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Insights in cushing's syndrome and disease

Edited by
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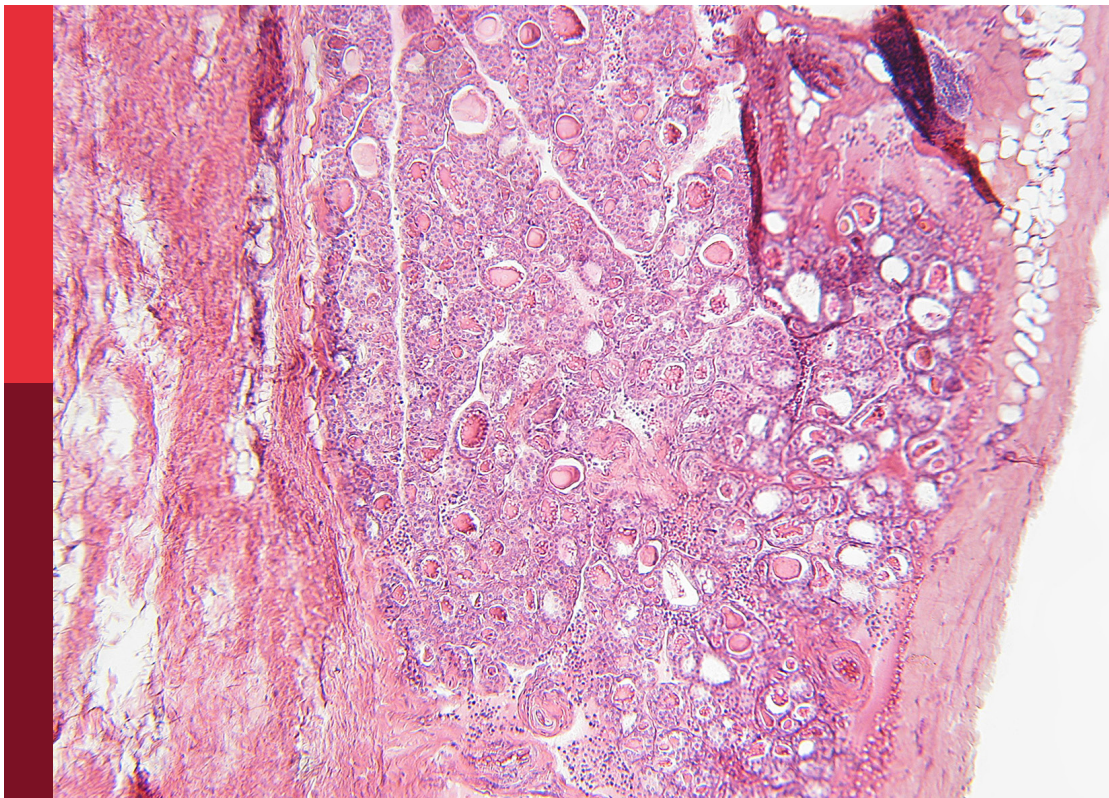

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Insights in cushing's syndrome and disease

Topic editors

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MINI REVIEW
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Cyclic Cushing's Syndrome – A Diagnostic Challenge

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Cyclic Cushing's syndrome (also known as intermittent or periodic) is a disease characterized by periods of transient hypercortisolemia shifting into periods of normo- and/or hypocortisolemia. Diagnosis of cyclic Cushing's syndrome is based on at least three periods of confirmed hypercortisolemia interspersed by two periods of normocortisolemia. Cyclic Cushing's syndrome is one of the greatest challenges in modern endocrinology due to its diverse clinical picture, unpredictable duration and frequency of phases, and various etiologies. We discuss a diagnostic algorithm for periodic hypercortisolemia with special regard to hair cortisol analysis and desmopressin stimulation test which both seem to be helpful in finding the correct answer.

Keywords: cyclic Cushing's syndrome, cyclic hypercortisolemia, desmopressin test, hair cortisol, diagnosis of Cushing's syndrome, DDAVP test

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INTRODUCTION

Cyclic Cushing's syndrome (also known as intermittent or periodic) is a disease characterized by periods of transient hypercortisolemia shifting into periods of normo- and/or hypocortisolemia. Just as classic Cushing's syndrome, cyclic hypercortisolemia may arise from hormonal activity of corticotropinoma (approximately 80% of all cases), ectopic adrenocorticotropic hormone (ACTH; corticotropin) release, or ACTH-independent causes (1–9). Retrospective analysis of 201 patients with Cushing's syndrome showed that 15% of them met the diagnostic criteria of intermittent hypercortisolemia and up to 70% showed evidence of cyclicity before the diagnosis (10). Some authors believe that cyclic Cushing's syndrome might be more common than previously assumed. Giorgi et al. demonstrated periodic nature of subclinical hypercortisolemia in 18% patients with hormonally active adrenal incidentalomas (3). The fluctuation of observed abnormalities may explain the difficulties in diagnostics of periodic hypercortisolemia and often ambiguous results (3, 11). These dilemmas seem to be especially pronounced in ACTH-dependent cases (6, 7, 12).

Diagnosis of cyclic Cushing's syndrome is based on at least three periods of confirmed hypercortisolemia interspersed by two periods of normocortisolemia (13, 14). Cyclic Cushing's syndrome is one of the greatest challenges in modern endocrinology due to its diverse clinical picture, unpredictable duration and frequency of phases, and various etiologies. Patients may present with different severity of signs and symptoms appearing in either transient or continuous pattern. Sometimes only a few manifestations, such as recurrent peripheral edema, cardiac arrythmia, or hypokalemia, are present (15–17). Most commonly, the suspicion of cyclic Cushing's syndrome arises in individuals suspected of hypercortisolemia but not meeting the full

diagnostic criteria of any particular disease. Periodic hypercortisolemia should be also considered in patients whose initial tests confirm autonomic corticosteroid production but normocortisolemia follows. Duration of phases can range from 12 hours to 86 days as shown by available case reports; disease-free periods are unpredictable and they can hover from days to months (13, 18).

PATHOPHYSIOLOGY

Pathophysiology of cyclic hypercortisolemia may involve hypothalamic dysfunction with different degree of corticotroph cell response to neurotransmitters such as corticoliberin (CRH), dopamine, neuroepinephrine, serotonine and/or γ-aminobutyric acid (GABA). Other possible causes of intermittent signs and symptoms include spontaneous bleeding into the pituitary tumor followed by disrupted hormone synthesis in the neoplastic corticotroph cells, and persistent tumor response to the hypothalamus-pituitary-adrenal regulatory mechanisms (negative feedback) (5, 13, 15, 18, 19).

DIAGNOSTICS OF ENDOGENOUS HYPERCORTISOLEMIA

Initial assessment of suspected endogenous hypercortisolemia includes first-line tests such as urinary free cortisol (UFC) – at least twice, late-night salivary cortisol – at least twice, and/or the low-dose overnight dexamethasone suppression test (DST). The Endocrine Society suggests further evaluation if at least one out of the aforementioned screening tests is positive (20). Certain scenarios necessitate the use of alternative initial tests such as late-night serum cortisol or two-day low-dose dexamethasone suppression test (LDDST). The latter one seems especially helpful due to improved specificity as compared with the overnight DST. If laboratory results are ambiguous, the second-line diagnostic approach may include LDDST with consecutive CRH administration (20, 21) (**Figure 1**). It is especially useful for distinguishing between Cushing's syndrome and non-neoplastic hypercortisolemia (previously referred to as pseudo-Cushing's syndrome). The cut-off values for all of the aforementioned tests are listed in the (**Table 1**).

Drug interactions should not be underestimated during the investigation. Some medications can alter dexamethasone metabolism by CYP3A4 induction or inhibition. Drugs such as for instance phenytoin, phenobarbital, carbamazepine, and rifampicine are known as cytochrome inducers, while itraconazole, fluoxetine, diltiazem, and ritonavir are examples of inhibitors. Circulating transcortin (CBG) concentration increases due to estrogen or mitotane use. Various xenobiotics can intensify cortisuria – notable examples are carbamazepine and fenofibrate.

Differential diagnostics of the origin of hypercortisolemia should follow after at least two initial tests come back positive since none of the screening tests alone is sensitive and specific enough to confirm endogenous hypercortisolemia. Differentiation of the origin of hypercortisolemia can be based on morning plasma ACTH, and CRH stimulation test. Sometimes – though nowadays it remains controversial – two-day high-dose dexamethasone suppression test (HDDDST) may be used (20, 21, 27). Interpretations of different tests can be found in **Table 1**. If no apparent hypophyseal mass is found in magnetic resonance and pituitary-derived ACTH-dependent hypercortisolemia is suspected, cavernous sinus catheterization can be considered.

Adrenal imaging is necessary whenever ACTH-independent hypercortisolemia is suspected.

DISTINCT FEATURES IN DIAGNOSTICS OF CYCLIC HYPERCORTISOLEMIA

Whenever cyclic hypercortisolemia is suspected, it is advised to start the investigation with UFC and/or late-night salivary cortisol (5, 13, 20, 28). If the initial results are within the normal limits but clinical suspicion remains strong, evaluation should be repeated for months, if not years, depending on the severity of presented symptoms. The low-dose overnight DST and two-day LDDST are generally contraindicated during remission since subjects can achieve full suppression. Importantly, hormonal suppression tests during relapses may generate paradoxical responses resulting in peaks of serum cortisol (15, 29).

Differential diagnosis of cyclic Cushing's syndrome should include: exogenous steroid use, mild autonomous hypercortisolemia (previously referred to as subclinical Cushing's syndrome), non-neoplastic hypercortisolemia, use of xenobiotics affecting hormonal tests, glucocorticoid resistance syndrome, and factitious disorder. It is crucial to continuously repeat biochemical testing to finally confirm the diagnosis (13). The chance of accurate diagnosis is the highest during the active phase or shortly after it concludes. According to the Endocrine Society, excessive exposure to exogenous steroids and iatrogenic Cushing's syndrome should be excluded before the proper diagnostics starts (20). Some characteristics typical for Cushing's syndrome such as menstrual irregularity, acne, and hirsutism, overlap with features of polycystic ovary syndrome (PCOS), insulin insensitivity, obesity or late-onset congenital adrenal hyperplasia. If there are any doubts regarding the origin of observed abnormalities, initial testing for Cushing's syndrome can be introduced. In obesity and PCOS the low-dose overnight DST and/or late-night serum cortisol are the tests of choice (30, 31).

Rare but possible explanation of apparent intermittent hypercortisolemia includes factitious disorder. Factitious disorder, also known as Münchhausen's syndrome, is a psychiatric disorder resulting in patients deliberately fabricating signs and symptoms of an illness. Descriptions of factitious Cushing's syndrome are infrequent and cases involve the use of various exogenous corticosteroids (32, 33). Steroids can be either conventionally used as drugs or added to already collected specimens (for example urine). Suppressed ACTH and DHEA-S levels may suggest exogenous steroid use. Professional laboratories sometimes offer synthetic glucocorticoid serum analysis.

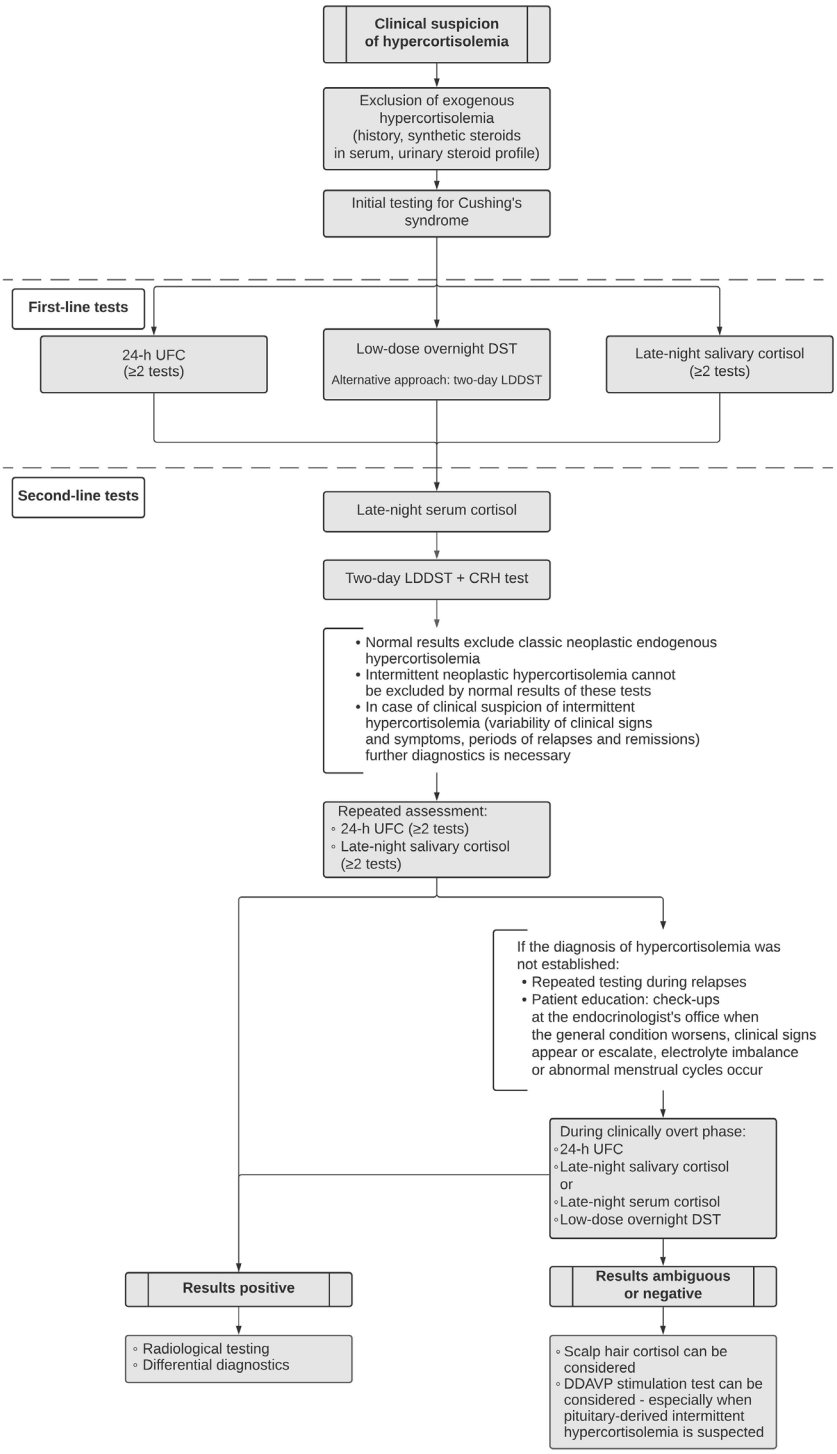

FIGURE 1 | Proposed diagnostic algorithm for suspected cyclic Cushing's syndrome.

