)	102.0 (2.8)	106.4 (1.9)	104.4 (4.8)
	medium	94.7 (1.8)	88.0 (3.0)	100.1 (5.5)	96.7 (2.9)
	high	93.5 (2.3)	92.6 (2.1)	100.8 (5.1)	100.2 (1.9)
PT	Low	96.1 (2.1)	95.7 (2.8)	100.9 (1.9)	99.1 (0.7)
	medium	99.2 (1.5)	99.8 (2.6)	101.0 (1.3)	97.3 (2.3)
	high	98.5 (2.5)	97.2 (1.7)	101.4 (2.6)	96.5 (3.2)
PD	Low	89.3 (1.4)	89.8 (1.5)	99.2 (1.4)	100.2 (0.4)
	medium	94.4 (0.9)	94.5 (4.1)	100.2 (1.5)	97.8 (1.6)
	high	90.7 (0.5)	91.4 (2.8)	101.8 (3.2)	96.9 (1.6)
5-PD	Low	94.3 (2.7)	95.9 (2.4)	99.3 (2.6)	100.1 (2.0)
	medium	94.7 (2.2)	92.8 (4.9)	104.0 (1.3)	100.0 (2.6)
	high	92.8 (1.4)	92.3 (2.4)	104.9 (2.3)	101.0 (2.0)

All values presented as relative concentration (% of day 0), mean (%CV)

Supplemental Table 3.4. Steroid stability for 3h at 55 °C. Triplicates of pooled urine after pre-heating were compared to untreated samples after enzymatic hydrolysis for 6h at 30 °C.

	Steroid concentrations [ng/mL], mean (SD), n=3		**Concentration ratio pre-heated samples/ untreated samples [%]**
	Pre-heated 3h at 55 °C	Untreated	
5-PT	1903 (5.8)	1907 (5.8)	99.8
DHEA	3550 (26.5)	3670 (52.9)	96.7
Cortisone	96.7 (1.8)	96.6 (3.0)	100.2
Cortisol	684 (8.7)	669 (4.4)	102.2
α-Cortolone	1613 (66.7)	1547 (25.2)	104.3
THS	2657 (176)	2530 (200)	105.0
Etio	2533 (83.3)	2470 (157)	102.6
Pregnenolone	174 (4.2)	179 (3.06)	97.6
PT	1907 (30.6)	1867 (3.1)	102.1
PD	4107 (35.1)	4130 (11.5)	99.4
5-PD	3123 (15.3)	3177 (49.3)	98.3

Supplemental Table 3.5. Pearson correlation between steroid excretion and tumor diameter of the urine samples of patients with adrenal tumor (n=19)

	Pearson r	p-Value.
5-PT	0.486*	0.035
DHEA	0.282	0.243
Cortisone	0.628**	0.004
Cortisol	0.700**	$8.37x10^{-4}$
α-Cortolone	0.042	0.865
THS	0.373	0.115
Etio	0.394	0.095
Pregnenolone	0.293	0.223
PT	0.460*	0.047
PD	0.126	0.606
5-PD	0.500*	0.029

* $p<0.05$, ** $p<0.01$

4 Classification of adrenocortical tumors using urinary steroid profiling

The content of this chapter has been submitted for publication. The results developed from a cooperation with the Chair of Bioinformatics of the University of Würzburg. For individual author contributions, see Table A7 and Table A10. Permissions for reprint were obtained from all co-authors.

A simplified diagnostic workflow for adrenocortical tumors using urinary steroid profiling by LC-MS

Nora Vogg[1,2], Tobias Müller[3], Andreas Floren[3], Thomas Dandekar[3], Anna Riester[4], Ulrich Dischinger[1], Max Kurlbaum[1,2], Matthias Kroiss[1,4]*, Martin Fassnacht[1,2]*

[1]Department of Internal Medicine I, Division of Endocrinology and Diabetes, University Hospital, University of Würzburg, Germany

[2]Central Laboratory, Core Unit Clinical Mass Spectrometry, University Hospital Würzburg, Germany

[3]Department of Bioinformatics, Biocenter, Am Hubland, University of Würzburg, Germany

[4]Department of Internal Medicine IV, University Hospital Munich, Ludwig-Maximilians-Universität München, Munich, Germany

* equal contribution

Abstract

Background: Preoperative identification of malignant adrenal tumors is challenging. 24-h urinary steroid profiling by mass spectrometry and machine learning has demonstrated high diagnostic power, but the unavailability of bioinformatic models for public use has limited its routine application. We here aimed at a simple classification model for the differentiation of adrenocortical adenoma (ACA) and adrenocortical carcinoma (ACC).

Methods: Eleven steroids (5-pregnenetriol, dehydroepiandrosterone, cortisone, cortisol, α-cortolone, tetrahydro-11-deoxycortisol, etiocholanolone, pregnenolone, pregnanetriol, pregnanediol, and 5-pregnenediol) were quantified by LC-MS/MS in 24-h urine samples from adrenal tumor patients (n=268, n(ACA)=217, n(ACC)=51). Random forest modelling and decision tree algorithms based on histologic diagnosis (n=161) or follow-up were applied and independently validated in 84 patients with paired 24-h and spot urine available.

Results: A decision tree using excretions of only 5-pregnenetriol and tetrahydro-11-deoxycortisol classified the three groups low, intermediate, and high risk for malignancy. 148/217 ACA were classified as being at low, 67 intermediate, and 2 high risk of malignancy. Conversely, none of the ACC demonstrated a low risk profile leading to a negative predictive value of 100% for malignancy in this group. This was confirmed in the independent validation cohort in both 24-h urine and spot urine. Based on these results, we propose a diagnostic workflow, in which first tier urine analysis could obviate adrenal-dedicated imaging in >65% of patients with ACA.

Conclusions: The LC-MS/MS-based classification model provided excellent results for the exclusion of ACC in 24-h urine. Analysis of spot urine led to similarly satisfactory results suggesting that urine collection might be dispensable.

Introduction

Adrenal tumors are among the most common neoplasms in humans with a prevalence between 3–10% increasing with age (1-4). The increasingly frequent use of cross sectional imaging led to a rise of incidentally detected adrenal tumors. Thus, the need for reliable diagnostic workup of these incidentalomas has increased substantially (5). Current guidelines aim at the diagnosis of (i) the malignant potential of a given lesion and (ii) autonomous hormone secretion (6). The differentiation between benign adrenocortical adenoma (ACA) and malignant adrenocortical carcinoma (ACC) can be challenging based on current diagnostic methods and techniques. Frequently occurring ACA mainly present with mass diameters <4 cm and require no therapeutic intervention in case autonomous hormone secretion is excluded (6). In contrast, ACC are very rare with an annual incidence of 0.5–2/1.000.000 (7). Treatment options are limited in advanced stages and result in a 5-year survival <15% (8). Hence, early diagnosis of ACC in localized stages might be lifesaving by enabling complete surgical tumor resection (9). Delayed surgery has been linked to time-consuming hormonal workup (10). Therefore, a simplified diagnostic test strategy in clinical routine is urgently needed.

Current international guidelines for the diagnostic workup of adrenal tumors recommend imaging and biochemical testing for hormone excess (6). Unenhanced abdominal computed tomography (CT) is the imaging method of choice whereby tumor tissue attenuation ≤10 (11) or ≤20 Hounsfield units (HU) (12) indicates absence of malignancy with high specificity but poor sensitivity. Magnetic resonance imaging (MRI) with chemical shift is probably similarly accurate, but the number of sound studies is limited (11). While the additional value of delayed wash-out CT has recently been found to be moderate (4, 13), also fluorodesoxyglucose positron emission tomography (FDG-PET) missed to identify 7 of 47 malignant tumors in a series of 117 indeterminate adrenal masses (14). In clinical practice, however, most adrenal tumors are detected in enhanced CT scans that cannot discriminate malignant from benign adrenal tumors. Thus, an additional adrenal-focused imaging is usually required during the diagnostic workup of adrenal incidentalomas.

Urinary steroid profiling by mass spectrometry-based techniques has previously demonstrated its value in the differential diagnostics of adrenal tumors (15). By using gas chromatography mass spectrometry (GC-MS), several groups found increased urinary steroid excretion with particularly high tetrahydro-11-deoxycortisol (THS) in ACC compared to benign adrenal tumors or controls (16-21). In a prospective study of more than 2000 patients with adrenal mass, the quantification of 15 steroids in 24-h urine samples and risk classification by machine-

learning was demonstrated to be diagnostically particularly useful when imaging results were included in the diagnostic algorithm. In combination, this strategy resulted in a positive predictive value (PPV) of 76.4% and a negative predictive value (NPV) of 99.7% (12) for the diagnosis of malignancy. Importantly, in that study, GC-MS as the standard technique for steroid profiling has been replaced by the clinically more readily applicable liquid chromatography mass spectrometry (LC-MS) (22, 23).

Urine sampling over 24 h is the current method of choice for the assessment of steroid output and is considered to usefully exploit the circadian rhythmicity of steroidogenesis in healthy subjects compared to patients with autonomous steroid secretion. All previous studies aiming at the diagnosis of malignancy in adrenal tumors have used this traditional sampling (12, 16-21, 24). However, the collection of 24-h urine is cumbersome, time-consuming, and prone to errors specifically when performed in patients with impaired capabilities to adhere to sampling procedures. Thus, in clinical routine incomplete collections occur in a proportion of 30% or higher (25, 26).

We here aimed to overcome the drawbacks of sampling and the use of proprietary algorithms that until now have limited the application of urinary steroid profiling for the differential diagnosis of malignancy in adrenal tumors outside very specialized institutions.

Materials and Methods

Study design and population

This is a retrospective study of prospectively collected urine samples from adult patients treated at two German referral centers (University Hospitals Würzburg and Munich). The study was conducted as part of the European Network for the Study of Adrenal Tumors (ENSAT) registry that has been approved by the local ethics committee (#88/11 and 379/10). All patients provided written informed consent. The inclusion criterion was presence of an adrenal tumor with a diameter ≥ 2 cm. Exclusion criteria were previous treatment of adrenal disease and diagnoses of pheochromocytoma, myelolipoma, or adrenal metastases from other malignancies based on clinical workup. The final diagnosis was based on current clinical practice guidelines for the management of adrenal incidentalomas and ACC (6, 8, 9) with post-operative histopathology and/or follow-up investigations as gold standards. Patients were instructed to collect their urine after discarding the first morning urine over the period of 24-h including the following morning urine. Spot urine samples were taken at a random time of day within 14 days before or after

24-h urine collection. Urine samples were collected between March 2010 and March 2022 and stored at –20°C until analysis.