Exogenous steroids might be cross-reactive with endogenous cortisol, therefore specific assays discerning their presence should be used. Urine samples can be assessed using high-performance liquid chromatography (33) and/or gas chromatography-mass spectrometry which proved to be useful in tracking a variety of steroids, and cortisol breakdown products can be measured in urine as well (13). As fatal cases of factitious hypercortisolemia were reported (32), patients suspected of

TABLE 1 | Diagnostic procedures commonly used in evaluation of hypercortisolemia.

Diagnostic procedure	Clinical use	Cutoff values
First- and second-line screening tests for hypercortisolemia		
24-h UFC	Suspicion of hypercortisolemia – screening test	Depends on laboratory and assay method
Low-dose overnight DST	Suspicion of hypercortisolemia – screening test	<50 nmol/l (1,8 µg/dl) in healthy subjects
Late-night salivary cortisol	Suspicion of hypercortisolemia – screening test	Cut-off values vary depending on population and assay method (20, 22–24)
Two-day LDDST	Suspicion of hypercortisolemia – screening test	<50 nmol/l (1,8 µg/dl) in healthy subjects
Late-night serum cortisol	Suspicion of hypercortisolemia	<50 nmol/l (1,8 µg/dl) in sleeping healthy subjects
Two-day LDDST with consecutive CRH test	Suspicion of hypercortisolemia, useful in non-neoplastic hypercortisolemia	Serum cortisol stays suppressed <38 nmol/l (1,4 µg/dl) after CRH administration in healthy subjects and non-neoplastic hypercortisolemia
DDAVP stimulation test	Suspicion of ACTH-dependent hypercortisolemia, useful in cyclic hypercortisolemia, useful in distinguishing between neoplastic and non-neoplastic hypercortisolemia (25)	Increase of plasma ACTH by at least 50% in ACTH-dependent cases
Hair cortisol	Suspicion of hypercortisolemia, useful in cyclic hypercortisolemia	Depends on laboratory and assay method
Evaluation of origin of hypercortisolemia		
Morning plasma ACTH	Evaluation of origin of hypercortisolemia (ACTH-dependent vs. ACTH-independent)	>20 pg/ml in ACTH-dependent cases <10 pg/ml in ACTH-independent cases
CRH stimulation test	Evaluation of origin of ACTH-dependent hypercortisolemia, useful in distinguishing between ACTH-dependent and ACTH-independent neoplastic hypercortisolemia when ACTH is indeterminate (10-20 pg/ml) (26)	Increase of plasma ACTH by 35-50% or serum cortisol by 14-20% in ACTH-dependent cases of pituitary origin
Two-day HDDST	Evaluation of origin of ACTH-dependent hypercortisolemia, *however its use remains controversial*	Drop of serum cortisol by at least 50% in corticotropinoma

DST, dexamethasone suppression test; LDDST, low-dose dexamethasone suppression test; HDDST, high-dose dexamethasone suppression test; ACTH, corticotropin; CRH, corticoliberin; DDAVP, desmopressin; UFC, urinary free cortisol.

Münchhausen's syndrome should be promptly examined by an experienced psychiatrist and appropriate assistance should be offered to them.

Well-established factors provoking non-neoplastic hypercortisolemia are alcohol abuse, poorly controlled diabetes mellitus, severe depression, obesity, and pregnancy (34). These conditions result in excessive activation of the hypothalamus-pituitary-adrenal (HPA) axis without autonomous hypercortisolemia. Even though observed abnormalities can closely resemble those arising from neoplastic hypercortisolemia, most signs and symptoms should resolve after the underlying condition fades (20, 21).

Identification of the origin of hypercortisolemia in cyclic Cushing's syndrome is based on the same tests as in the classic form of the disease: morning plasma ACTH, CRH stimulation test, rarely HDDST (**Table 1**). Just like in the classic Cushing's disease, if no apparent hypophyseal mass is found in magnetic resonance and pituitary-derived ACTH-dependent hypercortisolemia is suspected, cavernous sinus catheterization can be considered. If intermittent hypercortisolemia is suspected, the procedure should be performed during active phase of the disease. Otherwise, the results might come back negative with the patient unnecessarily exposed to an invasive procedure (1, 19).

MISCELLANEOUS PROCEDURES

Since diagnostics of intermittent hypercortisolemia can carry distinct difficulties, some experts suggest using less common procedures (35–44).

Over the past few years, scalp hair cortisol measurement was gaining an ever-growing attention. The assessment of hair cortisol can be especially helpful in identification of cyclic Cushing's syndrome. Average hair growth oscillates around 1 cm per month, which allows retrospective tracking of varying concentrations of circulating cortisol. Elevated concentration of hair cortisol in patients with endogenous hypercortisolemia was demonstrated on multiple occasions and can be therefore seen as a marker of exposition to systemic cortisol (35–40). More so, direct relationship between hair cortisol and disease activity was proven in Cushing's syndrome (38). Hair cortisol is so far the only method granting retrospective analysis of exposition to cortisol over the past months or years (depending on the hair length). Henceforth, it seems to be a highly helpful tool in validating periodic cortisol overproduction. Sample collection is simple, noninvasive, there is no need to store the hair in any special environment and the specimen can be transported to the testing laboratory *via* standard

mail. Hair sample should be obtained from the occipital region and cut as close to the skin as possible. Next, the hair should be stored in an envelope or a plastic bag in a cold, dark place. Hair cortisol concentration remains stable for months in room temperature. So far, multiple methods of preparing the hair and its analysis were published; their use varies depending on the laboratory. Enzyme immunoassay (EIA) and liquid chromatography tandem mass spectrometry (LC-MS) are among the most common techniques (36, 40).

Desmopressin (DDAVP) stimulation test is another helpful method in evaluation of cyclic Cushing's syndrome (41). Currently, it is not recommended in routine testing because uniform criteria of interpretation are still to be developed (21). Desmopressin stimulation test relies on measuring plasma ACTH concentration before and 10, 20, 30 minutes after intravenous administration of 10 μg desmopressin. Significant rise in ACTH concentration should occur in pituitary ACTH-dependent Cushing's syndrome, while it should not be observed in healthy individuals, non-neoplastic hypercortisolemia, ACTH-independent hypercortisolemia, and ectopic ACTH production (42–49). The aforementioned response to DDAVP is a result of V3 receptor overexpression in corticotroph neoplastic cells. Normal pituitary cells typically exhibit minimal or nonexistent response to desmopressin. The test might be used not only to establish the diagnosis, but to assess potential relapses – even as a promising early marker of long-term results of surgery (50, 51). Data showing typical response to desmopressin in ACTH-dependent Cushing's syndrome in a patient with intermittent hypercortisolemia is available and in that case testing was performed during remission (43). DDAVP stimulation test may be taken into consideration in cases of pituitary-derived cyclic hypercortisolemia presenting with negative or ambiguous results of conventional tests. More so, the DDAVP test may be suitable for distinguishing between neoplastic and non-neoplastic hypercortisolemia due to the pathophysiology of observed reaction (25, 51).

Both hair cortisol and DDAVP stimulation test are not routinely used in differential diagnostics of hypercortisolemia. However, periodic Cushing's syndrome is a clinical challenge often defying routine testing and therefore asks for alternative options. We would like to propose a diagnostic algorithm for cyclic Cushing's syndrome as shown in **Figure 1**.

TREATMENT

Once the diagnosis is finally confirmed, patients with cyclic Cushing's syndrome should follow conventional treatment suited for patients with endogenous hypercortisolemia. Surgery remains the most effective and preferable method of treatment in most hormonally active adenohypophyseal masses (with prolactinoma being an exception). Unfortunately, complete resection in clinically advanced disease might not be achievable. It is especially important for the patients to be cared for in experienced neurosurgical centers as it was proven to improve the outcomes (52). If resection is not complete or unobtainable at all, radiotherapy and/or continuous pharmacotherapy should be introduced. Decision regarding appropriate type of intervention should be discussed in a multidisciplinary medical team to provide personalized treatment.

Radiotherapy is a valuable second-line treatment in patients not meeting the criteria of full excision or with recurrent Cushing's disease. The most prevalent techniques are stereotactic radiosurgery (SRS) and fractionated external beam radiation therapy (EBRT) (53, 54). Qualification for radiotherapy should be performed by an experienced radiation oncologist to ensure that the chosen method is optimal for the patient. One of the main concerns is preserving the optic chiasm. After the treatment, the patient should be carefully evaluated for potential side effects, such as permanent neurological damage or hypopituitarism.

Currently, a vast choice of drugs is available: steroidogenesis inhibitors [metyrapone, ketoconazole, mitotane, etomidate, or recently FDA-approved orphan drug osilodrostat (55)], glucocorticoid receptor-directed agents (mifepristone), and pituitary-directed agents (cabergoline, pasireotide). If the condition is especially severe and/or recurrent, bilateral adrenalectomy remains a potentially life-saving option (20). As signs and symptoms of Cushing's syndrome tend to progress over time and the disease can eventually become fatal, appropriate treatment should not be postponed. After treatment, all patients should be screened for hormone deficiencies, as well as for signs and symptoms of a relapse.

Surgery remains the recommended modality of treatment in cyclic Cushing's syndrome arising from ectopic ACTH-secreting tumors, or hormonally active adrenal tumors (unilateral adrenalectomy).

SUMMARY

Cyclic Cushing's syndrome usually causes significant diagnostic problems. The diagnosis should be taken into consideration whenever clinical suspicion of hypercortisolemia meets normal results of hormonal tests. In that scenario, the work-up should be repeated, especially when clinical signs and symptoms (re)appear. Increased 24-h UFC, elevated late-night salivary and/or serum cortisol can confirm cyclic hypercortisolemia. DST may provoke paradoxical rise of serum cortisol in relapsing patients. In questionable and ambiguous cases, hair cortisol and DDAVP stimulation test should be kept in mind as a valuable option.

AUTHOR CONTRIBUTIONS

RŚ-S, AB, KaS: These authors contributed equally to this work and share first authorship: conceived the idea of the work, contributed to the design of publication and reference collection, and were responsible for preparing the manuscript. PK: involved in preparing the manuscript and reference collection. KS: proof-reading and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: A Challenging Localization of a Pulmonary Ectopic ACTH-Secreting Tumor in a Patient With Severe Cushing's Syndrome

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Background: Ectopic adrenocorticotropic syndrome (EAS) is a rare cause of endogenous ACTH-dependent Cushing's syndrome, usually associated with severe hypercortisolism as well as comorbidities. Tumor detection is still a challenge and often requires several imaging procedures. In this report, we describe a case of an ectopic ACTH secretion with a misleading localization of the responsible tumor due to a concomitant rectal carcinoma.

Case presentation: A 49-year-old man was referred to our Endocrinology Unit due to suspicion of Cushing's syndrome. His medical history included metastatic rectal adenocarcinoma, diagnosed 5 years ago and treated with adjuvant chemotherapy, radiotherapy and surgical resection. During follow-up, a thoracic computed tomography scan revealed two pulmonary nodules located in the superior and middle lobes of the right lung with a diameter of 5 and 10 mm, respectively. However, these nodules remained radiologically stable thereafter and were not considered relevant. All biochemical tests were suggestive of EAS (basal ACTH levels: 88.2 ng/L, nv 0–46; basal cortisol levels: 44.2 µg/dl, nv 4.8–19.5; negative response to CRH test and high dose dexamethasone suppression test) and radiological localization of the ectopic ACTH-secreting tumor was scheduled. The CT scan revealed a dimensional increase of the right superior lung nodule (from 5 to 12 mm). [^{68}Ga]-DOTA-TOC PET/CT scan was negative, while [^{18}F]-FDG-PET/CT showed a tracer accumulation in the superior nodule. After a multidisciplinary consultation, the patient underwent thoracic surgery that started with two atypical wedge resections of nodules. Frozen section analyses showed a neuroendocrine tumor on the right middle lobe nodule and a metastatic colorectal adenocarcinoma on the superior lesion. Then, a right superior nodulectomy and a right middle lobectomy with

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mediastinal lymphadenectomy were performed. The final histopathological examination confirmed a typical carcinoid tumor, strongly positive for ACTH. A post-surgical follow-up showed a persistent remission of Cushing's syndrome.