We followed the method outlined by Buderer (27) to compute sample sizes for expected sensitivity and specificity and took the larger required sample size of the two, which in our case is the sensitivity. Assuming a proportion of 20% ACC at specialized centers, a minimum sample size of 173 samples was required to yield a 10% width of a 2-sided 95% CI of an expected sensitivity of 90% (16).

A cohort of 268 patients provided a 24-h urine sample per patient that were used for classification model establishment (training/test cohort). ACC and ACA patients were randomly split into a training set (n=188, 70%) and a test set (n=80, 30%). An independent cohort of 84 patients provided both 24-h urine and corresponding spot urine and served as validation cohort, but also to compare the performance of spot urine to 24-h urine. Figure 4.1 visualizes the composition of the study cohort. In total, 71 patients had an ACC and 281 were diagnosed with ACA. These diagnoses were based on histology in all patients with ACC and in 145 patients with ACA. In the remaining patients, strict follow-up criteria (13) were applied to prove the benign nature of the lesion.

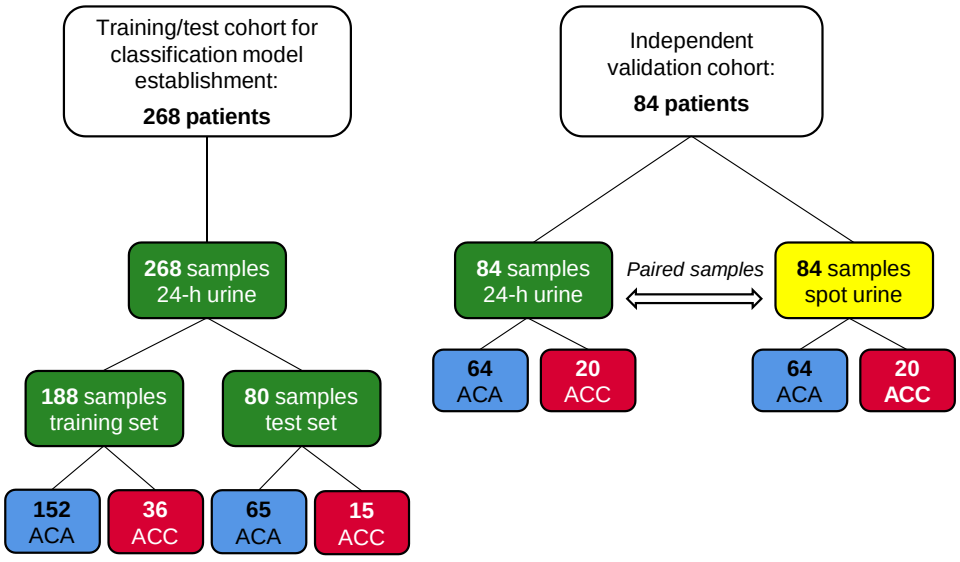


Figure 4.1. CONSORT diagram depicting the study cohorts. The classification model was established using 268 samples randomly assigned to the training (n=188) and test cohort (n=80). An independent cohort of 84 patients was used to validate the classification model with paired samples of 24-h urine and spot urine of each patient. ACC: adrenocortical carcinoma, ACA: adrenocortical adenoma.

Age, sex, and tumor diameter were recorded and imaging characteristics were classified as unsuspicious or suspicious. For this purpose, unsuspicious was defined as HU ≤20 in unenhanced CT (12), relative contrast washout >58% in delayed washout CT (13), loss of signal intensity between in- and out-of-phase images in MRI chemical shift analysis (28), or absence of FDG uptake or uptake less than the liver (6, 29).

Laboratory methods

The urinary concentrations of the following 11 steroids were quantified by LC-MS/MS as described in detail elsewhere (30): 5-pregnenetriol (5-PT), dehydroepiandrosterone, cortisone, cortisol, α-cortolone, tetrahydro-11-deoxycortisol (THS), etiocholanolone, pregnenolone, pregnanetriol, pregnanediol, and 5-pregnenediol. Briefly, 150 µl urine underwent enzymatic hydrolysis of steroid conjugates with arylsulfatase/glucuronidase from *Helix pomatia* and steroids were extracted via solid-phase extraction.

Creatinine measurements were performed using a Cobas® 8000 immunoassay (Roche Diagnostics GmbH).

Establishment of classification models and statistical analysis

Statistical analyses were performed using IBM SPSS 28 and R version 4.0.2 (31). Groups were compared using non-parametric tests and p-values were adjusted for multiple testing according to Benjamini-Hochberg (32) with adjusted $p<0.05$ considered significant.

For classification, we used conditional inference decision trees (33) as implemented in the R package partykit (34). The ctree algorithm recursively performs univariate splits of the dependent variable based on values on a set of covariates and therefore avoids the variable selection bias of other decision tree algorithms. Unlike the others, the ctree algorithm uses a significance test procedure in order to select variables. The derived significances are based on permutation tests. For implementation of the random forest algorithm (as implemented in the cforest function), we relied on conditional inference decision trees. We applied both functions with defaults settings, but with an increased number of resamplings (n=99999) and set the proportion of observations needed to establish a terminal node to minprob=0.1.

Results

Patient characteristics and steroid excretions

Urine samples of 352 patients with adrenal tumors were included in this study within a training/test and validation cohort. Demographic and clinical characteristics of the patient cohorts are summarized in Table 4.1.

Table 4.1. Demographic and clinical characteristics of the patients with ACC vs. ACA for training/test cohort and validation cohort

	Training/test cohort		**Validation cohort**	
	ACC (n=51)	**ACA (n=217)**	**ACC (n=20)**	**ACA (n=64)**
Sex				
Men	16 (31.4%)	63 (29.0%)	6 (30.0%)	21 (32.8%)
Women	35 (68.6%)	154 (71.0%)	14 (70.0%)	43 (67.2%)
Age [years]	53 (46–65)	59 (51–68)	53 (47–58)	59 (52–65)
Tumor diameter [cm]	10.0 (7.5–14.0)	3.2 (2.7–4.1)	10.3 (8.9–12.1)	3.0 (2.4–3.9)
2 to ≤4cm	2 (3.9%)	160 (73.7%)	1 (5.0%)	54 (84.4%)
> 4cm	49 (96.1%)	57 (26.3%)	19 (95.0%)	10 (15.6%)
Imaging characteristic				
Unsuspicious	0	157 (72.4%)	0	42 (65.6%)
Suspicious	51 (100.0%)	56 (25.8%)	20 (100.0%)	21 (32.8%)
Not specified	0	4 (1.8%)	0	1 (1.6%)

Data presented as n (%) or median (IQR).

The comparison between ACA and ACC using Wilcoxon test was performed both for urinary steroid concentrations normalized on creatinine and on collection volume as 24-h sum excretion (Supplemental Figure 4.1). As both normalization approaches were comparable with more significant differences between ACC and ACA using steroid-to-creatinine ratios and with the aim of applying the classification model to spot urine, creatinine-normalized steroid excretion was investigated for further analyses.

Between groups, we observed significantly higher ($p<0.001$) excretion of 8/11 steroids in patients with ACC compared to patients with ACA after correction for multiple testing (Supplemental Figure 4.2).

Establishment of the classification model and application to the validation cohort

We applied both a decision tree strategy and random forest classification to differentiate between ACA and ACC. The excretion of the two steroids 5-PT and THS that were selected automatically by the ctree algorithm performed surprisingly well for clinical diagnosis. Even the threshold of 5-PT at 275.7 µg/g creatinine alone resulted in a total training error of only 6.4%: 7/36 patients with ACC and 5/152 patients with ACA were misclassified (Figure 4.2). In comparison, the training error of the more complex random forest model based on all 11 steroid excretions was 5.3%. We found strong correlation between all steroids in a 5-PT and THS dominated cluster, respectively (Supplemental Figure 4.3).

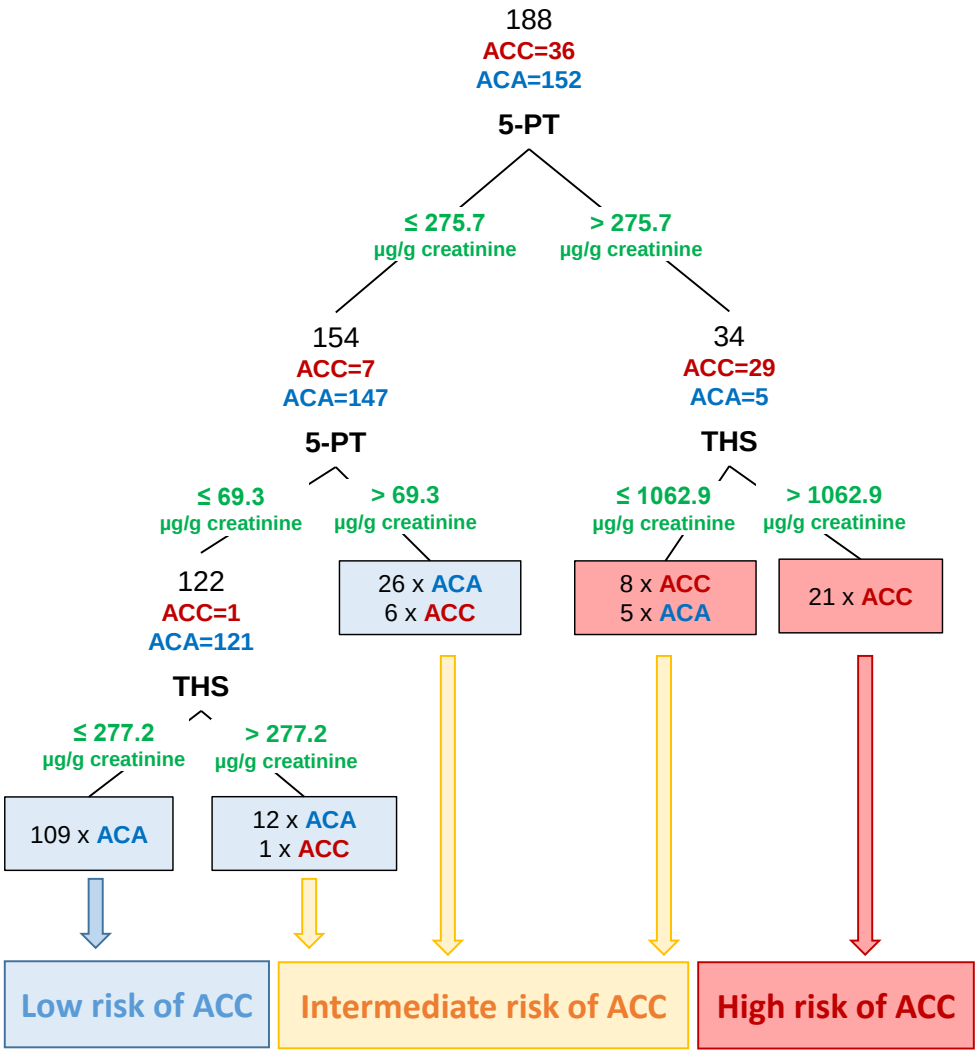


Figure 4.2. The decision tree based on 5-PT and THS excretions was trained on 188 24-h urine samples from patients with adrenal tumor to discriminate ACA from ACC. Two high accuracy branches for the classification of ACA and ACC were identified. Patients with 5-PT ≤69.3 µg/g creatinine and THS ≤277.2 µg/g creatinine have a low risk for ACC. The arm classifying patients at high risk includes samples with 5-PT >275.7 µg/g creatinine and THS >1062.9 µg/g creatinine.

The decision tree revealed a valuable substructure regarding classification accuracy in its branches. Both the left and the right branch were accurate with 0.0% training error. However, the three intermediate branches together exhibited a training error of 20.7%, including 15 ACC and 43 ACA. Therefore, we defined the three classes of low (5-PT ≤ 69.3 µg/g creatinine and THS ≤ 277.2 µg/g creatinine), intermediate, and high risk of ACC (5-PT > 275.7 µg/g creatinine and THS > 1062.9 µg/g creatinine).

Subsequently the decision tree was applied to the 24-h urines of the test set (n=80). 48 samples were classified with either high or low risk of ACC while 32 samples had an intermediate risk

of ACC. Two of the nine samples with high risk had the final diagnosis of ACA and all 39 samples with low risk were ACA (Table 4.2).

Table 4.2. Diagnostic performance of the decision tree applied to the test set of 80 patients

	ACC	**ACA**	**Total**	**Predictive values (%), 95% CI**
High risk of ACC	7	2	9	77.8 (PPV), 40.2–96.1
Intermediate risk of ACC	8	24	32	
Low risk of ACC	0	39	39	100.0 (NPV) 88.8–100.0
Total	15	65	80	
Accuracy (%), 95% CI	46.7 (TPR), 22.3–72.6	60.0 (TNR), 47.1–71.7	57.5, 45.9–68.5	

TPR: True positive rate, TNR: True negative rate, PPV: Positive predictive value, NPV: negative predictive value

The independent cohort of 84 patients with adrenal tumors was used to validate the decision tree. Classification between ACA and ACC resulted in a total accuracy of 66.7% when considering the high risk class as correctly classified for ACC and the low risk class for ACA, respectively. The true positive rate (sensitivity) was 70.0% and the true negative rate (specificity) was 65.6%. A proportion of 87.5% of patients classified with high risk of ACC had the final diagnosis of ACC whereas low risk classification excluded ACC with 100.0% NPV.

Proposal of a modified algorithm to diagnose adrenal tumors

All 352 patients who provided a 24h-urine sample were included in a cross tabulation combining urinary steroid analysis and cross sectional imaging characteristics (Figure 4.3A). The 190 patients who were classified with low risk of ACC by the urinary steroid decision tree all had the final diagnosis of ACA. Of the 116 samples classified with intermediate risk by urinary analysis, 29 were ACC and 87 were ACA. In 58 of these 87 patients with ACA, the tumor appeared unsuspicious on imaging. 46 Patients had high risk urinary steroids and 42

thereof actually had an ACC while four patients had an ACA. Three of these four ACA lacked typically benign imaging features and one was unsuspicious on imaging.

Motivated by the clinical scenario that the majority of adrenal incidentalomas are nowadays detected in a contrast enhanced CT scan indicated for other purposes and hence requires additional adrenal-focused imaging (e.g. unenhanced CT, chemical-shift MRI etc.), we developed a sequential diagnostic approach using urinary steroid profiling in 24-h urine followed by imaging (Figure 4.3B). With this algorithm, 249 of 281 patients with ACA (88.6%) could be classified correctly with excellent exclusion of ACC in our sample set. The most obvious advantage of this approach, however, is that additional imaging could have been dismissed in 190 patients with ACA (67.6% of all ACA) after urinary analysis.

Evaluation of spot urine as surrogate of 24-h urine

As the independent validation cohort of 84 patients provided paired samples of 24-h urine and spot urine, we aimed to directly compare steroid excretions in both sample types. No significant differences in steroid excretions of 5-PT and THS normalized to creatinine were detectable between both collection approaches using Wilcoxon testing after adjustment for multiple testing (Figure 4.4A).

The decision tree model was applied to both sample types. Direct comparison of the classification using 24-h urine and spot urine samples resulted in a slightly lower performance of spot urine with a total accuracy of 56.0% compared to 66.7% in 24 h urine. 26 Samples of 24-h urine and 35 samples with spot urine were classified with intermediate risk. However, PPV in spot urines was almost as high as in 24-h urine samples (86.7% vs. 87.5%) and NPV was 100.0% in both urine types (Figure 4.4B–E).

In addition to the 34 patients who were correctly classified as ACA using spot urine, the tumors of 20 more patients with ACA were unsuspicious on imaging. Hence, when applying the combined diagnostic workflow using spot urine analysis followed by additional imaging for patients with intermediate and high risk, the considerable proportion of 54 of all 64 patients with ACA (84.4%) was identified correctly. Moreover, all 20 ACC were classified with either intermediate or high risk in urinary steroid analysis and suspicious imaging characteristics (Supplemental Figure 4.4).

A

Urinary steroids \ Imaging	ACC				ACA			
	Suspicious	Unsuspicious	n/a	Total	Suspicious	Unsuspicious	n/a	Total
High risk of ACC	42	0	0	42	3	1	0	4
Intermediate risk of ACC	29	0	0	29	29	58	0	87
Low risk of ACC	0	0	0	0	45	140	5	190
Total	71	0	0	71	77	199	5	281

B

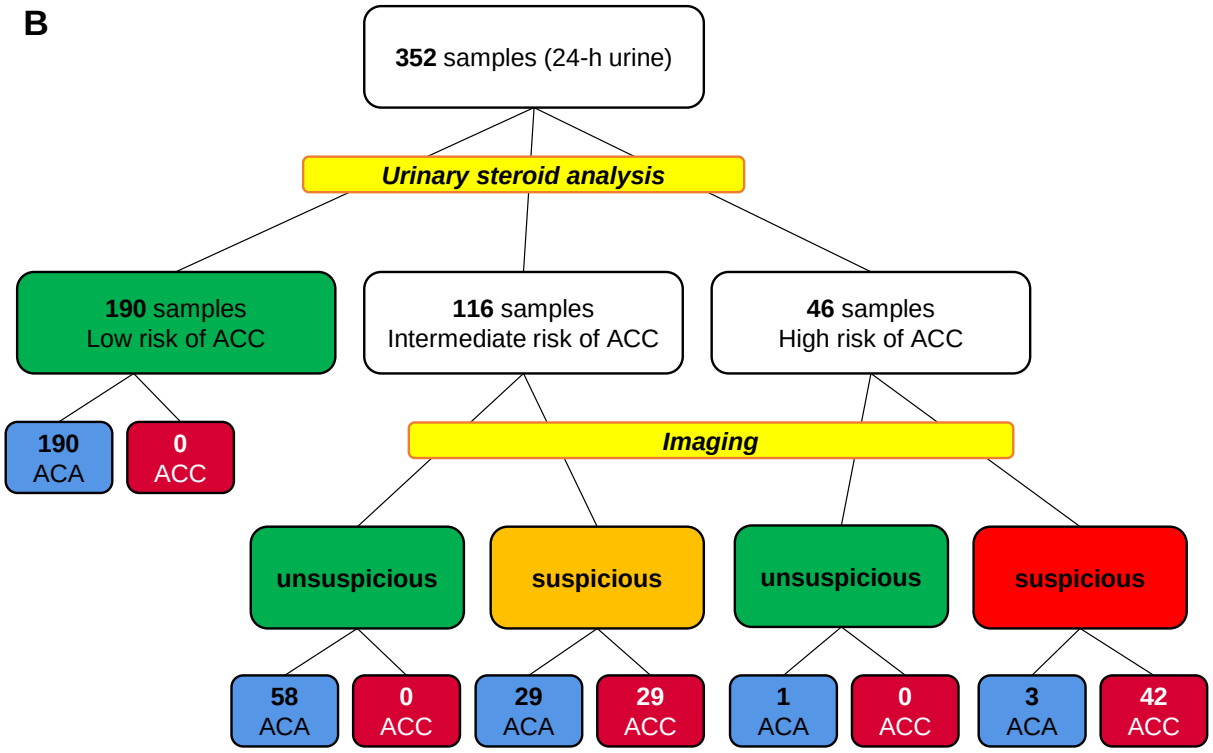
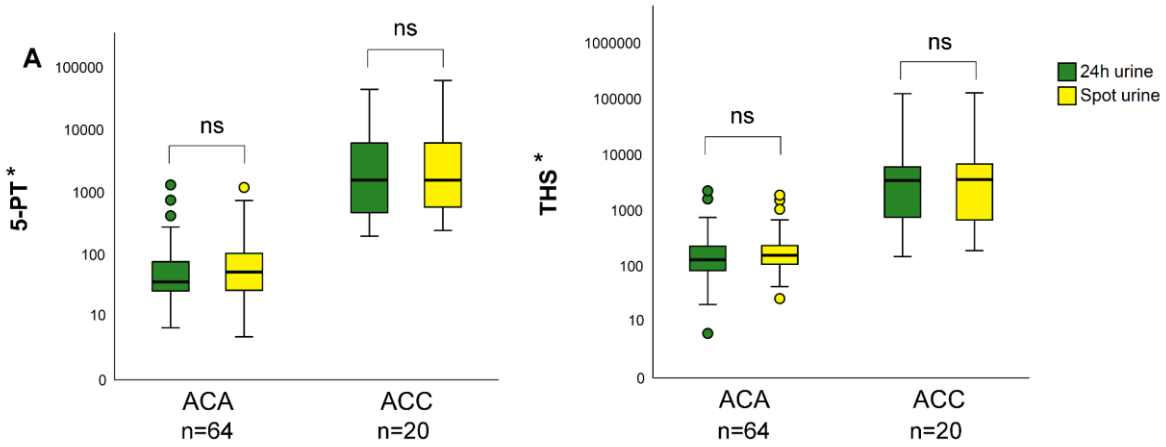
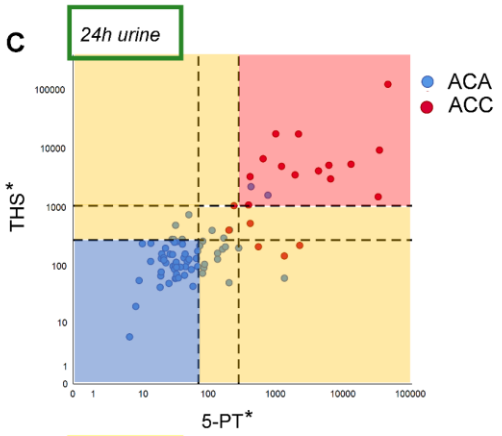