Conclusions: The present report describes a case of severe hypercortisolism due to EAS not detected by functional imaging methods, in which the localization of ACTH ectopic origin was puzzled by a concomitant metastatic rectal carcinoma. The multidisciplinary approach was crucial for the management of this rare disease.

Keywords: Cushing's syndrome, ectopic ACTH syndrome, pulmonary carcinoid, rectal carcinoma, hypercortisolism

INTRODUCTION

Cushing's syndrome (CS) is caused by a chronic exposure to supraphysiological levels of glucocorticoids leading to several comorbidities and high mortality if not adequately treated (1). An ectopic adrenocorticotropic (ACTH) syndrome (EAS) is an infrequent form of endogenous ACTH-dependent CS (2), usually associated with intense hypercortisolism (3) as well as severe comorbidities such as hypokalemia, diabetes mellitus, infections and vertebral fractures (4–7). Since the recommended first line treatment of EAS is the surgical removal of the ectopic ACTH-secreting tumor (EAT) (8), its prompt localization is crucial. However, the first imaging study succeeds in identifying the EAT in only 50–60% of cases, while the ectopic origin of ACTH may remain occult for several years in up to one-fifth of patients (9). A failure in the localization of the ACTH ectopic source requires additional imaging procedures, second-line therapies (ranging from pharmacological treatment to bilateral adrenalectomy) and a close follow up.

Here we describe the case of a severe CS due to ectopic ACTH secretion with a misleading localization of the responsible tumor due to a concomitant rectal carcinoma.

CASE DESCRIPTION

A 49-year-old Caucasian man was referred to our Endocrinology Unit in June 2020 from the Hepatology Unit with suspicion of CS. The patient reported a recent occurrence of severe backache and progressive muscular weakness, leading to gait impairment and the need to use crutches. His past medical history was relevant for a left knee chondroblastoma, diagnosed and surgically treated at 14 years of age, and a rectal adenocarcinoma with liver metastasis, diagnosed in 2015 and treated with adjuvant chemotherapy, radiotherapy and surgical resection. Thereafter the patient underwent a regular oncologic follow-up and in June 2017, a thoracic computed tomography (CT) scan revealed two pulmonary nodules located in the superior and middle lobes of the right lung with a diameter of 5 and 10 mm, respectively. However, since these two nodules remained stable during radiological follow-up, including CT scans in 2018 and 2019, and did not show fluorodeoxyglucose ([^{18}F]-FDG) uptake at positron emission tomography (PET) performed in September 2017, they were not considered relevant.

The patient also had arterial hypertension treated with monotherapy, diagnosed at the age of 34, diabetes mellitus diagnosed 3 months earlier, was in diet therapy and had a hepatitis C virus infection found in 2016 and had it subsequently eradicated through an antiviral treatment with Ombitasvir/Dasabuvir plus weight-based Ribavirin.

Physical examination showed the patient being overweight (BMI: 26.6 kg/m^2) with central adiposity, a moon face and a buffalo hump. The patient also presented proximal miopathy and scattered bruises, while no striae rubrae was present. The blood pressure was at 170/120 mmHg and the fasting capillary glycemia was at 310 mg/dl. The other biochemical features are summarized in **Table 1**.

The diagnostic work-up included four major steps: 1) Biochemical confirmation of CS; 2) Classification as ACTH-dependent CS; 3) Differential diagnosis between Cushing's Disease (CD) and EAS; and 4) Localization of ACTH-secreting tumor and evaluation of its extension. The biochemical diagnosis was rapidly posed as all the tests were suggestive of EAS (**Table 1**) (10, 11). The pituitary magnetic resonance imaging (MRI) revealed a small pituitary adenoma of 1.9 mm, which was interpreted as irrelevant in the final diagnosis. Bilateral inferior petrosal sinus sampling was not performed, as the clinical and the biochemical findings were considered sufficient to support the diagnosis of EAS. A radiological localization of the ectopic ACTH-secreting tumor was scheduled. The CT scan revealed a significant increase of the lung nodule located in the right superior lobe (from 5 to 12 mm), while the nodule in the right middle lobe was unchanged (**Figure 2**). However, a [^{68}Ga]-DOTA-TOC PET/CT scan did not show any pathologic tracer accumulation. On a [^{18}F]-FDG-PET/CT (capillary glycemia <150 mg/dl before examination), the right superior nodule took-up the radiopharmaceutical with a maximum standardized uptake value (SUV) of 2.4, while the other pulmonary nodule did not accumulate [^{18}F]-FDG (**Figure 2**). It must be underlined that the smaller nodule could have been close to the spatial resolution limit of the PET scanner.

The assessment of the CS-associated comorbidities revealed multiple vertebral and costal fractures, the former accounting for the patient's back pain; rapidly worsening glucose control, which required basal-bolus insulin therapy; severe hypokalemia and refractory hypertension which needed a progressive increase of canrenone of up to 300 mg/day in addition to three other antihypertensive drugs (**Table 2**).

TABLE 1 | Biochemical features at presentation and laboratory diagnostic work-up.

	Patient's value	Reference interval
Hemoglobin (g/dl)	15.5	13.5–17.5
Leucocytes (10^9/L)	10.05	4.8–10.8
Neutrophyles (10^9/L)	8.07	1.5–6.5
PCR (mg/dl)	5.4	<0.5
APTT (ratio)	0.8	0.86–1.2
Glycemia (mg/dl)	291	70–110
HbA1c (mmol/mol)	71	20–42
Na^+ (mEq/L)	141	135–145
K^+ (mEq/L)	2.5	3.3–5.1
ALT (U/L)	269	9–59
AST (U/L)	74	10–35
GGT (U/L)	883	8–61
TGL (mg/dl)	584	<150
Total cholesterol (mg/dl)	196	<190
HDL cholesterol (mg/dl)	26	
Basal cortisol (8.00 AM, µg/dl)	44.2	4.8–19.5
Basal ACTH (8.00 AM, ng/L)	88.2	0–46
Cortisol (µg/dl) after 1 mg dexamethasone suppression test	44.8	<1.8
Urinary free cortisol (µg/dl)	770	<60
LH (mIU/L)	<0.3	1.7–8.6
Testosterone (µg/L)	0.59	2.8–8.4
TSH (mIU/L)	0.47	0.28–4.3
FT4 (ng/L)	6.4	8–17
PRL (µg/L)	27.8	1.7–16
IGF1 (µg/L)	121	50–200

Biochemical tests for diagnosis of EAS

CRH stimulation test:
- morning basal ACTH: 72 ng/ml → ACTH peak: 80.4 (+11%)
- morning basal cortisol: 39 mcg/dl → cortisol peak: 42.9 mcg/dl (+10%)

High dose Dexamethasone Suppression Test
- cortisol: 37.6 mcg/dl (–3.5% *vs* basal)

Immediately after the laboratory and radiological assessment, medical therapy with metyrapone, a steroidogenesis enzyme inhibitor, was started. Due to severe and refractory hypokalemia and hypertension, the initial dose was at 500 mg/day, which quickly increased to 1,000 mg/day. This dose allowed a rapid and important reduction of basal cortisol levels and a normalization of free urinary cortisol (**Figure 1**).

In the meantime, the case was discussed in a multidisciplinary team that included an endocrinologist, a thoracic surgeon, an anaesthesiologist, a neurosurgeon, and a radiologist. After that, it was decided to refer the patient to a thoracic surgery with the aim to analyze the pulmonary nodules through frozen section and then proceed with thoracic surgery, according to the results. The patient was informed by the multidisciplinary team on the procedure and its associated risks, including the possible need for a chronic oxygen therapy because of his low pulmonary function. The patient accepted the surgery and pulmonary rehabilitation, in addition to continuous-positive airway pressure (CPAP) therapy, which was immediately started. One week later, the patient underwent a minimally invasive thoracic surgery. Surgical procedure started with two atypical wedge resections of nodules. Frozen section analyses resulted positive for neuroendocrine tumor on right middle lobe nodule and for metastatic colorectal adenocarcinoma on the superior lesion. For oncological reasons a right middle lobectomy and a mediastinal lymphadenectomy were performed. The postoperative course was uneventful and the patient was discharged on post-operative day 4 in good clinical condition. The final histopathological examination confirmed the frozen section procedure results: a colorectal carcinoma metastasis in the right superior pulmonary nodule and a typical carcinoid tumor (according to the WHO 2015 classification), strongly positive for ACTH immunostaining, in the middle lobe (**Figure 2**). After surgery, low levels of morning plasmatic ACTH and cortisol (ACTH <5 pg/ml, cortisol 1.52 µg/dl) confirmed EAS remission. In the following days, the general clinical status of the patient significantly improved, thus allowing the withdrawal of insulin therapy, canrenone, potassium chloride and the reduction of anti-hypertensive drugs dosage (**Table 2**). Three months after surgery, a biochemical evaluation showed a persistent remission of the disease and the patient was able to walk without crutches.

PATIENT PERSPECTIVE

"I remember my first endocrinological visit at Fondazione Ca' Granda, Ospedale Maggiore Policlinico, I was feeling tired, very anxious, also depressed, with mood swings and I was having difficulties to concentrate. My back was aching very hard and I was able to walk only a few steps with two crutches and my face was swollen and reddish. My symptoms began three four months before and worsened quickly. The weeks after the surgical procedure I was feeling very tired, my appetite lacked, but my mind was clear and I was feeling more optimist. After three months my back still hurts, but less than before and I can easily walk with one crutch, so now I'm autonomous. My appetite is normal, I feel energetic, my mind is clear and I have begun to do small jobs. Overall, I can say that my quality of life significantly improved after the surgical intervention."

DISCUSSION

The present case report describes an unusual association of a lung carcinoid tumor responsible for a severe EAS with a metastatic rectal cancer that puzzled the localization of ectopic ACTH source. Although the state of intense hypercortisolism is not precisely defined in the literature, it was seen that the CS complications are more frequent and rapidly evolving when UFC is increased five times above the upper limit of normal range (ULN) (12). In these cases, many patients need urgent control of hypercortisolism. Our patient had UFC levels approximately 11 times above ULN and presented rapid occurrence and worsening of several CS comorbidities already described in the literature (1).

As more than a half of ectopic ACTH-secreting tumors are located in the chest (45% pulmonary NET, 6.5% thymic NET) (9, 13) and taking into account patient's medical history, imaging investigation started with a thorax CT scan that revealed a significant increase of the right superior lung nodule in the last year. Overall, the CT scan has a sensitivity of 82% (77–85%)

TABLE 2 | Associated comorbidities: treatment and progression.

Comorbidities	Medical treatment		3 months after surgical cure
Hypertension	Perindopril, Amlodipin, Bisoprolol	+ Metyrapone 1,000 mg/day	Improved
Hypokalemia	Canrenone, Potassium Chloride		Remitted
Diabetes mellitus	Basal-bolus Insulin		Remitted
Dyslipidemia	Diet		Normalized
NASH	Diet		Improved
Osteoporosis with vertebral and costal fractures	Zoledronate, Calcium Carbonate, Cholecalciferol, Orthopaedic brace		–
Mixed anxiety-depressive disorder	Escitalopram Bromazepam		Improved
Mandibular abscess	Amoxicillin + Clavulanic Acid, Dental Treatment		Remitted
Increased thromboembolic risk*	Enoxaparin		APTT normalised
Central hypogonadism	Not treated		Remitted
Central hypothyroidism	Not treated		Remitted

**Reduced APTT, reduced mobility, infection, very high UFC levels.*

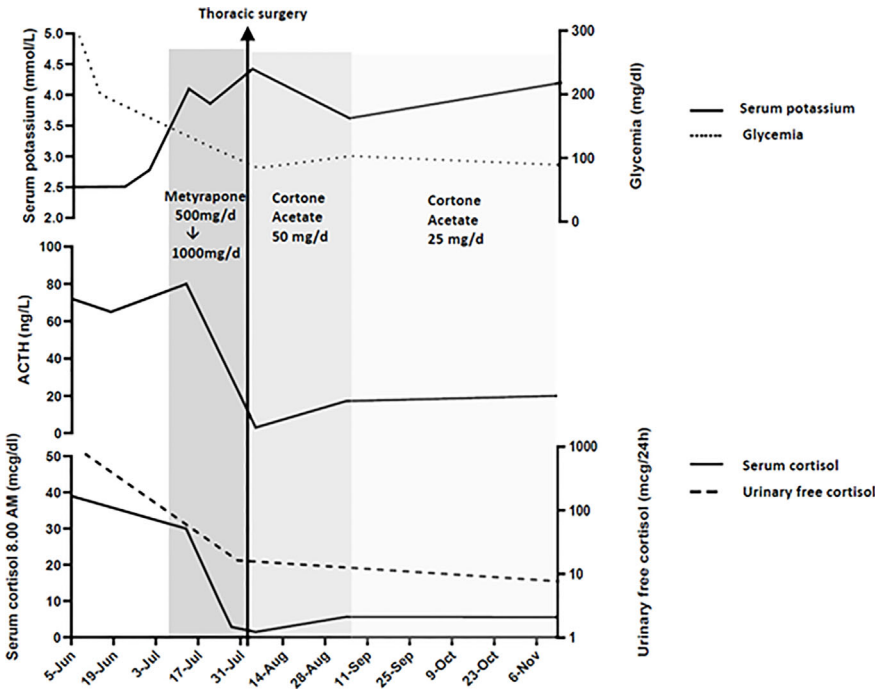

FIGURE 1 | Serum cortisol, ACTH, glycemia, potassium and urinary free cortisol levels at presentation, during medical therapy and after thoracic surgery.

when used to detect different types of NET (14). In the case of EAS, a systematic review conducted by Isidori and colleagues showed that only in a half of cases cross-sectional imaging was positive at presentation, while in the rest of cases the tumor was detected during follow-up, also through functional imaging (29% cases), or it was never found (18%) (9). In our particular case, considering also the previous diagnosis of rectal carcinoma, the detection of two distinct nodules at the thorax CT made the origin of ectopic ACTH secretion unclear. Therefore, the patient underwent a [^{68}Ga]-DOTA-TOC PET/CT scan, that resulted negative, and a [^{18}F]-FDG-PET/CT scan that showed a radiopharmaceutical uptake of the nodule located in the superior lobe of right lung. The second nodule was not reported as [^{18}F]-FDG avid, although the small size lowered the sensitivity of the examination. The performance of [^{68}Ga]-DOTA-TOC PET/CT and [^{18}F]-FDG-PET/CT for the detection of EAT is still debated. [^{68}Ga]-DOTA-TOC PET/CT is usually preferred in occult tumors, that are often non-metastatic and well-differentiated NETs. In fact, the slow growth rate of these tumors can determine a negative result to [^{18}F]-FDG-PET/CT (15), which is more useful to characterize the behavior of tumor detected at CT, since a positive result is more frequently associated with atypical or aggressive NETs (16).

[^{68}Ga]-DOTATOC PET/CT provides a high sensitivity (88–93%) and specificity (88–95%) for the diagnosis of carcinoid tumor (14), although a recent systematic review reported a

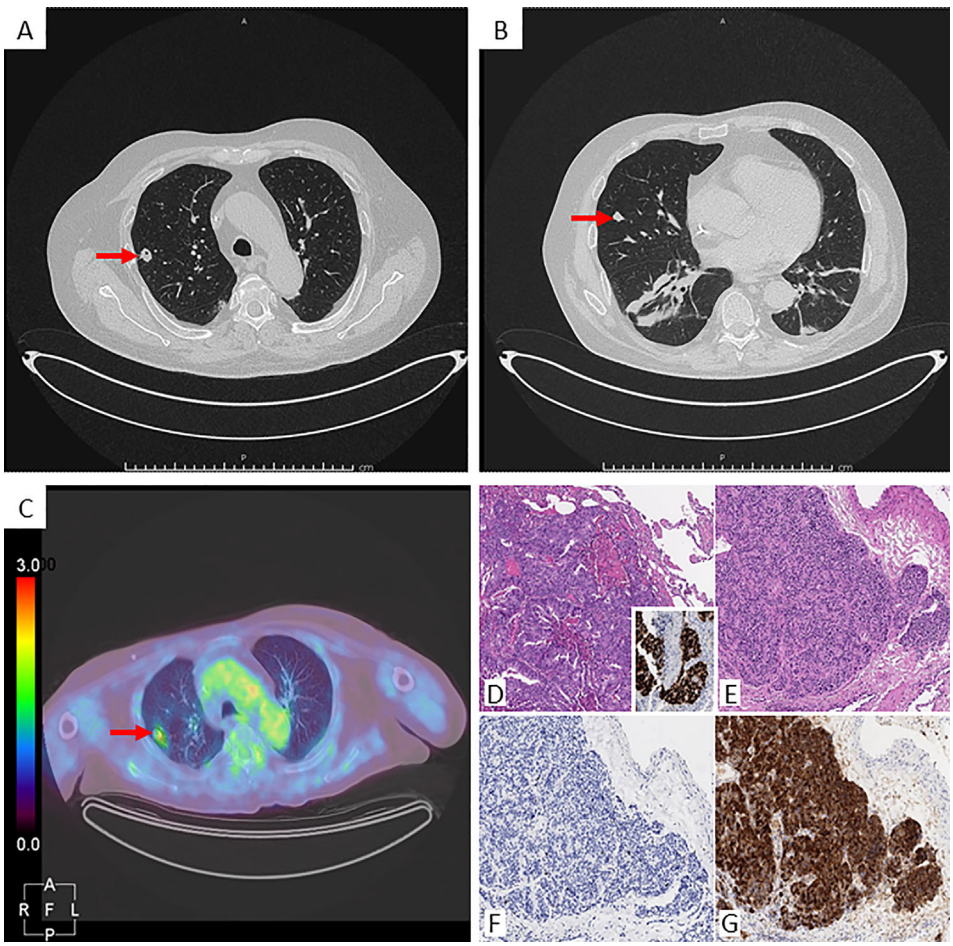

FIGURE 2 | Radiological images and histological panel of the lung nodules. Chest CT scan: **(A)** nodule in the right superior lobe (arrow), axial projection; **(B)** nodule in the right middle lobe (arrow), axial projection. [^{18}F]-FDG-PET/CT: **(C)** uptake of the right superior nodule (arrow), axial projection. Histological panel: the nodule from the superior right lobe (**D** Hematoxylin-Eosin, 100x) consists of a metastatic carcinoma, with its colo-rectal primitivity confirmed upon CDX2 positivity at immunohistochemistry (inset). Detail from the middle lobe nodule lobe (**E** Hematoxylin-Eosin, 100x) depicts an epithelial neoplasm growing in an organoid fashion, composed of bland-looking cells with low mitotic rate and proliferative index (**F** Ki67/Mib1, 100x), estimated within a 1-2% range, and featuring intense positivity for ACTH at immunohistochemistry (**G** 100x).

significantly lower overall sensitivity of 76.1% for EAS (17). Furthermore, its detection rate may be lower in high grade NET. A study conducted by Binderup and colleagues showed that in neuroendocrine tumors with proliferation index >15%, somatostatin receptor scintigraphy reached a sensitivity of only 69%, much lower than 92% of [^{18}F]-FDG-PET/CT (18). It must be underlined that somatostatin receptor scintigraphy has a lower sensitivity when compared to somatostatin receptor PET and these results are underestimating the accuracy of [^{68}Ga]-DOTA-TOC PET/CT (19). In a different manner, the lack of pathological uptake to [^{68}Ga]-DOTA-TOC may also be related to the finding that glucocorticoids downregulate the somatostatin receptors (20, 21). Moreover, in two cases of EAS, the reduction of cortisol levels using mifepristone permitted the localization of ACTH ectopic origin with ^{111}In-pentetreotide (22). Of note, at the time of imaging, the patient had extremely elevated levels of urinary free cortisol (x11 ULN, see **Table 1**).

As a whole, according to CT scan and functional imaging, we defined three possible scenarios: i. occult EAT with two lung nodules of uncertain origin (metastasis of rectal carcinoma in superior lobe)?; ii. EAT of right middle lobe with false negative [^{68}Ga]-DOTA-TOC PET/CT associated with probable metastasis at superior lobe; and iii. High grade NET of right superior lobe with false negative [^{68}Ga]-DOTA-TOC PET/CT associated with middle lobe indeterminate lesion.

As stated before, an indication to thoracic surgery was confirmed after a multidisciplinary discussion and motivated by the following arguments: first, the patient suffered from a severe and life-threatening form of CS; second, metyrapone therapy is able to inhibit adrenal steroidogenesis but it does not target the primary cause of cortisol excess and may have various side effects such as hypertension, hypokalaemia, and adrenal insufficiency (23); and third, the biopsy of two pulmonary nodules was already indicated in the context of previous diagnosis of rectal carcinoma.

The frozen section examination revealed an EAT in the middle lobe of the right lung and a rectal carcinoma metastasis in the right superior lobe. An atypical resection of the right superior pulmonary lobe for the rectal metastasis and a middle lobectomy lymphadenectomy for the ACTH positive carcinoid tumor were performed accordingly. Then, in the present case the pulmonary carcinoid tumor was not detected in either functional imaging methods.

In conclusion, the present report describes a case of severe hypercortisolism due to EAS in which the localization of ACTH ectopic origin was puzzled by a concomitant metastatic rectal carcinoma. The biochemical diagnosis of EAS was instrumental in the reassessment of apparently benign lung nodules and the past history of metastatic rectal cancer imposed a rapid surgical exploration of both lesions. A multidisciplinary approach as well as a clear and close communication with the patient played a crucial role in the management of this case.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AS, GM, EF, MA, MR, MC, and ACh: diagnostic approach of the case and writing the paper. AS and GC: editing images. ACr, RI, and BM: sample collection and preparation and patient follow-up. LR, PM, and MN: surgical treatment of the patient and writing the paper. GC: histopathological examination. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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Effectiveness of Medical Treatment of Cushing's Disease: A Systematic Review and Meta-Analysis

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Objective: The objective of this systematic review was to evaluate the effectiveness and safety of pasireotide, cabergoline, ketoconazole, levoketoconazole, metyrapone, osilodrostat, and temozolomide for the treatment of Cushing's disease (CD).

Methods: The primary outcomes were the proportion of CD control, adverse events (AE), and reduction of urinary free cortisol. Search strategies were applied to Embase, Medline, and CENTRAL. Independent reviewers assessed the study eligibility, extracted data, and evaluated risk of bias. Standardized mean difference was calculated with 95% confidence interval (CI) for continuous data (*i.e.*, pre- and post-intervention). Random meta-analyses for the proportion of CD control and AE were conducted.

Results: Twenty-nine controlled and non-controlled studies were included. No study with temozolomide and levoketoconazole and one study with osilodrostat fulfilled the inclusion criteria. The meta-analyses of proportion of CD control was 35% for cabergoline (95% CI: 27–43%, six studies, 141 participants), 44% for pasireotide (95% CI: 25–35%, eight studies, 522 participants), 41% for ketoconazole (95% CI: 36–46%, six studies, 450 participants), 66% for metyrapone (95% CI: 46–87%, four studies, 66 participants), and of 66.4% for osilodrostat (95% CI: 57.9, 74.3, 97 participants, one study). One study compared two different treatments (cabergoline *vs*. ketoconazole), and no statistical difference was observed in CD control (RR: 0.53, 95% CI: 0.15 to 1.87, 14 participants, very low certainty of evidence). The most frequent AE associated with pasireotide was hyperglycemia, dizziness and nausea with cabergoline and metyrapone, and elevated transaminases with ketoconazole.

Conclusion: The superiority of one drug over another could not be determined due to lack of controlled studies, but the proportion of disease control identified in our meta-analysis may support clinical decision. New therapeutic options should be investigated

due to the limited efficacy and tolerability of the currently available medical treatment for patients with Cushing's disease.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020205567, identifier CRD42020205567.

Keywords: Cushing's disease, pasireotide (SOM230), cabergoline, ketoconazole, metyrapone, systematic literature review, meta-analysis

INTRODUCTION

Cushing's disease (CD) results from an ACTH-secreting pituitary adenoma and is the main cause of endogenous hypercortisolism in adults. The incidence of CD is 1.2 to 2.4 patients per million each year (1). The first-line treatment for CD is transsphenoidal surgery (TSS), which can lead to disease control in 68 to 98% of patients (2). Late recurrence of the disease after TSS has been reported to occur in 15 to 66% of patients at 5 to 10 years after surgery, which was considered successful (3, 4). Patients who underwent TSS without success and those with contraindications for surgical treatment might benefit from medical treatment (5). Medical treatment is also recommended to control severe hypercortisolism before surgery or while awaiting the effects of radiotherapy treatment (6, 7).

Three main categories of medical treatment can be identified according to the mechanism of action: pituitary-, adrenal-, and glucocorticoid receptor (GR)-directed drugs (8). Mifepristone is the main GR-directed drug, and although it improves the clinical burden of chronic hypercortisolism, it does not affect cortisol secretion (9).

Pituitary-directed drugs, namely, pasireotide, cabergoline, and temozolomide, target the corticotroph pituitary tumor directly. Pasireotide is a somatostatin analogue with high affinity for the SST5 receptor that decreases ACTH production. Two formulations of pasireotide are available, subcutaneously administered twice daily (600 and 900 mcg), and have a long-acting release formulation, requiring a single intramuscular administration every 4 weeks (10 and 30 mg). Disease control might be achieved in 20 to 60% of patients with CD who remained uncontrolled after surgery (10, 11).

Cabergoline is a long-acting dopamine agonist that might inhibit ACTH secretion by acting on dopamine receptor subtype 2. The control of hypercortisolism might be achieved in up to 40% of patients (12), while others have found cabergoline to be of little value in the therapy of CD (13).

Adrenal-directed drugs induce a decrease of cortisol secretion through the inhibition of steroidogenesis. Ketoconazole is an imidazole derivative that inhibits several enzymes, such as 17,20-lyase and 11β-hydroxylase. Ketoconazole induces control in 30 to 80% of patients with Cushing's syndrome (14). Additionally, metyrapone is an adrenal enzyme blocker, mainly acting on 11b-hydroxylase, and has been extensively studied for the treatment of Cushing's syndrome, showing an average control rate of 75.9% (14).

New adrenal-directed drugs have been recently developed, such as levoketoconazole and osilodrostat. Levoketoconazole is the cis-2S,4R steroisomer of the classical racemic ketoconazole, showing a similar enzymatic inhibitory profile and found to be more potent in experimental models (15). Osilodrostat potently inhibits the adrenal enzymes aldosterone synthase and 11b-hydroxylase, therefore inducing a decrease in glucocorticoid and mineralocorticoid production and secretion (16). Moreover, the efficacy of retinoic acid, which acts on the proopiomelanocortin gene transcription and inhibits corticotropinoma development, was assessed in a small cohort (17).