Figure 4.3. (A) Cross tabulation of 24-h urinary steroid classification to the imaging characteristics suspicious or unsuspicious (or n/a) for ACC and ACA patients of the entire cohort (n=352). (B) Flow chart of a sequential diagnostic approach using urinary steroid analysis followed by adrenal-focused imaging only for patients with intermediate or high risk of ACC. Green indicates classification as ACA, red indicates classification as ACC and orange indicates undetermined classification.



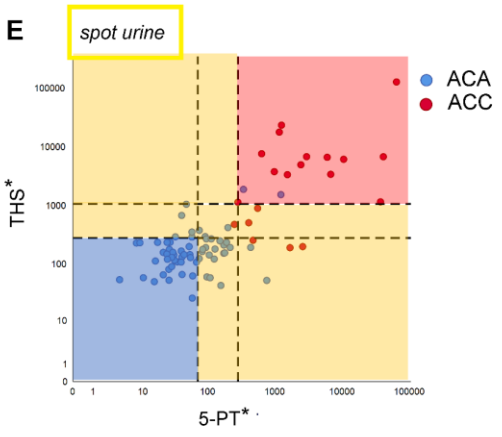
B

24h urine	ACC	ACA	Total	Predictive values (%)
High risk of ACC	14	2	16	87.5 (PPV)
Intermediate risk of ACC	6	20	26	
Low risk of ACC	0	42	42	100.0 (NPV)
Total	20	64	84	
Accuracy (%)	70.0 (TPR)	65.6 (TNR)	66.7	



D

spot urine	ACC	ACA	Total	Predictive values (%)
High risk of ACC	13	2	15	86.7 (PPV)
Intermediate risk of ACC	7	28	35	
Low risk of ACC	0	34	34	100.0 (NPV)
Total	20	64	84	
Accuracy (%)	65.0 (TPR)	53.1 (TNR)	56.0	



*steroid excretion in [µg steroid / g creatinine]

Figure 4.4. (A) Comparison of 5-PT and THS excretion in 24-h urine and spot urine within ACA and ACC patients showed no significant differences. (B-E) Diagnostic performance of the independent validation cohort. Cross tabulation (B, D) and visualization as scatterplot (C, E) of the 24-h urine sample set (B, C) and the spot urine sample set (D, E).

Discussion

This study demonstrates the performance of a simple classification model using the excretion of two urinary steroids for the differentiation between ACA and ACC. Importantly, the application of the more complex random forest algorithm only slightly improved overall accuracy by 1–2% compared to a decision tree. This supports our aim to propose a relatively simple model with the perspective of facilitating interpretation and reducing complexity for clinical routine implementation. After normalization of steroid concentrations to creatinine, a direct interpretation can be carried out considering only 5-PT, the urinary metabolite of 17-hydroxypregnenolone, and THS, the urinary metabolite of 11-deoxycortisol. A major strength of the decision tree is its substructure of the five classification branches, allowing for determination of the three classes of high, intermediate, and low risk of ACC. High risk and low risk classes are both of high diagnostic accuracies with very low error rates. Not unexpectedly, the 0.0% training error of the high risk class could neither be reproduced in the test set, nor in the independent validation cohort though, as two ACA patients were classified with high risk of ACC in the test and validation set, respectively. All these four cases were preoperatively suspected ACC but identified as ACA in postoperative histopathology. Two of these ACA were oncocytic ACA. However, as all four cases had clinically significant hormone excess, adrenalectomy was indicated (6). In contrast, tumors classified as “low risk” had a 0.0% error rate, which was reproducible in the test set as well as in the independent validation cohort for both 24-h urine and spot urine. With a NPV of 100%, ACC could be excluded with high certainty in more than half of patients with ACA even using spot urine.

Previous studies using LC-MS/MS in 24-h urine quantified 26 (24) and 15 steroids (12). Our LC-MS/MS method is validated to quantify 11 urinary steroids (30) but the finally selected decision tree-based classification requires only excretion of two steroids leading to reduced time and effort for data interpretation. Inclusion of additional steroids are unlikely to deliver greatly improved classification performance due to high correlations between each other in a THS and 5-PT dominated cluster. Complex classification algorithms such as random forest can thus be obviated in clinical routine.

Imaging characteristics are a key tool in adrenal tumor diagnostics and a tumor diameter >4 cm is generally considered suspicious (6, 35). However, both small ACC and large ACA occur with measurable frequency (36), indicating that the tumor diameter alone has limited diagnostic accuracy. In our entire cohort, 67 ACA had a diameter >4 cm and three ACC measured ≤4 cm.

These three ACC did not show typical features of benign tumors on imaging and all urine samples were classified with intermediate risk.

In the clinical situation, the lack of adequate imaging and the need for repeated or alternative imaging studies is common, leading to increased cost, delay in diagnostic workup and increased radiation exposure. In the present study, a subgroup of 190 of all 281 ACA (67.6%) had urinary steroid excretions with low risk of ACC indicating that these patients might not have required further imaging in the diagnostic workup. In the intermediate and high risk groups according to urinary steroid analysis, unsuspicious imaging characteristics were present in 59 additional patients with ACA which is likewise an accurate exclusion criterion for ACC.

In Figure 4.3B, we propose a sequential approach for the implementation of steroid urinary steroid profiling in clinical routine to reduce the number of patients that require additional adrenal-dedicated imaging. Creatinine-normalized urinary steroid excretion would constitute the first tier test for the workup of incidentalomas. Patients with low risk of ACC according to urine analysis would not undergo further imaging, whereas samples classified with intermediate und high risk of ACC would trigger additional imaging. Still, in patients with high risk of ACC further imaging might also be dispensable before surgery. Even in our series with a higher proportion of ACC than in a non-expert center, we would have been able to reduce the number of required imaging in ACA patients by more than 65% using 24-h urine. Given the higher proportion of ACA in a community setting, the absolute number of spared imaging would be significantly higher.

To our knowledge, this study is the first to systematically compare 24-h urine samples in patients with adrenal tumors to corresponding spot urine. Bileck et al. previously found poor correlation of 40 steroids normalized to creatinine between 24-h urine and spot urine but did not study the value of spot urine for the differential diagnosis of adrenal masses (37). Paired Wilcoxon test of 5-PT and THS excretions did not reveal significant differences between the two sample types in our validation cohort. More samples were classified as intermediate risk in spot urine though, which is likely caused by circadian variations of steroid excretion in spot urine samples. However, the stringent cutoffs for 5-PT and THS in the classification model hardly affected NPV and PPV. Depending on patient factors and practical considerations, spot urine might serve as an easily accessible sample for urinary steroid analysis and has the potential to replace 24-h urine in the future. In case of classification with intermediate risk after spot urine analysis, a subsequent 24-h urine collection may be taken into consideration to reduce the intermediate risk proportion. Patients with biochemical evidence of low risk of ACC or

unsuspicious characteristics on imaging require no adrenalectomy. In contrast, patients with high risk urinary steroid profile and suspicious tumor appearance on imaging should undergo adrenalectomy without any further delay. Patients with intermediate risk of ACC and suspicious tumor appearance should be treated individually, but in case of doubt rather undergo surgery as the ACC proportion of these cases was 50% in our study cohort.

Several limitations apply to our study: Despite the acquisition of samples in two adrenal tumor referral centers, the sample size is still limited, in particular regarding spot urines. Further, the proportion of ACC in the entire cohort is relatively high and does not reflect the proportions in unselected cohorts with adrenal tumors. On the other hand, patients were thoroughly characterized including available histopathology in 216 patients and reliable diagnostic criteria in patients who have not undergone surgery. Another drawback is the limitation of the classification model to adrenal lesions with exclusion of extra-adrenal malignancy. Moreover, quantification of 5-PT and THS by LC-MS/MS is not available in every laboratory. Over the years, however, LC-MS/MS has increasingly replaced alternative techniques and is currently available at many centers.

In conclusion, we present a classification model to be used as part of the diagnostic workup of adrenal tumors that consists of simple and non-invasive sample acquisition and a straightforward interpretation of steroid excretion data with high practical applicability. The exclusion of ACC with high certainty using only two urinary steroids even in spot urine is a major advance of our study and highly relevant in clinical practice. Nevertheless, prospective validation studies with larger sample sizes are required, especially for the use of spot urine as sample matrix.