There is still uncertainty on the effectiveness and safety of the alternative medications to patients with CD. Therefore, the aim of this systematic review was to assess the effectiveness and safety of medical treatment for patients with uncontrolled CD who underwent TSS or who had contraindications to surgery as first-line treatment, with at least 6 months of follow-up.

MATERIALS AND METHODS

This systematic review is reported according to the PRISMA statement (18) and was registered at PROSPERO (CRD42020205567).

Eligibility Criteria

We included randomized and non-randomized controlled trials and non-controlled studies that were in accordance with the criteria below.

Patients

Adults with diagnosis of CD, who did not fulfill control criteria after TSS, who presented a recurrence of Cushing's after a postoperative period of eucortisolism, or who had contraindications for surgery as first-line treatment were included in the study. We considered as having CD patients with clinical manifestations of the disease associated with at least two positive screening tests for hypercortisolism, a baseline plasma ACTH level >20 pg/ml, and confirmed ACTH-secreting pituitary adenoma after surgery. For symptomatic patients who did not undergo surgery or whose tumor could not be identified after surgery, we considered as diagnostic criteria a bilateral inferior petrosal sinus catheterization or a magnetic resonance image evidencing a pituitary adenoma >6 mm (19).

Intervention and Comparison

Monotherapy with pasireotide, cabergoline, ketoconazole, levoketoconazole, metyrapone, osilodrostat, and temozolomide was considered.

Outcomes

The primary outcomes were as follows: proportion of disease control as defined by the authors and proportion of adverse

events (AE), with the latter reported according to the Common Terminology Criteria for Adverse Events (20). The secondary outcomes were improvement of urinary free cortisol (UFC) and comorbidities associated to CD (*i.e.*, weight loss, improvement of diabetes mellitus, waist circumference, hypertension, and cholesterol). Serious adverse events (SAE) were those that resulted in death, hospitalization, or prolongation of existing hospitalization, a persistent or significant incapacity, substantial disruption of the ability to conduct normal life functions, or a congenital anomaly (21).

Exclusion Criteria

To minimize the risk of selection bias, at least 10 patients had to be included in the studies. In case of overlapping populations, the article with the largest sample and more complete reporting of data was included.

Search Strategy

Three general search strategies were developed for the main electronic health databases: Embase (1980–August 20, 2020), PubMed/Medline (1966–August 20, 2020), and Cochrane Collaboration Controlled Trials Register (1982–August 20, 2020). A second search on all databases was conducted on January 16, 2021. The strategies for PubMed and Embase were reviewed by a medical librarian (MD) using the PRESS 2015 Evidence-Based Checklist tool (22). The search strategies included the following descriptors and synonyms: Cushing's disease, cabergoline, pasireotide and ketoconazole, osilodrostat, levoketoconazole, metyrapone, and temozolomide. The complete search strategy for Pubmed/Medline is provided in the supplementary material (**Supplementary Tables S1A**, **S2A**). To search for gray literature, we checked for ongoing studies on ClinicalTrials.gov, references of articles selected for full reading, and annals of congress. There was no language restriction.

Selection of Studies

Two reviewers (JSCG and VSNN) independently reviewed the titles and abstracts. Potentially eligible studies were selected for full reading, to be assessed for adequacy to the PICO previously established. In case of disagreement, a consensus meeting was made.

Data Extraction and Risk of Bias of the Included Studies

Two reviewers (JSCG and ANSCN) used a standardized form to independently extract relevant data of the included studies and to assess the risk of bias of the included studies. In case of disagreement, a consensus meeting was made. To assess the risk of bias of the included studies, the critical appraisal tool from Joanna Briggs's Institute was adapted to check the included studies with regard to the following aspects: (i) clear inclusion criteria, (ii) diagnostic criteria stated, (iii) description of valid biochemical assays to measure hypercortisolism, (iv) consecutive and complete inclusion of participants, (v) complete reporting of baseline information, (vi) complete reporting of outcomes, (vii) complete reporting of demographics of the site, and (viii) appropriate statistical analysis. For each aspect, we assigned yes, no, or unclear (23).

Synthesis and Analysis of Data

Homogeneous endpoints in at least two studies were plotted in meta-analyses using the Stata Statistical Software 16 (Stata Statistical Software: Release 16, College Station, TX, StataCorp LLC, USA). Proportional meta-analyses were performed for dichotomous data. We used the updated command *metaprop_one* and fit the logistic-normal random-effects model to the data (20). Continuous data were expressed as means and standard deviation (SD), and the pre- and post-intervention standardized mean difference (SMD) were calculated with respective 95% confidence interval (CI).

Inconsistencies between the results of the studies included were ascertained by a visual inspection of forest plots and by applying the Higgins statistic (I^2) and the chi-square test (χ^2). Moderate heterogeneity was ascertained if I^2 >35%. For χ^2, statistic heterogeneity was considered if p <0.10 (21). In order to explore the potential sources of heterogeneity, meta-regression was performed using logit transformed outcomes and logit transformed with study SD. The study sample size, study design (*i.e.*, randomized, prospective), mean age of the study participants, and doses of the intervention were considered as potential explanatory variables. The Knapp–Hartung correction was used to calculate the significance of the meta-regression coefficients (24).

Prediction interval (PI) was calculated for the random-effect meta-analysis, if χ^2 p <0.1 or I^2 >35% and more than five studies. PI predicts the possible treatment effect in an individual study setting, whereas the random effect meta-analysis summarizes the average effect across the studies (25). Because the potential treatment effect when applied within an individual study setting may differ from the average effect, the PI provides interesting insights for clinical practice (25).

Quality of the Evidence

For the outcomes from controlled studies, the quality of evidence for estimating the effect of intervention was generated in accordance with the Grading of Recommendations Assessment, Development, and Evaluation Working Group (26).

RESULTS

Study Selection

The search strategies resulted in 2,102 and 1,712 articles after duplicates were removed using the Endnote software. We selected 55 articles for full reading, of which 27 (1,405 patients) were included. Although we set out to include patients with CD only, we included for full reading studies that sampled other etiologies of Cushing's syndrome and tried contacting authors to retrieve the data for CD only. Among the excluded studies, nine studies had overlapping population with other already included studies (27–35), seven studies were cohorts of less than 10 patients (36–42), three did not match the study population (43–45), six did not comply with the outcomes (46–51), and three assessed outcomes before six months of follow-up (13, 52, 53). The selection process is summarized in **Figure 1**.

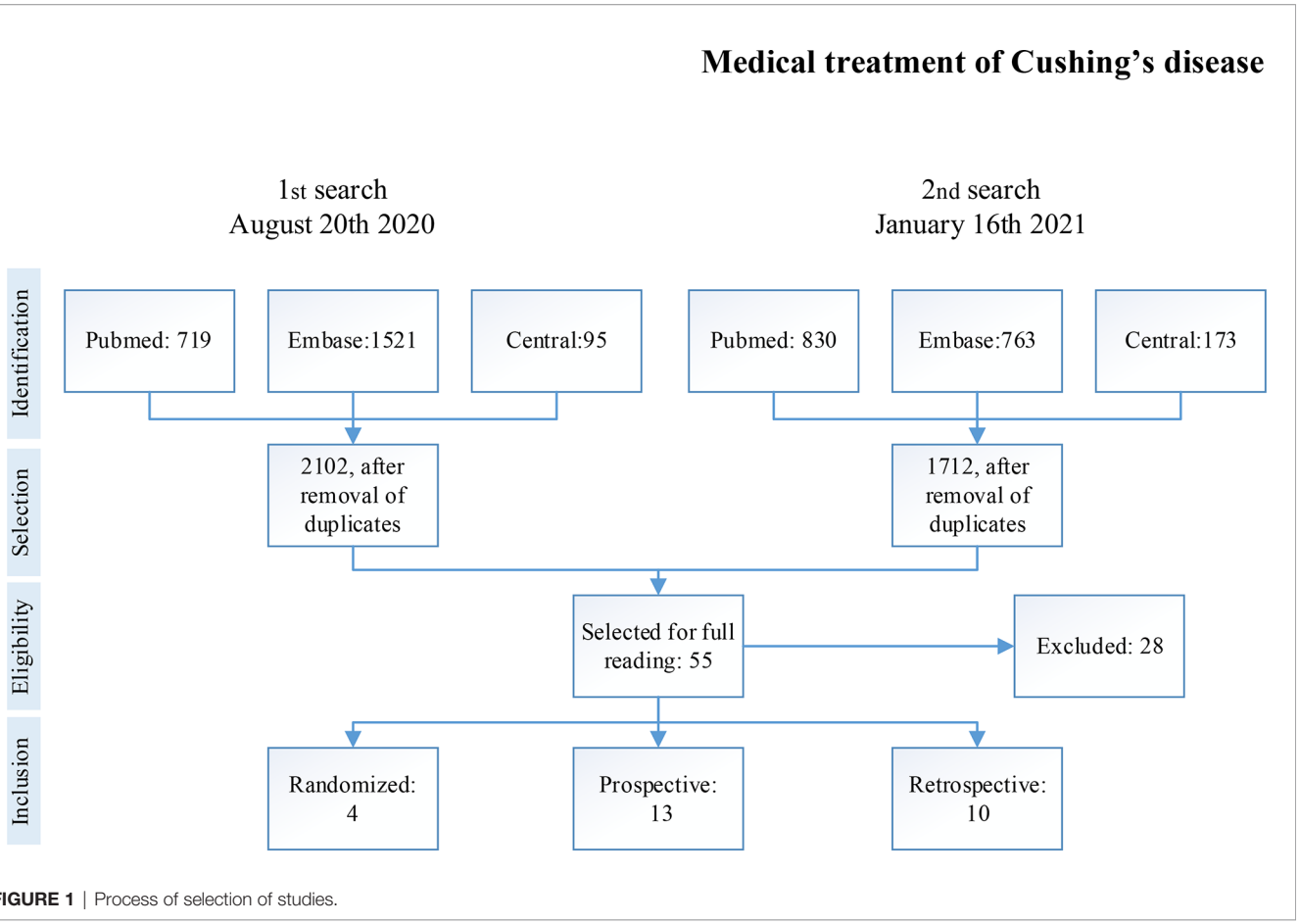

FIGURE 1 | Process of selection of studies.

Characteristics of the Included Studies

The characteristics of the included studies are reported in the supplementary material (**Supplementary Table S3A**). We included eight studies on pasireotide (518 patients) (10, 11, 54–59), six studies on cabergoline (139 patients) (12, 60–64), 10 studies on ketoconazole (559 patients) (60, 65–73), five studies on metyrapone (160 patients) (66, 69, 74–76), and one study on osilodrostat (36 patients) (77).

There were four randomized controlled studies (10, 54, 60, 77) and 23 single-arm studies, from which 13 were prospective (11, 12, 55–58, 61, 62, 65, 67, 68, 73, 75) and 10 were retrospective (59, 63, 64, 66, 69–72, 74, 76). To confirm the pituitary origin of Cushing's syndrome, the selected studies considered dynamic tests, in addition to the criteria pre-established in our review protocol. Four studies considered the 8-mg/day high-dose dexamethasone suppression test (12, 63, 71, 72), four studies considered the corticotropin-releasing-hormone test (10, 68, 73, 75), and 10 considered both (59–61, 64–67, 69, 70, 74).

Among the three randomized controlled studies included, only one compared two different medications. Data plotted in the meta-analysis refers to the 6-month follow-up of the study by Barbot et al., in which both cabergoline and ketoconazole were used as monotherapy. No statistical difference was observed in the CD control (relative risk: 0.53, 95% CI: 0.15 to 1.87, 14 participants, very low certainty of evidence). The quality of evidence was rated down due to imprecision (*i.e.*, wide CI and no achievement of optimal information size) and high risk of selection bias (60). Two randomized studies compared different dosages of pasireotide (*i.e.*, Colao et al. compared 600 *vs.* 900 mcg and Lacroix et al. compared 10 *vs.* 30 mg long-acting release) (10, 54).

Risk of Bias of the Included Studies

The description of inclusion criteria was adequate in all the included studies. In 65% of the studies, it was unclear if the inclusion of patients was consecutive and complete. Reporting was unsatisfactory in 24% of the included studies regarding baseline information and in 48% regarding outcomes. Statistical analysis was considered inappropriate in 20% of the studies. **Table 1** shows the risk of bias of the included studies.

Proportion of Patients With Disease Control

The treatment effects of pasireotide, cabergoline, ketoconazole, and metyrapone on disease control, as defined by the individual included studies, were pooled in the proportional meta-analyses. Although UFC was most commonly used to measure disease control, Lila et al. used midnight salivary cortisol (MNSC) and low-dose dexamethasone suppression (LDSC) test to define

TABLE 1 | Risk of bias of the included studies.