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Institutional Review Board Statement

This study was part of the European Network for the study of adrenal tumors (ENSAT) registry, which has been approved by the local ethics committee of the University of Würzburg (#88/11). All patients included in this study provided written informed consent.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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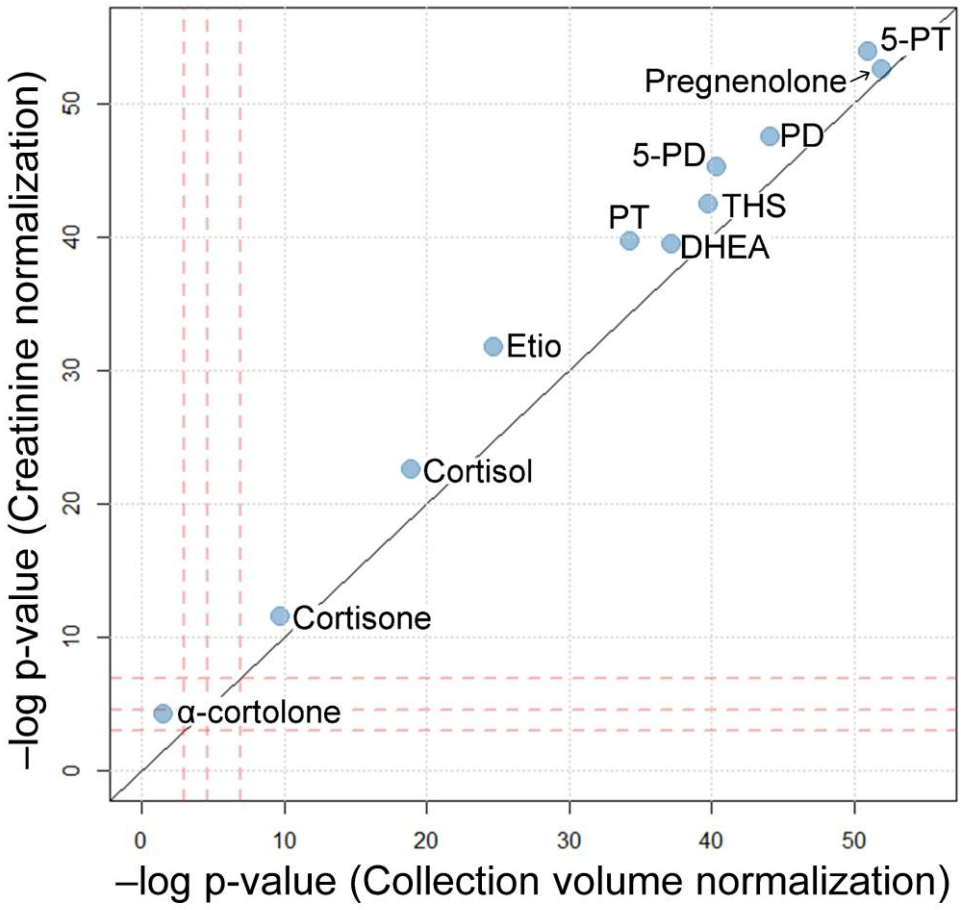
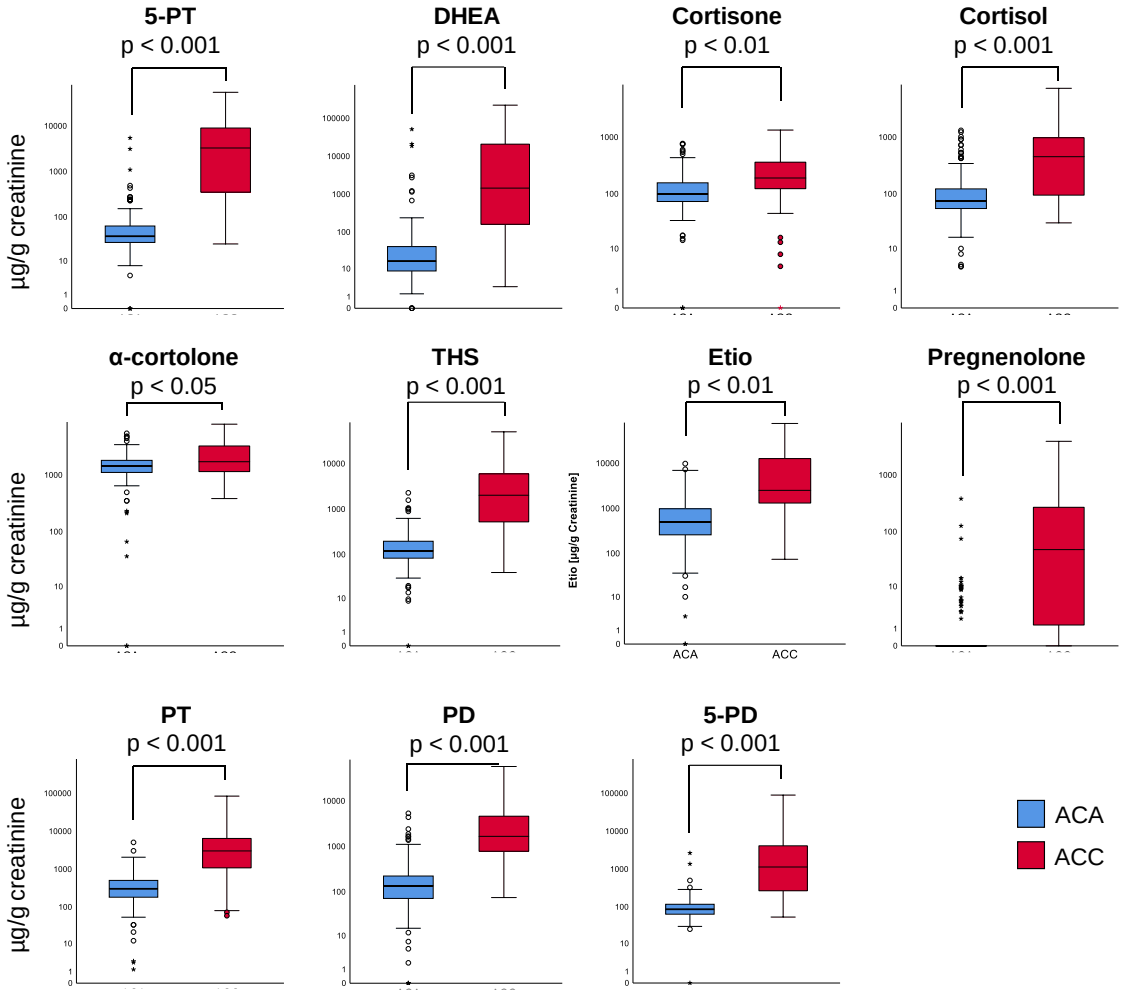
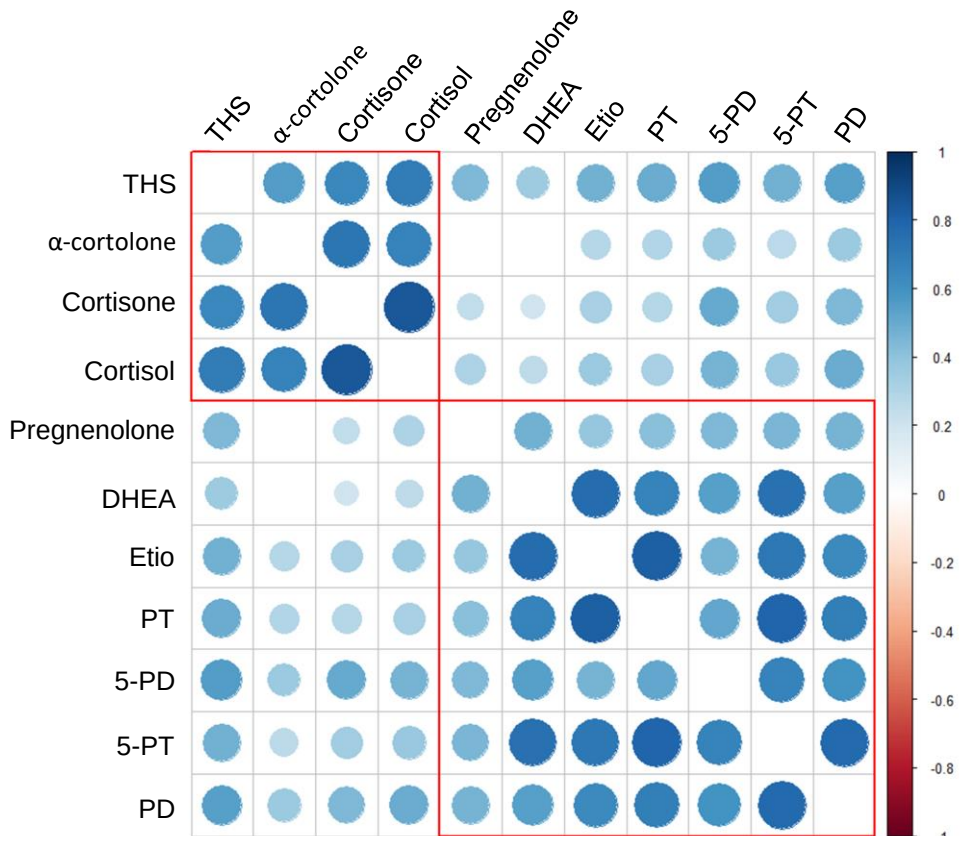
Supplemental Figures



Supplemental Figure 4.1. Comparison of ACC vs. ACA differentiation using p-values based on the Wilcoxon test for the two normalization approaches of steroid concentrations to creatinine and collection volume. Red lines indicate the significance levels 0.05, 0.01, and 0.001. All steroids have lower p-values using normalization to creatinine.



Supplemental Figure 4.2. Steroid excretions in 24-h urine samples of the training/test cohort. Patients with ACA (n=217, blue boxplots) show significantly lower steroid excretions than patients with ACC (n=51, red boxplots) after comparison with Wilcoxon rank-sum test and multiple testing correction. 5-PT: pregnenetriol, DHEA: dehydroepiandrosterone, THS: tetrahydro-11-deoxycortisol, Etio: etiocholoanolone, PT: pregnanetriol, PD: pregnanediol, 5-PD: 5-pregnenediol.



Supplemental Figure 4.3. Correlation plot showing Spearman correlation coefficients of significant correlations between the steroids. The two major clusters identified by hierarchical cluster analysis are marked in red, with one cluster containing THS and the other cluster containing 5-PT.

Validation cohort: spot urine

	ACC				ACA			
Imaging / *Urinary steroids*	Suspicious	Unsuspicious	n/a	Total	Suspicious	Unsuspicious	n/a	Total
High risk of ACC	13	0	0	13	1	1	0	2
Intermediate risk of ACC	7	0	0	7	9	19	0	28
Low risk of ACC	0	0	0	0	11	22	1	34
Total	20	0	0	20	21	42	1	64

Supplemental Figure 4.4. Cross tabulation of urinary steroid classification to the imaging characteristic suspicious or unsuspicious (or n/a) for patients with ACC and ACA of the independent validation cohort (n=84) using spot urine.

5 Discussion

Currently applied diagnostic methods for the investigation of adrenal tumors are lacking diagnostic accuracy. Two LC-MS/MS methods were developed, validated, and applied to biological samples of patients with adrenal tumors.

5.1 Optimization of the DST

The first part of this thesis comprises the development and validation of a method for the quantification of Dex and cortisol in DST serum samples, the application to patient samples, and the establishment of method-specific thresholds to filter out false positive test results. By measuring serum samples of 400 patients after 1 mg DST, a considerable inter-individual variation in Dex exposure was found. Serum Dex concentrations varied from undetectable to 20.2 ng/mL with a median concentration at 4.8 ng/mL. Demographic and clinical features were investigated for potential effects on the serum Dex concentration. A decreased eGFR and diabetes mellitus were found to be significantly associated with an increased serum Dex concentration. In contrast, BMI, sex, age, nicotine consumption, and the use of oral contraceptives did not significantly affect Dex exposure. The newly established cutoff concentration for the minimal necessary serum Dex concentration at 1.8 ng/mL helps to rule out false-positive tests due to non-compliance or insufficient Dex exposure for pharmacokinetic reasons. In our cohort of 400 patients, 31 patients (7.75 %) exhibited serum Dex <1.8 ng/mL, indicating insufficient exposure for the DST which is a substantial proportion that needs to be considered. Moreover, the method-specific adaption of the commonly applied cortisol threshold from 1.8 µg/dL to 2.4 µg/dL remarkably improved clinical test specificity from 71.7 % to 92.4 % (Vogg et al. 2021).

5.2 Improved and simplified discrimination of ACA and ACC

The second method was developed and validated to quantify 11 deconjugated steroids in urine and aims to improve the differentiation between ACC and ACA. Analytical performance was validated according to the guidelines of the European Medicines Agency (2011) and the Food and Drug Administration (2018). The focus was set on an improved analytical accuracy and urinary steroid stability, which is highly relevant for sample transport in clinical practice and was extensively validated (Vogg et al. 2022).

This method was applied to 268 24-h urine samples of patients with adrenal tumor and a simple classification model, suitable for clinical routine implementation, was established. The final decision tree used for classification was based on the two steroids 5-PT and THS. By lower and upper threshold excretions for both steroids, the decision tree results in five branches. Thereof, two branches were able to diagnose ACC and ACA with high accuracy and the final classification was consequently grouped into low risk, intermediate risk, and high risk of ACC. The diagnostic performance of this model showed good results in the test cohort with a PPV of 77.8 % and a NPV of 100.0 %. In an independent validation cohort of 84 patients who provided both a 24-h urine and a spot urine sample, the classification model showed even better results with a PPV of 87.5 % and a NPV of 100.0 % using 24-h urine. The direct comparison of 24-h urine to spot urine in the validation cohort revealed an only marginally worse performance of the spot urine samples with PPV and NPV of 86.7 % and 100.0 %, respectively. As steroid cutoff concentrations for classification of low and high risk groups were set stringently in the proposed model, the predictive values of the urine test were hardly affected by diurnal variations of steroids. This finding is an important first step towards the possible replacement of 24-h urine by spot urine, however, prospective confirmation in a larger setting is required.

5.3 Implications for clinical routine diagnostics

The method for quantification of Dex and cortisol is intended to be applied to clinical samples very frequently as the DST is a widely performed screening test for autonomous cortisol secretion. According to international guidelines, every patient with adrenal incidentaloma undergoes the DST, as well as patients with suspected CS of adrenal or extra-adrenal origin like Cushing's disease or ectopic CS (Nieman et al. 2008, Fassnacht et al. 2016, Fleseriu et al. 2021). Usually, this test is performed just by determination of the serum cortisol concentration. However, the presented results clearly demonstrate that the evaluation of Dex exposure is important due to the strong inter-individual variability in Dex response which should be considered during test interpretation. By measuring Dex, false positive tests with lacking cortisol suppression due to insufficient serum Dex can be identified. These patients should undergo a different diagnostic test for the investigation of autonomous cortisol secretion, like determination of midnight salivary cortisol or cortisol in 24-h urine, or require a higher dosage of oral Dex for the DST. The validated LC-MS/MS method is short, simple and ready-to-use in clinical practice. In September 2021, the method was implemented as routine determination for DST samples at the University Hospital Würzburg. However, since cortisol quantification by

immunoassays is cheaper and faster, a sequential approach could be considered. Determination of cortisol and Dex by LC-MS/MS might be performed only for the positive tests with cortisol >1.8 µg/dL in the immunoassay measurement. Some individuals might still sufficiently suppress cortisol despite a serum Dex <1.8 ng/mL, which is demonstrated by one patient in the AI-ACS cohort in our study with both cortisol and Dex below their threshold concentrations. In this case, the Dex cutoff should be interpreted individually, and autonomous cortisol secretion can be excluded.

In contrast to the Dex-cortisol method, urinary steroid profiling will be applied more specifically for patients with adrenal tumor as part of the diagnostic workup. Determination of urinary steroid excretions is not (yet) part of the guidelines for the diagnosis of ACC (Fassnacht et al. 2018, Fassnacht et al. 2020). However, there are no doubts that an improvement of currently performed routine diagnostics is required. Recently, the application of urinary steroid profiling in combination with machine learning showed promising results for the detection of malignancy in adrenal tumors in a large prospective multicenter study with >2000 included patients (Bancos et al. 2020). With our newly developed LC-MS/MS method in combination with the decision tree for classification, we aimed for a simplified data evaluation and direct interpretation without the need of complex machine learning algorithms, which will simplify the application in clinical practice. The decision tree based on the excretions of 5-PT and THS shows a good diagnostic performance classifying into low risk, intermediate risk, and high risk of ACC. Especially the low risk class with 0.0 % error in training, test, and validation cohort performs excellent in the exclusion of ACC. There is a good chance to implement the method into clinical routine in the nearer future.

Not only from a patients' perspective the replacement of 24-h urine by spot urine could significantly simplify the diagnostic workup. If our promising results can be confirmed, urinary steroid profiling might even become a screening test not only for any incidentaloma but also for follow-up investigations for the detection of potential recurrence of ACC, which was already investigated by Chortis et al. using a 19-steroid panel (Chortis et al. 2020).

5.4 Comparison of biological sample matrices

The first LC-MS/MS method quantifies Dex and cortisol in serum whereas the second method quantifies steroid metabolites in 24-h urine. As both methods pursue different purposes within

the adrenal tumor investigation, the utilized sample matrix is difficult to compare directly. For both methods, the respective sample matrix has its eligibility and advantage for the intended purpose. The aim of the DST is to evaluate the functionality of the negative feedback mechanism within the HPA axis by the suppressibility of cortisol after Dex intake (Wood et al. 1997). Therefore, the physiological response to Dex is quickly detectable via the cortisol concentration in blood, which is in this case qualified as the ideal sample matrix for the DST.

For steroid profiling, various biomaterials may be used as sample matrix. Serum, plasma, and 24-h urine were most frequently tested for the diagnosis of adrenal tumors (Fanelli and Di Dalmazi 2019, Araujo-Castro et al. 2021). Blood sampling for the determination of steroids in serum or plasma is performed quickly, but patients usually need to travel to the hospital or the doctor's office. It is invasive and possibly associated with pain and a hematoma at the puncture site. By steroid profiling in blood, the hormonal balance is reflected as a snapshot at the time of sampling. This can be a severe drawback, as most steroids underlie a circadian rhythm with high concentration variations within a day, which complicates the reproducibility and comparability of results. Taylor et al., Schweitzer et al., and Berke et al. recently published studies proposing serum or plasma steroid profiling for the diagnosis of ACC (Taylor et al. 2017, Schweitzer et al. 2019, Berke et al. 2022). 24-h urine represents the total urine excretion during a day, which overcomes diurnal variations, as the time integral over 24 hours is assessed. The urine collection is noninvasive and patients are able to collect urine at home and send the sample to the laboratory by mail. On the other hand, the collection process of 24-h urine is very elaborate and inconvenient. Moreover, the collection is often incomplete or incorrectly performed which might falsify the actual steroid excretion (Mann and Gerber 2019).

Both blood and 24-h urine samples have their advantages and limitations (Table 5.1). The ideal sample matrix would be noninvasive, sampled quickly, and without strong diurnal variations. Potential alternative matrices that approximate the ideal matrix might be saliva, spot urine, or overnight urine with normalization to a reference compound like urinary creatinine to compensate dilution effects. However, these matrices have not yet been tested in the diagnostic workup of adrenal tumors in a large setting. Within this thesis, a set of 84 spot urine samples of patients with adrenal tumor was investigated in comparison to the corresponding 24-h urine samples. The spot urine performed surprisingly well in diagnostic accuracy, however, these findings need confirmation in a larger, prospective setting.

Table 5.1. Comparison of serum/plasma vs. 24-h urine as matrix for steroid profiling

	Serum / Plasma	24-h Urine
Advantages	✓ Fast sampling	✓ noninvasive ✓ time integral over 24h
Drawbacks	× invasive × diurnal variations due to circadian rhythm of steroid secretion × patients need to visit doctor's office or hospital for sampling	× time-consuming × inconvenient × complete collection afflicted with errors × difficult for children, elderly, and disabled patients

5.5 Pre-analytical and analytical considerations

Before LC-MS/MS measurement, samples need to undergo a certain pre-treatment dependent of their matrix. Consequently, sample pre-treatment differs for the DST serum samples and the urine samples utilized for steroid profiling.