Author	Year	1. Clear inclusion criteria	2. Diagnostic criteria stated	3. Valid biochemical assay to measure hypercortisolism	4. Consecutive and complete inclusion of participants	5. Complete reporting of baseline information	6. Complete reporting of outcomes	7. Complete reporting of site demographics	8. Appropriate statistical analysis	Overall risk of bias[a]
Barbot et al. (60)	2014	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Colao et al. (10)	2012	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Lacroix et al. (54)	2018	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Albani et al. (55)	2018	✓✓	✓✓	✓✓	⚠	✕	✓✓	✓✓	✓✓	High risk
Barbot et al. (11)	2018	✓✓	✓✓	✓✓	✓✓	✕	✓✓	✓✓	✕	Some concerns
Boscaro et al. (56)	2014	✓✓	✓✓	✓✓	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Fleseriu et al. (57) (pasireotide)	2018	✓✓	✓✓	✓✓	⚠	✓✓	✕	✓✓	✓✓	High risk
Pivonello et al. (58)	2019	✓✓	✓✓	✓✓	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Vilar et al. (61)	2010	✓✓	✓✓	✓✓	⚠	✓✓	✕	✓✓	✓✓	High risk
Lila et al. (62)	2010	✓✓	✓✓	✕	⚠	✓✓	✕	✓✓	✓✓	High risk
Pivonello et al. (12)	2009	✓✓	✓✓	✓✓	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Castinetti et al. (72)	2008	✓✓	✓✓	⚠	✓✓	✕	✕	✓✓	✕	High risk
Castinetti et al. (71)	2014	✓✓	✓✓	⚠	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Invitti et al. (70)	1999	✓✓	✓✓	✓✓	⚠	✕	✕	✓✓	✕	High risk
Valassi et al. (69)	2012	✓✓	✓✓	✓✓	⚠	✕	✕	✓✓	✕	High risk
Godbout et al. (63)	2010	✓✓	✓✓	⚠	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Ferriere (64)	2016	✓✓	✓✓	⚠	⚠	✓✓	✓✓	✓✓	✓✓	Some concerns
Trementino et al. (59)	2016	✓✓	✓✓	✓✓	✓✓	✕	✕	✓✓	⚠	High risk
Luisetto et al. (68)	2001	✓✓	✓✓	⚠	⚠	✓✓	✕	✓✓	⚠	High risk
Ghervan et al. (67)	2015	✓✓	✓✓	✓✓	⚠	✓✓	✕	✓✓	⚠	High risk
Ceccato et al. (75)	2018	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Pivonello et al. (77)	2020	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Van der Bosch et al. (66)	2014	✓✓	✓✓	✓✓	⚠	✓✓	✕	✓✓	⚠	High risk
Moncet et al. (73)	2007	✓✓	✓✓	⚠	✕	✕	✕	✓✓	⚠	High risk
Sonino et al. (65)	1991	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	Low risk
Verhelst et al. (74)	1991	✓✓	✓✓	✕	⚠	✓✓	✕	✓✓	⚠	High risk

✕, *high risk of bias;* ✓✓, *low risk of bias;* ⚠, *unclear.*

[a]*For overall risk of bias, criteria 4, 5, and 6 were taken into consideration. Overall risk of bias was low if all three were low risk. If one of the three criteria were unclear or high risk, the overall assessment was "some concerns"; if two were unclear or high risk, the overall assessment was "high risk".*

disease control (62), and Daniel et al. used mostly 9 AM cortisol and mean cortisol from a cortisol day-curve (76). The morning serum cortisol was also used by three studies (66, 67, 74).

Figure 2 shows the proportional random meta-analysis on disease control. The pooled proportion of control was 35% (27–43%) for cabergoline and 41% (36–46%) for ketoconazole, with low heterogeneity. Although statistic heterogeneity was not confirmed for metyrapone (p = 0.12), the small sample of included studies yielded a large CI for`66% of the observed disease control (95%CI: 46–87%, four studies, 66 patients). A subgroup analysis considering studies that considered UFC as the only criteria of disease control was performed for cabergoline (36%, 95% CI: 28–45%, p = 0, five studies, 121 patients) and ketoconazole (41%, 95% CI: 36–45%, P = 0, 5 studies, 434 patients). With regard to pasireotide, 44% of patients had disease control (30–60%, χ^2 = 21.3, p = 0, PI = 18–74%, eight studies, 522 participants; **Supplementary Figure S1A**). To investigate the heterogeneity, meta-regression was conducted, and it showed that the number of included patients was the variable that explained 50% of heterogeneity. A meta-analysis with the two larger randomized studies showed that the proportion of disease control after pasireotide was 29% (25–35%, χ^2 = 0, **Figure 2**).

Improvement of UFC

Because different units of measure were applied to report UFC (*i.e.*, nmol/24 h, μg/24 h, or number of times above the upper limit of normality), the SMD was used as a measure of effect size to plot pre- and post-intervention data in a meta-analysis. The meta-analysis of studies with pasireotide, cabergoline, and ketoconazole consistently showed a reduction on UFC, although with high heterogeneity. The pooled results for cabergoline, ketoconazole, and pasireotide are shown on **Table 2** (forest plots in **Supplementary Figure S2A**).

The high heterogeneity on the meta-analysis for cabergoline could be explained by one outlier study (12), in which a higher weekly dose (7 mg) was used. The visual analysis of the forest plot for ketoconazole likewise had one outlier study (65), which had a high risk of bias. A sensitivity analysis was performed to explore the heterogeneity for pasireotide. Considering randomized studies only, pasireotide reduced the UFC in -0.94 SD (CI: -1.14, -0.74, I^2 = 7%) (**Supplementary Figures S2A–C**).

Improvement of Comorbidities

Pre- and post-intervention data on systolic and diastolic blood pressure, cholesterol, triglycerides, body mass index, and waist circumference were extracted when available. Among these secondary outcomes, improvement of blood pressure (BP) levels was the most commonly reported, although there was variability in reporting and measurement (*i.e.*, proportion of controlled BP, reduction of the parameter itself). Hence, due to the lack of data and heterogeneity on the reporting of outcomes, a meta-analysis for pasireotide was performed considering the reduction of the parameter itself, disregarding the number of medications, which was poorly described in most studies. **Table 3** shows a metanalysis on the improvement of BMI, waist circumference, and systolic and diastolic BP with pasireotide.

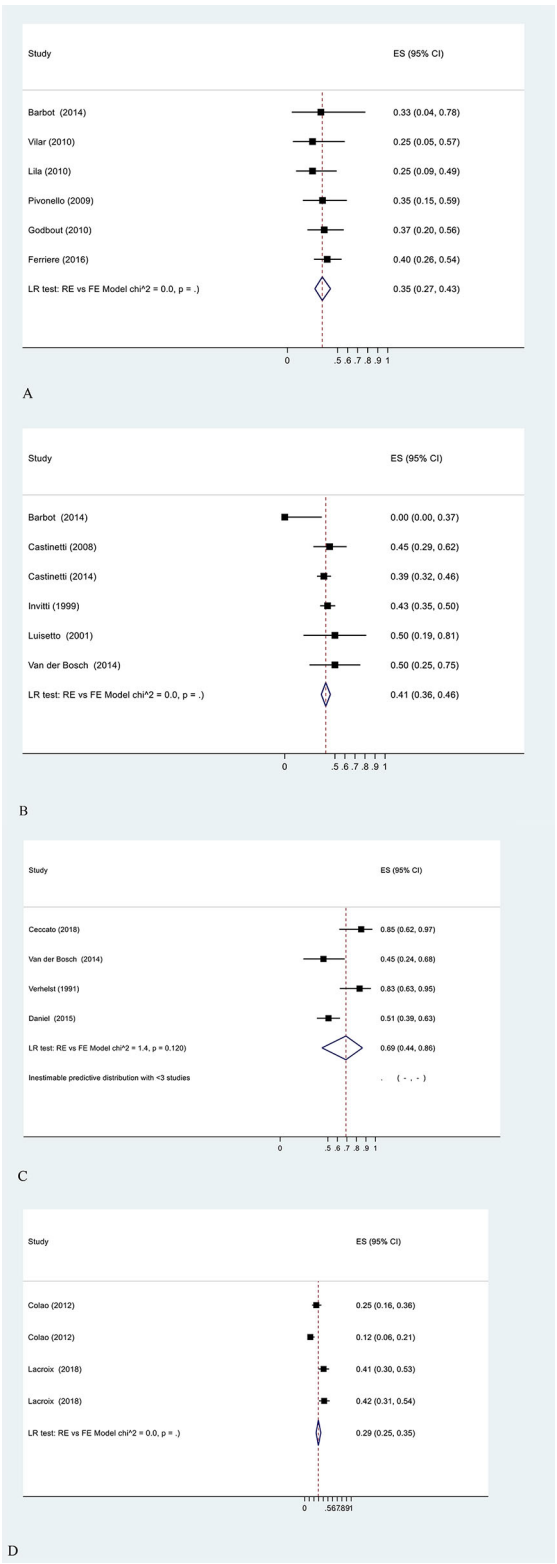

FIGURE 2 | Meta-analysis on the proportions of disease control after treatment with **(A)** cabergoline, **(B)** ketoconazole, **(C)** metyrapone, and **(D)** pasireotide.

TABLE 2 | Summary of meta-analysis on the reduction of urinary cortisol pre- and post-intervention.

	SMD	95% CI	I^2	*P*	PI	Included studies (*n*)	Included patients (*n*)
Pasireotide[a]	-0.94	-1.17, -0.71	51.9%	0.358	-1.60, -0.28	7	503
Randomized studies only	-0.94	-1.14, -0.74	7%	0.028	-1.44, -0.45	2	312
Cabergoline	-2.4	-4.5, -0.25	95%	–	–	4	68
Ketoconazole	-2.88	-5.18, -0.58	96.6%	–	–	4	246

I^2, Higgins test of heterogeneity; CI, confidence interval; PI, predictive interval; SMD, standard mean deviation.
[a]Randomized and prospective studies.

TABLE 3 | Summary of meta-analysis on the improvement of comorbidities with pasireotide.

Clinical parameters	SMD	95% CI	I^2	Included studies (*n*)	Included patients (*n*)
BMI	-1.49	-2.08, -0.90	81.4%	5	381
Randomized studies only	-1.44	-2.07, -0.82	90.5%	2	312
Waist circumference	-3.54	-4.84, -2.24	55%	5	381
Randomized studies only	-3.32	-5.2, -1.43	77%	2	312
Systolic blood pressure	-6.30	-8.46, -4.13	41.8%	7	448
Randomized studies only	-7.18	-10.49, -3.87	51%	2	312
Diastolic blood pressure	-4.32	-5.83, -3.01	0%	6	432
Randomized studies only	-3.95	-5.8, -2.31	0%	2	312

I^2, Higgins test of heterogeneity; CI, confidence interval; PI, predictive interval; SMD, standard mean deviation.

With regard to cabergoline, Lila et al. observed that four out of 18 patients showed a decrease of 20 mmHg on SBP and 10 mmHg on DBP after 5 months of treatment with cabergoline (62). In another series, a mean reduction on SBP from 141.5 to 118 mmHg after 12 months of follow-up was found (12). Moreover, ketoconazole was also reported to reduce the mean blood pressure from 148/105 mmHg at baseline to 115/85 mmHg after a mean follow-up of 23 months (72). A second study reported that 40% of patients had controlled hypertension after treatment with ketoconazole (71).

Safety

The proportion of different AE associated with pasireotide, cabergoline, ketoconazole, and metyrapone was also plotted in a proportional meta-analysis, as summarized in **Table 4**. SAEs were reported exclusively for pasireotide, probably because two very low bias randomized studies on pasireotide were included, whereas for other medications, the included studies were mostly prospective or retrospective cohorts.

In the proportional meta-analyses of main AE associated with pasireotide (*i.e.*, diabetes, hyperglycemia, cholecystitis, nausea, abdominal pain, and headache), a high heterogeneity was identified. The meta-regression showed that the type of study was the explanatory variable for this heterogeneity, and sensitivity analysis including only randomized trials was performed.

Cabergoline and metyrapone were mainly associated with vertigo and nausea, with low heterogeneity in the meta-analysis. Studies with ketoconazole reported mainly elevated transaminases, rash, and adrenal insufficiency. Diarrhea and/or abdominal pain were assessed as a composed outcome in the meta-analysis.

Studies Not Included in the Meta-Analyses

No study with temozolomide and levoketoconazole fulfilled our inclusion criteria. Osilodrostat was evaluated in one prospective, open-label, single-arm study with a placebo randomized withdrawal period (77). At 48 weeks, 91 (66.4%, 95% CI: 57.9, 74.3) enrolled patients had a complete response. Sixty-four out of 97 patients (66%) who were treated with osilodrostat throughout the 48 weeks and had a complete response and maintained a complete response for at least 6 months. The most common adverse events included nausea (42%), headache (34%), fatigue (28%), and adrenal insufficiency (28%). Moreover, symptomatic hypocortisolism was reported by 70 (51%) patients, and 58 (42%) patients reported adverse events related to adrenal hormone precursors.

DISCUSSION

Therapeutic guidelines recommend medical treatment for patients with CD who are not surgical candidates or who have a persistent disease after TSS, although with no preference for either medical treatment (5). Our systematic review set out to assess the effectiveness and safety of medical treatment for CD. The proportional meta-analyses showed a similar proportion of CD control between cabergoline (27–43%), pasireotide (25–35%), and ketoconazole (36–46%). A meta-analysis of metyrapone resulted in 66% of disease control, but with broad CI (46–87%) because most studies were small retrospective cohorts. Moreover, the proportion of disease control with metyrapone may be overestimated because most studies considered the morning serum cortisol as criterion for disease control.

In contrast with other pituitary tumors such as prolactinomas, in which an optimistic response to medical treatment is expected (78), medical treatment for CD induced disease control in less than 50% of patients. Moreover, there are several AE associated with these medications as shown in our meta-analyses. Therefore, new treatment alternatives have been studied, such as osilodrostat and levoketoconazole. A single study on osilodrostat included in our review reported a

TABLE 4 | Proportional meta-analysis of the frequency of adverse events.