The first step in serum sample preparation is a protein precipitation step with cold acetonitrile. After two centrifugation steps and dilution with mobile phase, the autosampler injects the prepared sample directly onto an online-SPE column for extraction of the analytes. As the online-SPE column output is connected to the analytical column via a switching valve, extraction and separation of analytes are performed automatically in direct succession. In contrast, pre-treatment of urine samples contains an enzymatic deconjugation step, followed by offline-SPE. The incubation for hydrolysis of sulfate and glucuronide moieties lasts 3 hours at 55 °C. Offline-SPE takes approximately 2–3 hours, depending on the sample size and includes two equilibration steps, sample loading, two washing steps, analyte elution, and complete solvent evaporation, followed by reconstitution.

Strengths of online-SPE are the automated extraction associated with a simple and short sample preparation process for the laboratory staff. Offline-SPE in the contrary requires a lot of manual work, rendering it a labor- and time-intensive extraction technique. Nevertheless, offline-SPE represents a good option for very low concentrated analytes requiring concentration enrichment for sufficient sensitivity. Offline-SPE provides operational flexibility regarding the type of sorbent and the amount and type of washing and elution solvents. Thereby, the extraction efficiency can be optimized easily and offline-SPE is a good choice for method development and research (Liska 1993). The optimized sample preparation for the extraction of urinary

steroids reported within this thesis enables excellent recoveries and a very reproducible extraction performance for all steroids using offline-SPE (Vogg et al. 2022).

5.6 Conclusion and outlook

In conclusion, mass spectrometry-based quantification of steroids clearly improves the hormonal workup of adrenal tumors. The analytical methods are associated with a great potential to become part of clinical routine diagnostics with an increasing implementation of LC-MS/MS in clinical laboratories.

As the determination of serum Dex concentrations after 1 mg DST revealed strong inter- and even intra-individual variations, the influencing factors beyond the already evaluated demographic and clinical features, require further investigation. How significant is the effect of time variations of Dex intake at night and/or blood sampling in the next morning? Are there any effects by food intake or medication? These research questions still need to be answered in the future. Moreover, an expansion of the LC-MS/MS method with other steroids beyond cortisol and Dex is conceivable. Eisenhofer et al. found the simultaneous determination of 15 adrenal steroids in plasma to be helpful for subtyping CS in a retrospective study (Eisenhofer et al. 2018). This approach could also be combined with the DST. By measuring a panel of adrenal steroids in DST samples, cortisol suppressibility, sufficient Dex exposure, and possibly subtypes of CS might be investigated within a single LC-MS/MS measurement. Lastly, it is worth considering a general method-specific cortisol cutoff adaption for the DST. The LC-MS/MS specific cutoff at 2.4 µg/dL significantly increases clinical specificity. However, routine cortisol measurements are most frequently performed using immunoassays which are reported to determine overall higher concentrations compared to mass spectrometry-based methods (Turpeinen and Hamalainen 2013). Therefore, individual cortisol threshold concentrations for the different assays are strongly advisable.

Steroid profiling in 24-h urine was already prospectively proven to be valuable for the diagnosis of ACC when combined with a complex machine learning algorithm (Bancos et al. 2020). Within this thesis, a simple and comprehensible decision tree based on two urinary steroids was trained and tested on 268 24-h urine samples from patients with adrenal tumors. Furthermore, a good performance of spot urine compared to 24-h urine was shown for the first time in a limited sample size of 84 patients. In the future, further studies investigating larger sample sizes are required to confirm these findings. If the suitability of spot urine can be prospectively

validated, urinary steroid profiling might become an even more important pillar of the diagnostic workup of adrenal tumors due to the simplicity of sampling.

Both reported LC-MS/MS methods are in-house methods using threshold-based classification. For a widespread multicenter application, inter-laboratory comparability is crucial and standardization of the steroid hormone assays is necessary (Stanczyk et al. 2007). The effects of varying laboratory staff and different LC-MS/MS instrumentation between laboratories need to be minimized. Transferring the methods to commercial kit solutions would simplify assay standardization and the implementation to clinical routine.

Besides targeted metabolic profiling, untargeted metabolomics provides the possibility to discover new biomarkers for diagnosis and prognosis of adrenal tumors. Sun et al. identified the sulfated estrogens estradiol sulfate and estrone-3-sulfate to be significantly associated with prognosis and discovered the presence of estradiol-3,17-disulfate in a subset of tumors with poor overall survival (Sun et al. 2019). The diagnostic performance of sulfated steroids is still to be investigated (Mueller et al. 2021). Even beyond steroid hormones, other metabolites might be useful in the differentiation between ACC and benign adrenal tumors. For example, Patel et al. found that urinary creatine riboside was elevated 2.1-fold, and L-tryptophan, trimethyl-L-lysine, and 3-methylhistidine were lower 0.33-fold, 0.56-fold, and 0.33-fold, respectively, in patients with ACC (Patel et al. 2017). The investigation of novel biomarkers in the era of metabolomics and machine learning based data evaluation is definitely a promising tool for the preoperative diagnostic workup of adrenal tumors. Besides diagnosis, mass-spectrometry based methods might provide further information on prognosis and therapy responses of patients with ACC.

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Index of abbreviations

ACA	Adrenocortical adenoma
ACC	Adrenocortical carcinoma
ACTH	Adrenocorticotropin
AI-ACS	Adrenal incidentaloma with (possible) autonomous cortisol secretion
CE	Collision energy
CRH	Corticotropin releasing hormone
CT	Computed tomography
CXP	Cell exit potential
CYP	Cytochrome P450
Dex	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DST	Dexamethasone suppression test
DP	Declustering potential
eGFR	Estimated glomerular filtration rate
ENSAT	European Network for the Study of Adrenal Tumors
EP	Entrance potential
ESI	Electrospray ionization
Etio	Etiocholanolone
GC-MS	Gas chromatography mass spectrometry
HPA	hypothalamic-pituitary-adrenal
HPLC	High-performance liquid chromatography
HSD	Hydroxysteroiddehydrogenase
HU	Hounsfield units
IS	Internal standard
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
MRI	Magnetic resonance imaging

MRM	Multiple reaction monitoring
m/z	mass-to-charge ratio
NPV	Negative predictive value
PET	Positron emission tomography
PD	Pregnanediol
5-PD	5-Pregnenediol
PPV	Positive predictive value
PT	Pregnanetriol
5-PT	5-Pregnenetriol
QC	Quality control
ROC	Receiver operation characteristic
SD	Standard deviation
SPE	Solid-phase extraction
THS	Tetrahydro-11-deoxycortisol
ULOQ	Upper limit of quantification

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Appendix

Instruments and chemicals

Table A1: Chemicals and consumables

Product	Supplier	Catalogue number	Method applied
Acquity UPLC Premier HSS T3 1.8µm 2.1x50mm	Waters GmbH, Eschborn, Germany	186009467	Urinary steroids
SepPak tC18 100mg 96-well Plate	Waters GmbH, Eschborn, Germany	186002321	Urinary steroids
XBridge BEH C18, 2.5 µm, 3.0x75 mm	Waters GmbH, Eschborn, Germany	186006034	Dex-cortisol
Oasis HLB 15 µm 2.1x20 mm online SPE column	Waters GmbH, Eschborn, Germany	186002035	Dex-cortisol
MS-grade water	VWR International GmbH, Darmstadt, Germany	83645.320P	Both
MS-grade methanol	VWR International GmbH, Darmstadt, Germany	83638.320P	Both
Acetic acid	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	5.33001	Both
Formic acid	Fisher Scientific GmbH, Schwerte, Germany	15675840	Urinary steroids
Ammonium acetate	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	73594	Both
Acetonitrile	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	1.00029	Dex-cortisol
Sigmatrix steroid-free urine matrix	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	SAE0074	Urinary steroids
β-Glucuronidase/ Arylsulfatase	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	10127698001	Urinary steroids

Table A2: Analytical standards

Standard	Supplier	Catalogue number	Method applied
Cortisol	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	PHR1014	Both
Cortisol-d4	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	705594	Both
Dexamethasone	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	D0700000	Dex-cortisol
Dexamethasone-d5	Toronto Research Chemicals Inc., Toronto, Canada	D298802	Dex-cortisol
Mass*Chrom*® Steroids in Serum/Plasma quality controls	Chromsystems Instruments & Chemicals GmbH, Gräfelfing, Germany	0341/ 0342/ 0343	Dex-cortisol
Etiocholanolone	Steraloids Inc., Newport, RI, USA	A3610-000	Urinary steroids
5-Pregnen-3b,20a-diol (5-PD)	Steraloids Inc., Newport, RI, USA	Q4460-000	Urinary steroids
5b-Pregnan-3a,20a-diol (PD)	Steraloids Inc., Newport, RI, USA	P6000-000	Urinary steroids
5b-Pregnan-3a,17,20a-triol (PT)	Steraloids Inc., Newport, RI, USA	P9450-000	Urinary steroids
Cortisone	Steraloids Inc., Newport, RI, USA	Q2500-000	Urinary steroids
a-Cortolone	Steraloids Inc., Newport, RI, USA	P9150-000	Urinary steroids
5-Pregnen-3b,17,20a-triol (5-PT)	Steraloids Inc., Newport, RI, USA	Q5890-000	Urinary steroids
Dehydroepiandrosterone (DHEA)	Steraloids Inc., Newport, RI, USA	A8500-000	Urinary steroids
Pregnenolone	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	PHR2564	Urinary steroids
Tetrahydro-11-deoxycortisol (THS)	Cayman Chemical, Ann Arbor; MI, USA	26501	Urinary steroids
DHEA-d6	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany	709549	Urinary steroids
THS-d5	IsoSciences, Ambler, PA, USA	15272	Urinary steroids

Etio-d5	IsoSciences, Ambler, PA, USA	14319	Urinary steroids
PT-d5	IsoSciences, Ambler, PA, USA	14321	Urinary steroids
PD-d5	Toronto Research Chemicals Inc., Toronto, Canada	P705132	Urinary steroids
Pregnenolone-d4	Toronto Research Chemicals Inc., Toronto, Canada	P712202	Urinary steroids