	Frequency of AE	95% CI	Chi-square	*p*	PI	Included studies (*n*)	Included patients (*n*)
Pasireotide							
SAE	0.17	0.04, 0.49	0.6	0.219	–	8	522
Diabetes	0.21	0.15, 0.28	2.1	0.076	0.11, 0.36	8	522
Randomized only	0.25	0.21, 0.30	0	–	–	2	312
Hyperglycemia	0.29	0.15, 0.49	18.4	0	0.06, 0.72	8	522
Randomized only	0.48	0.42, 0.53	0	–	–	2	312
Diarrhea	0.3	0.16, 0.48	17.7	0	0.08, 0.68	5	467
Cholecystitis	0.13	0.02, 0.54	73.2	0	0, 0.92	5	467
Randomized only	0.38	0.33, 0.44	0	–	–	2	312
Nausea	0.21	0.12, 0.33	7.8	0.003	0.06, 0.50	5	467
Randomized only	0.29	0.24, 0.34	0	–	–	2	312
Abdominal pain	0.29	0.14, 0.49	0.8	0.18	–	3	331
Randomized only	0.21	0.16, 0.25	0	–	–	2	312
Headache	0.24	0.19, 0.28	0	–	–	3	331
Randomized only	0.23	0.18, 0.28	0	–	–	2	312
Fatigue	0.2	0.16, 0.25	0	–	–	4	363
Cabergoline							
Escape from treatment	0.14	0.09, 0.21	0	–	–	6	143
Vertigo	0.12	0.07, 0.19	0	–	–	6	143
Nausea	0.1	0.06, 0.16	0	–	–	6	143
Fatigue	0.07	0.03, 0.18	0.1	0.373	–	6	143
Ketoconazole							
Elevated transaminases[a]	0.14	0.11, 0.18	0	–	–	8	366
Diarrhea and/or abdominal pain	0.08	0.04, 0.18	2.6	0.052	–	8	366
Rash	0.03	0.01, 0.09	2.4	0.06	–	8	366
Adrenal insufficiency	0.06	0.04, 0.10	0.2	0.327	–	8	366
Metyrapone							
Nausea	0.18	0.07, 0.40	1.9	0.085	–	4	89
Vertigo	0.17	0.10, 0.26	0	–	–	4	89
Hirsutism	0.17	0.10, 0.26	0	–	–	4	89
Fatigue	0.07	0.01, 0.40	0.1	0.351	–	4	89
Hypokalemia	0.09	0.05, 0.17	0	–	–	4	89

AE, adverse events; CI, confidence interval; Chi-square, heterogeneity; PI, predictive interval; SAE, serious adverse events.
[a]*Includes an increase in alanine aminotransferase and alkaline phosphatase.*

proportion of disease control of 66% after 48 weeks of follow-up (77). However, osilodrostat is not yet available in most countries, and its safety needs further assessment in larger trials. The only study on levoketoconazole included patients with Cushing's syndrome of all etiologies and therefore was excluded from our review. This non-controlled study induced disease control in 36% of the 95 patients at the 6-month follow-up (45).

Temozolomide is an orally active alkylating agent that has been used in patients with aggressive corticotroph tumors. Two retrospective case series evaluated temozolomide for patients with aggressive pituitary adenomas and carcinomas (43, 44). Complete remission ocurred in 13% (three out of 23) and 50% (10 out of 20) of the patients. Both studies were excluded from the review because the diagnostic criteria was not clearly reported. Moreover, the outcome measurement for diasease remission was imprecise.

Some limitations of our systematic review must be acknowledged. First, our results were predominantly from uncontrolled studies. Among the controlled studies, Barbot et al. compared cabergoline and ketoconazole (60), while two studies compared two different dosages of pasireotide (10, 54). The only study to compare two different drugs had a high risk of bias due to the incomplete reporting of the randomization process and high uncertainty of the effect size due to the small sample (60). Therefore, lack of controlled studies limits the conclusions with regard to the comparative effectiveness of the medications studied. A second limitation was the low quality of evidence of the included studies, which were mainly small cohorts.

Two similar systematic reviews were published, but with significant differences (14, 79). Gadelha et al. did not perform a meta-analysis due to paucity of the studies included (79). Broersen et al. performed a comprehensive systematic review addressing the medical treatment for Cushing's syndrome of all etiologies (14). Nevertheless, recently, a large trial on pasireotide was published (54). Therefore, the contribution of our meta-analysis provides an updated overview on the effectiveness of the medical treatment for CD. In addition to disease control, this is the first study to pool the effect on UFC reduction, comorbidities, and AE.

Disease control was defined by most of the included studies as UFC below the upper limit of normality, with few exceptions. Lila et al. considered MNSC and LDSC, while four studies considered the morning serum cortisol (66, 67, 74, 76). Among the four studies included in the proportional meta-analyses of disease control of metyrapone, only one considered UFC as a criterion for disease control (75). There is a good correlation between the normalization of UFC and the improvement of signs

and symptoms of hypercortisolism (32). Moreover, the normalization of UFC is associated with a low recurrence risk, and therefore some studies advocate that it should be considered as the main criterion to determine control (3).

Despite being not within the scope of this review, a combination of drugs has shown promising results for CD. In the second phase of the randomized study by Barbot et al., the combination of cabergoline and ketoconazole achieved UFC normalization in 79% of patients and a significant improvement in the symptoms of hypercortisolism. These results persisted for at least 6 months, with a few adverse events (60). Feelders et al. treated 17 patients first with pasireotide as monotherapy, then combined pasireotide with cabergoline, and then added ketoconazole if the patients did not achieve control (52).

The small sample, however, limits the conclusion with regard to the effectiveness of the combined treatment. A phase II, open-label, multicenter clinical trial on the combination of pasireotide and cabergoline, the CAPACITY study, will assess the efficacy and safety in CD patients (80).

In conclusion, medical treatment is a valid treatment alternative for patients who had a recurred hypercortisolism after TSS or who had contraindications for surgery. The proportion of disease control after treatment with cabergoline, ketoconazole, and pasireotide identified in our meta-analysis may support the clinical decision. New therapeutic options should be investigated due to the limited efficacy and tolerability of the currently available medical treatment for patients with Cushing's disease.

PRACTICAL IMPLICATIONS

Pasireotide is approved for CD within the Brazilian Unified Health System, while cabergoline and ketoconazole are used as off-label medications. These results may support the inclusion of cabergoline and ketoconazole as alternative second-line treatment for patients with CD in Brazil as well as in other countries.

SYSTEMATIC REVIEW REGISTRATION

This systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO CRD42020205567).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

VS and CB conceptualized and designed the study. JS and MD developed the search strategies. VS and JS independently screened eligible studies. JS and AN extracted data from the included studies and assessed the individual risk of bias. VS and JS assessed in pairs and independently the risk of bias. VS performed the meta-analysis. VS supervised all the phases of this review and refereed any disagreement to avoid errors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.732240/full#supplementary-material

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Cushing's Disease: Assessment of Early Cardiovascular Hemodynamic Dysfunction With Impedance Cardiography

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Background: Cushing's disease is a rare condition associated with a high cardiovascular risk and hypercortisolemia-related hemodynamic dysfunction, the extent of which can be assessed with a noninvasive method, called impedance cardiography. The standard methods for hemodynamic assessment, such as echocardiography or ambulatory blood pressure monitoring may be insufficient to fully evaluate patients with Cushing's disease; therefore, impedance cardiography is being currently considered a new modality for assessing early hemodynamic dysfunction in this patient population. The use of impedance cardiography for diagnosis and treatment of Cushing's disease may serve as personalized noninvasive hemodynamic status assessment and provide a better insight into the pathophysiology of Cushing's disease. The purpose of this study was to assess the hemodynamic profile of Cushing's disease patients and compare it with that in the control group.

Material and Methods: This observational prospective clinical study aimed to compare 54 patients with Cushing's disease (mean age 41 years; with 64.8% of this population affected with arterial hypertension) and a matched 54-person control group (mean age 45 years; with 74.1% of this population affected with arterial hypertension). The hemodynamic parameters assessed with impedance cardiography included the stroke index (SI), cardiac index (CI), systemic vascular resistance index (SVRI), velocity index (VI), (ACI), Heather index (HI), and thoracic fluid content (TFC).

Results: The Cushing's disease group was characterized by a higher diastolic blood pressure and a younger age than the control group (82.9 *vs.* 79.1 mmHg, p=0.045; and 41.1 *vs.* 44.9 years, p=0.035, respectively). Impedance cardiography parameters in the Cushing's disease group showed: lower values of SI (42.1 *vs.* 52.8 ml/m^2; p ≤ 0.0001), CI (2.99 *vs.* 3.64 l/min/m^2; p ≤ 0,0001), VI (42.9 *vs.* 52.1 1/1000/s; p=0.001), ACI (68.7 *vs.* 80.5 1/100/s^2; p=0,037), HI (13.1 *vs.* 15.2 Ohm/s^2; p=0.033), and TFC (25.5 *vs.* 27.7

1/kOhm; p=0.006) and a higher SVRI (2,515 *vs.* 1,893 dyn*s*cm^{-5}*m^2; p ≤ 0.0001) than those in the control group.

Conclusions: Cushing's disease is associated with significantly greater vasoconstriction and left ventricular systolic dysfunction. An individual assessment with impedance cardiography may be useful in Cushing's disease patients in order to identify subclinical cardiovascular complications of chronic hypercortisolemia as potential therapeutic targets.

Keywords: Cushing's disease, impedance cardiography, cardiovascular complications, arterial hypertension, left ventricular systolic dysfunction

INTRODUCTION

Cushing's disease is a rare chronic disorder due to excessive secretion of adrenocorticotropic hormone (ACTH) by a pituitary adenoma. Cushing's disease-associated hypercortisolemia has been linked to significant functional and structural systemic abnormalities, with changes in the hemodynamic profile and a considerably increased cardiovascular risk (1–3). Some adverse effects of chronic hypercortisolemia include hemodynamic disturbances associated with excessive vascular constriction and elevated blood pressure (BP), obesity, impaired carbohydrate metabolism, and dyslipidemia, all of which may contribute to substantial cardiovascular remodeling (4–7). Subclinical effects of hypercortisolemia may be undetectable with standard hemodynamic assessment methods. Therefore, novel diagnostic tools should be sought to help detect abnormalities in Cushing's disease patients as early as possible and improve the chances of their optimal targeted treatment and, as a result, lower the cardiovascular risk. One noninvasive and well-validated tool for assessing cardiovascular hemodynamics is impedance cardiography, which helps assess such cardiovascular hemodynamic parameters as arterial stiffness, intravascular volume, and cardiac function, which are useful in clinical evaluation of Cushing's disease patients, particularly those with concomitant arterial hypertension (8–11). Therefore, the purpose of this study was to use this method for assessing cardiovascular function to evaluate the hemodynamic profiles of patients with Cushing's disease and compare them with those in the control group.

MATERIALS AND METHODS

Study Population

Two age-matched groups were compared in this observational, prospective clinical study. The Cushing's disease group comprised 54 patients with Cushing's disease (including 12 males; mean age 41 years; with 64.8% of this population with controlled arterial hypertension – mean blood pressure 126/83 mmHg), and the control group comprised 54 individuals (including 19 males; mean age 45 years; with 74.1% of this population with controlled arterial hypertension – mean blood pressure 121/79 mmHg). Neither study group included patients with significant comorbidities.

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP). The study protocol had been approved by the Bioethics Committee of Military Institute of Medicine in Warsaw. All study participants had provided their written informed consent.

Cushing's Disease

The Cushing's disease group included both male and female patients newly diagnosed with Cushing's disease, defined based on a standard hormone blood test and imaging study results in accordance with the European Society of Endocrinology guidelines: symptoms of hypercortisolemia combined with serum cortisol suppression or a decrease in urinary free cortisol by >50% during a high-dose dexamethasone suppression test (HDDST; 8 mg over 48 h) or a positive ACTH stimulation test with the use of corticotropin-releasing hormone (CRH; 100 mg intravenously) and evidence of pituitary adenoma in magnetic resonance imaging (MRI) (12). Inferior petrosal sinus sampling was performed in all cases of a microadenoma smaller than 6 mm, inconclusive MRI results, or contradictory responses to dynamic testing. All patients from the Cushing's disease group underwent standard hormone level tests for ACTH, follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone and history-taking for any concomitant impaired carbohydrate metabolism (type 2 diabetes mellitus, impaired fasting glycemia, and impaired glucose tolerance) diagnosed previously or during the first study visit. Since none of the Cushing's disease group patients had been taking any drugs affecting the hypothalamic–pituitary–adrenal axis, their medical treatment had no effect on their hemodynamic assessments.

Control Group

The control group-from which a subgroup of 54 individuals, matched for key clinical variables (age, sex, body mass index (BMI), mean blood pressure (MBP), proportion of arterial hypertension cases), had been selected for a comparative analysis—comprised the subjects from the government-funded study "Non–invasive hemodynamic assessment in hypertension (FINE-PATH)" (ClinicalTrials.gov Identifier NCT01996085), *conducted at the Military Institute of Medicine.* There were 120 initially recruited patients of both sexes, with arterial hypertension treated for at least 12 months, and 35 healthy

individuals of both sexes, without cardiovascular conditions or any other clinically significant internal medical conditions.