Table A3: Instruments

Product	**Manufacturer**	**Method applied**
Agilent 1290 Infinity HPLC system	Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany	Both
QTRAP 4500 MD	AB Sciex Germany GmbH, Darmstadt, Germany	Dex-cortisol
QTRAP 6500+	AB Sciex Germany GmbH, Darmstadt, Germany	Urinary steroids
Immulite® 2000 XPi	Siemens Healthcare GmbH, Erlangen, Germany	Dex-cortisol
Cobas® 8000	Roche Deutschland Holding GmbH, Grenzach-Whylen, Germany	Urinary steroids
Microcentrifuge Mikro 200R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany	Dex-cortisol
Benchtop centrifuge Universal 320	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany	Urinary steroids
Nitrogen evaporator EVA-EC2-48-S	VLM Korrosions-Prüftechnik, Labortechnik & Dienstleistungen GmbH, Bielefeld, Germany	Urinary steroids
Precision balance ABJ 120-4M	Kern & Sohn GmbH, Balingen-Frommern, Germany	Dex-cortisol
Analytical balance XS205	Mettler-Toledo GmbH, Gießen, Germany	Both
Vortex V-1 plus	A. Hartenstein, Würzburg, Germany	Both
pH meter inoLab pH 7110	Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany	Dex-cortisol

Table A4: Software

Product	**Company**
Excel 2016	Microsoft Corporation, Redmond, WA, USA
PowerPoint 2016	Microsoft Corporation, Redmond, WA, USA
Word 2016	Microsoft Corporation, Redmond, WA, USA
SPSS Statistics 28	IBM Corporation, Armonk, NY, USA
OriginPro	OriginLab Corporation, Northampton, MA, USA
Analyst 1.6.3	AB Sciex Germany GmbH, Darmstadt, Germany

Statement of individual author contribution and of legal second publication rights to manuscripts included in the dissertation

Table A5: Individual author contribution to manuscript 1

Manuscript 1: Vogg, N., Kurlbaum, M., Deutschbein, T., Gräsl, B., Fassnacht, M., & Kroiss, M. (2021). Method-specific cortisol and dexamethasone thresholds increase clinical specificity of the dexamethasone suppression test for Cushing syndrome. Clinical Chemistry, 67(7), 998-1007.						
Participated in	**Author Initials,** Responsibility decreasing from left to right					
Study Design Methods Development	MKr NV	MKu MKu	MF BG	NV		
Data Collection	NV	BG	TD			
Data Analysis and Interpretation	NV	MKr	MKu	MF	TD	
Manuscript Writing Writing of First Draft Writing of Introduction Writing of Materials & Methods Writing of Discussion Review & Editing	 NV NV NV MKr all authors	 MKr MKr NV	 MF MF MF	 MKu MKu MKu		

Table A6: Individual author contribution to manuscript 2

Manuscript 2: Vogg, N., Mueller, T., Floren A., Dandekar, T., Scherf-Clavel, O., Fassnacht, M., Kroiss, M., Kurlbaum, M. (2022). Targeted metabolic profiling of urinary steroids with a focus on analytical accuracy and sample stability. Journal of Mass Spectrometry and Advances in the Clinical Lab, 25, 44–52.						
Participated in	**Author Initials,** Responsibility decreasing from left to right					
Study Design Methods Development	MKu NV	NV MKu	MKr	MF		
Data Collection	NV	MKu	MKr	MF		
Data Analysis and Interpretation	NV	MKu	MKr	MF		
Manuscript Writing Writing of First Draft Writing of Introduction Writing of Materials & Methods Writing of Discussion Review & Editing	 NV NV NV NV all authors	 MKu MKu MKu	 MKr OSC MKr	 MF MF		

Table A7: Individual author contribution to manuscript 3

Manuscript 3: Vogg, N., Mueller, T., Floren A., Dandekar, T., Riester, A., Dischinger, U., Kurlbaum, M., Kroiss, M., Fassnacht, M. A simplified diagnostic workflow for adrenocortical tumors using urinary steroid profiling by LC-MS. *Submitted manuscript.*						
Participated in	**Author Initials,** Responsibility decreasing from left to right					
Study Design Methods Development	MF NV	MKr TM	MKu MKu	NV	TM	
Data Collection	NV	MF	MKr	MKu	UD	AR
Data Analysis and Interpretation	TM	NV	MF	MKr	MKu	AF
Manuscript Writing Writing of First Draft Writing of Introduction Writing of Materials & Methods Writing of Discussion Review & Editing	 NV NV NV NV all authors	 MKr TM MF	 MF MKu MKr	 MF MKu	 MKr	 AF

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The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

Nora Vogg Würzburg

Doctoral Researcher's Name Date Place Signature

Prof. Dr. Martin Fassnacht Würzburg

Primary Supervisor's Name Date Place Signature

Statement of individual author contribution to figures/tables of manuscripts included in the dissertation

Table A8: Individual author contribution to figures and tables of manuscript 1

Manuscript 1: Vogg, N., Kurlbaum, M., Deutschbein, T., Gräsl, B., Fassnacht, M., & Kroiss, M. (2021). Method-specific cortisol and dexamethasone thresholds increase clinical specificity of the dexamethasone suppression test for Cushing syndrome. Clinical Chemistry, 67(7), 998-1007.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
2.1	NV	MKr			
2.2	NV	MKr			
2.3	NV	MKr	MF	MKu	
2.4	NV				
Table					
2.1	NV	MKr			
2.2	NV				
Supplemental					
S. Figure 2.1	NV				
S. Figure 2.2	NV				
S. Figure 2.3	NV	MKr			
S. Table 2.1	BG	NV			
S. Table 2.2	NV	BG			
S. Table 2.3	NV				
S. Table 2.3	NV				
S. Table 2.5	NV				

Table A9: Individual author contribution to figures and tables of manuscript 2

Manuscript 2: Vogg, N., Mueller, T., Floren, A., Dandekar, T., Scherf-Clavel, O., Fassnacht, M., Kroiss, M., Kurlbaum, M. (2022). Targeted metabolic profiling of urinary steroids with a focus on analytical accuracy and sample stability. Journal of Mass Spectrometry and Advances in the Clinical Lab, 25, 44–52.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
3.1	NV	TM			
3.2	NV	MKu			
3.3	NV	MKr	MKu		
Table					
3.1	NV				
3.2	NV	MKu			
3.3	NV	MF	MKr		

Supplemental					
S. Figure 3.1	NV				
S. Figure 3.2	NV	MKu			
S. Figure 3.3	NV				
S. Table 3.1	NV	MKu			
S. Table 3.2	NV	MKu			
S. Table 3.3	NV	MKu			
S. Table 3.4	NV	MKu			
S. Table 3.5	NV				

Table A10: Individual author contribution to figures and tables of manuscript 3

Manuscript 3: Vogg, N., Mueller, T., Floren A., Dandekar, T., Riester, A., Dischinger, U., Kurlbaum, M., Kroiss, M., Fassnacht, M. A simplified diagnostic workflow for adrenocortical tumors using urinary steroid profiling by LC-MS. *Submitted manuscript.*					
Figure	**Author Initials,** Responsibility decreasing from left to right				
4.1	NV				
4.2	TM	NV			
4.3	NV	MF	MKr		
4.4	NV				
Table					
4.1	NV	MF	MKr		
4.2	NV				
Supplemental					
S. Figure 4.1	TM	NV			
S. Figure 4.2	NV	TM			
S. Figure 4.3	TM	NV			
S. Figure 4.4	NV				

I also confirm my primary supervisor's acceptance.

Nora Vogg Würzburg

Doctoral Researcher's Name Date Place Signature

Publications and conference contributions

Publications

Mueller, J. W., **Vogg, N.**, Lightning, T. A., Weigand, I., Ronchi, C. L., Foster, P. A., & Kroiss, M. (2021). Steroid sulfation in adrenal tumors. The Journal of Clinical Endocrinology & Metabolism, 106(12), 3385-3397.

Vogg, N., Kurlbaum, M., Deutschbein, T., Gräsl, B., Fassnacht, M., Kroiss, M. (2021). Method-specific cortisol and dexamethasone thresholds increase clinical specificity of the dexamethasone suppression test for Cushing syndrome. Clinical Chemistry, 67(7), 998-1007.

Vogg, N., Mueller, T., Floren, A., Dandekar, T., Scherf-Clavel, O., Fassnacht, M., Kroiss, M., Kurlbaum, M. (2022). Targeted metabolic profiling of urinary steroids with a focus on analytical accuracy and sample stability. Journal of Mass Spectrometry and Advances in the Clinical Lab, 25, 44–52.

Vogg, N., Mueller, T., Floren A., Dandekar, T., Riester, A., Dischinger, U., Kurlbaum, M., Kroiss, M., Fassnacht, M. A simplified diagnostic workflow for adrenocortical tumors using urinary steroid profiling by LC-MS. (*submitted manuscript*)

Conference contributions

03/2020 German Congress of Endocrinology, Gießen, Germany: Oral presentation "Improving the diagnostic value of dexamethasone suppression test (DST) by simultaneous quantitation of serum dexamethasone and cortisol via LC-MS/MS"

10/2020 EUREKA symposium, GSLS Würzburg, Germany (online): Poster presentation "Impact of serum dexamethasone concentration on the dexamethasone suppression test for Cushing's syndrome"

06/2021 MedEins Klausurtagung, Würzburg, Germany: Flash talk „Simultane LC-MS/MS Messung von Dexamethason und Cortisol im DST"

09/2021 ENSAT Scientific Meeting, Brescia, Italy (online): Oral presentation "Method Comparison and Threshold Evaluation for Cortisol measured with CLIA and LC-MS/MS in Dexamethasone Suppression Test"

03/2022 German Congress of Endocrinology, Baden-Baden, Germany (online): Oral presentation "Establishment of a new method to quantify urinary steroid profiles for the diagnosis of adrenocortical carcinoma"

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Affidavit

I hereby confirm that my thesis entitled “Mass spectrometry-based quantification of steroids for the diagnostic workup of adrenal tumors” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg,

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eid statt, die Dissertation „Massenspektrometrische Quantifizierung von Steroiden zur Diagnostik von Nebennierentumoren“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt hat.

Würzburg,

Ort, Datum Unterschrift