Exclusion Criteria

The study exclusion criteria were conditions significantly affecting cardiovascular system function and those that could confound the obtained results, namely: coronary artery disease, including history of myocardial infarction; chronic heart failure with mid-range ejection fraction and heart failure with reduced ejection fraction, with left ventricular ejection fraction <50%; history of pulmonary embolism; documented history of stroke or transient ischemic attack; severe chronic obstructive pulmonary disease, with the Tiffeneau index (or forced expiratory volume in 1 second expressed as percentage of vital capacity, FEV1) <50% of the predicted value; respiratory failure (partial pressure of oxygen [PaO2] in blood <60 mmHg and/or increased partial pressure of carbon dioxide [PaCO2] >45 mmHg); status post head injury; pregnancy; lack of consent; any conditions making the patient unable to follow the study protocol.

Clinical Study

The clinical examination was conducted with a particular focus on cardiovascular risk factors (including family history of cardiovascular disease; cardiovascular symptoms; comorbidities; nicotine dependence; impaired carbohydrate metabolism; lifestyle; office BP measurement, including systolic and diastolic blood pressure (SBP and DBP); heart rate (HR); and anthropometric measurements (height, body weight, BMI). The office BP measurement was performed with the use of an automatic device (Omron M4 Plus, Japan) in accordance with European Society of Cardiology guidelines (13).

Impedance Cardiography

Hemodynamic parameters were measured at rest in a supine position *via* impedance cardiography with a *Niccomo*™ device (Medis, Ilmenau, Germany) during a 10-minute assessment. These 10-minute impedance cardiography recordings were used to analyze (*Niccomo Software*) in detail the mean hemodynamic parameters, such as HR [bpm]; SBP [mmHg], DBP [mmHg]; stroke volume (SV) [ml]; stroke index (SI) [ml/m^2], cardiac output (CO) [l/min]; cardiac index (CI) [l/min/m^2]); systemic vascular resistance (SVR) [dyn*s*cm^{-5}]; systemic vascular resistance index (SVRI) [dyn*s*cm^{-5}*m²]; velocity index (VI) [1/1000/s]: VI=1000*dZmax*Z0^{-1}, expressing peak aortic flow velocity; acceleration index (ACI) [1/100/s^2]: ACI=100*dZmax*dt^{-1}, expressing peak aortic flow acceleration; Heather index (HI) [Ohm/s^2]: HI=dZmax*TRC, expressing the ratio of peak systolic outflow to the time interval from the Q/R wave peak in ECG to the impedance cardiography wave peak and reflecting both the cardiac inotropic function and thoracic fluid content (TFC) [1/kOhm]. According to the data obtained from the PREDICT study, which helped identify the different risk groups based on the stroke index and thoracic fluid content values, for our study we adopted the cutoff values of SI at <35 ml/m^2 and of TFC at >35 1/kOhm (14).

Impedance cardiography is a non-invasive method designed for monitoring hemodynamic parameters on the basis of analysis of thoracic electrical resistance. During the examination, voltage changes associated with changes in blood volume and velocity in large vessels during systole and diastole are analyzed. This enables the calculation of parameters, including stroke volume and cardiac output, which is a particular advantage of the method. This method has also some limitations. The diagnostic value of the impedance cardiography is questionable in the following clinical situations: tachycardia> 250/min, significant arrhythmias, severe aortic regurgitation, extremely high blood pressure, intra-aortic counterpulsation, severe septic shock, post-sternotomy condition, very short or tall stature, severe obesity or severe malnutrition. Moreover, the quality of measurement strongly depends on skin preparation and movement artifacts (8).

Statistical Methods

Electronic filing and statistical analysis of data were conducted with *MS Office Excel 2016* and *Statistica 12.0* software (*StatSoft Inc., Tulsa, USA*). Continuous variables were expressed as means ± standard deviation (SD), medians, and interquartile ranges, and categorical (qualitative) variables were expressed as counts (n) and proportions (%). Continuous variable distribution was assessed visually with the Shapiro–Wilk test. For each comparative analysis, the propensity score matching method was used to select from the control group a subgroup matched for key clinical variables (age, sex, BMI, MBP, arterial hypertension rates), which may have a considerable impact on the evaluated variables. The differences in absolute values of normally distributed continuous variables were analyzed with a t-test, and the Mann–Whiney U test was used for the variables that were not normally distributed. Categorical (qualitative) variables were analyzed with the chi-square and Fisher's tests. Statistical significance was adopted at $p < 0.05$.

RESULTS

Demographic and Clinical Data of the Cushing's Disease Group

Detailed patient characteristics of the Cushing's disease group are presented in **Table 1**. Arterial hypertension rates were similar in the Cushing's disease and control groups (64.8% *vs.*74.1%; p=0.296), with all arterial hypertension patients undergoing medical treatment, usually with one or two antihypertensive drugs. Over 70% of Cushing's disease patients had abnormal body weight, with 22 patients obese (40.7%). A total of 20 out of 54 patients with Cushing's disease (37%) had been diagnosed with type 2 diabetes mellitus, 5 (9.3%) with prediabetes, and 29 (46.3%) exhibited normal glucose tolerance. Out of Cushing's disease patients with type 2 diabetes mellitus, 14 had been receiving metformin, 5 metformin and insulin, and 1 insulin. Forty-three out of the 54 patients with Cushing's disease had normal anterior pituitary lobe function. Eleven patients with an invasive corticotropic-releasing tumor had thyroid-stimulating hormone deficiency, but it was well controlled with a stable dose of L-thyroxin.

TABLE 1 | Comparison of the control group and Cushing's disease group in terms of patient characteristics and the hemodynamic parameters assessed with impedance cardiography.

VARIABLES	Controls mean ± SD	Patients with Cushing's disease means ± SD	p-value
PATIENT CHARACTERISTICS			
Age [years]	44.9 ± 9.4	41.1 ± 13.5	**0.035**
Male sex, n [%]	19/54 (35.2)	12/54 (22.2)	0.136
Body mass index [kg/m^2]	29.2 ± 4.9	29.7 ± 6.5	0.988
Heart rate [bpm]	69.8 ± 10.1	71.9 ± 11.4	0.322
Systolic blood pressure [mmHg]	121.6 ± 10.1	126.1 ± 16.8	0.096
Diastolic blood pressure [mmHg]	79.1 ± 8.0	82.9 ± 11.6	**0.045**
Arterial hypertension, n [%]	40/54 (74.1)	35/54 (64.8)	0.296
Creatinine [mg/dL]	0.82 ± 0.15	0.87 ± 0.27	0.836
Left ventricular ejection fraction [%]	66,69 ± 3,27	65,8 ± 4,2	0.323
IMPEDANCE CARDIOGRAPHY			
Heart rate [bpm]	70.2 ± 11.8	71.4 ± 11.9	0.581
Systolic blood pressure [mmHg]	121.2 ± 11.1	125.6 ± 17.0	0.113
Diastolic blood pressure [mmHg]	78.9 ± 9.0	82.5 ± 11.7	0.076
Mean blood pressure [mmHg]	89.9 ± 9.1	93.3 ± 12.0	0.093
Pulse pressure [mmHg]	42.3 ± 6.5	43.2 ± 11.2	0.507
Stroke index [ml/m^2]	52.8 ± 9.0	42.1 ± 10.4	**<0.0001**
Cardiac index [l/min/m^2]	3.64 ± 0.61	2.99 ± 0.78	**<0.0001**
Systemic vascular resistance index [dyn*s*cm^{-5}*m^2]	1,893 ± 379.1	2,515 ± 737.7	**<0.0001**
Velocity index [1/1000/s]	52.1 ± 14.9	42.9 ± 13.4	**0.001**
Acceleration index [1/100/s^2]	80.5 ± 31.6	68.7 ± 25.7	**0.037**
Heather index [Ohm/s^2]	15.2 ± 4.7	13.1 ± 5.0	**0.033**
Thoracic fluid content [1/kOhm]	27.7 ± 4.1	25.5 ± 3.9	**0.006**
Stroke index < 35 ml/m^2, n [%]	2/54 (3.7)	14/51 (27.5)	**0.001**
Thoracic fluid content >35 1/kOhm, n [%]	2/54 (3.7)	0/52 (0)	0.161

SD, standard deviation.
In bold: Statistical significance was adopted at p < 0.05.

Hemodynamic Data of the Cushing's Disease Group

Impedance cardiography parameters in the Cushing's disease group and the common abnormalities in impedance cardiography assessments are presented in **Table 1**. During impedance cardiography, the Cushing's disease group had an average blood pressure of 126/83 mmHg, mean blood pressure of 93 mmHg, and a mean heart rate of 71 bpm. Fourteen patients (27.5%) had a low stroke index (<35 ml/m^2). No patients from the study group showed increased thoracic fluid content (of >35 1/kOhm).

A Comparison Between Cushing's Disease Patients and Controls in Terms of Patient Characteristics and Hemodynamic Parameters Assesses *via* Impedance Cardiography

Table 1 compares patient characteristics in the Cushing's disease and control groups. The average blood pressure was normal in both groups. The study groups differed significantly only in terms of patient age and diastolic blood pressure values. Despite only slight differences in the evaluated key hemodynamic parameters (heart rate, systolic blood pressure, diastolic blood pressure), a comparison of the remaining impedance cardiography variable values demonstrated a number of differences between the study groups. Impedance cardiography showed markedly lower values of cardiac function in patients with Cushing's disease in comparison with those in controls, namely a lower stroke index ($p < 0.0001$) and cardiac index ($p < 0.0001$); significantly lower indices of myocardial contractility, namely velocity index ($p = 0.001$), acceleration index ($p = 0.037$), Heather index ($p = 0.033$); a significantly lower thoracic fluid content ($p = 0.006$); and a significantly higher systemic vascular resistance index ($p < 0.0001$). A total of 3.7% of controls and 27.5% of patients with Cushing's disease had a low stroke index (< 35 ml/m^2; $p = 0.001$). There were no statistically significant differences between the study groups in terms of the other evaluated parameters.

DISCUSSION

This study demonstrated hemodynamic abnormalities in patients with newly diagnosed Cushing's disease, despite an optimal blood pressure control in most of them. Comprehensive hemodynamic assessments with impedance cardiography showed that the hemodynamic profile of Cushing's disease patients differs from that of individuals without hypercortisolemia. We would like to emphasize that the Cushing's disease patients included in this study had no clinically overt cardiovascular dysfunction, and patients with severe comorbidities were excluded.

The demographic, history-related, and cardiovascular function data in our study differed from those obtained in other studies on cardiovascular dysfunction in Cushing's disease patients (15–17). This was due to the fact that the patients recruited to those other studies had not been selected; instead, they were patients with Cushing's disease of various duration and at various stages of

treatment. We would like to emphasize that our thorough cardiovascular hemodynamics assessment with impedance cardiography is one of the first attempts of utilizing this modality in patients with Cushing's disease.

Our hemodynamic assessment with impedance cardiography showed significantly decreased cardiac function, indicators of myocardial contractility, and thoracic fluid content, along with increased systemic vascular resistance in patients with Cushing's disease in comparison with those in control group, which confirms an unfavorable hemodynamic profile in Cushing's disease patients (pronounced vasoconstriction and impaired left ventricular hemodynamic function). The observed subclinical hemodynamic abnormalities support an additive effect of long-term hypercortisolemia on cardiovascular function in patients with Cushing's disease. One of the most common adverse complications of long-term tissue exposure to excess glucocorticoids is arterial hypertension, which develops in over 70% of patients with Cushing's disease and is an independent risk factor for mortality in this group of patients (15, 16, 18, 19). Interestingly, the hypothalamic–pituitary–adrenal axis, which is responsible for the circadian rhythm of endogenous cortisol secretion, also contributes to the regulation of the circadian blood pressure rhythm, and its dysregulation is one of the main factors associated with primary arterial hypertension (20–22). Clinical measurements of arterial hypertension in patients with Cushing's disease may be difficult in cases of hypercortisolemia. Current guidelines stress the need for personalized antihypertensive treatment (18, 23, 24) since arterial hypertension is an independent risk factor for mortality in patients with Cushing's disease (18, 19, 25). There is a linear relationship between arterial hypertension and both adverse cardiovascular events and mortality (25). Therefore, early detection of cardiovascular complications (including arterial hypertension, even before it becomes clinically manifest) may be of clinical significance and may help reduce cardiovascular mortality in patients with Cushing's disease. Out of the 54 patients with Cushing's disease in our study 64.8% were diagnosed with arterial hypertension. The pathophysiology of arterial hypertension in Cushing's disease patients is multifactorial and not fully understood. Arterial hypertension in these patients seems to be an effect of both hypercortisolism and increased activation of the renin–angiotensin–aldosterone system (23, 24, 26–29). These complex processes result in secondary endothelial dysfunction (4, 30–32) and increased carotid artery intima–media thickness, which is associated with atherosclerotic plaque developing earlier than in healthy individuals (15, 30, 33). Therefore, the increased arterial stiffness and abnormal vasoconstriction may play a key role in the pathophysiology of arterial hypertension in this patient population (34).

Our observations regarding this issue, based on the data obtained in our study with the use of a noninvasive method, i.e. impedance cardiography, are consistent with those reported by other authors. The results of our study showed that patients with Cushing's disease have considerably higher values of afterload indicators, which suggests that vasoconstriction abnormalities play a fundamental role in arterial hypertension pathophysiology in these patients, despite their relatively well-controlled blood pressure. This indicates that a hemodynamic assessment with impedance cardiography has an added value, which has been also suggested by the results of other studies. For instance, normal systolic blood pressure values in a group of heart failure patients were not equivalent to an optimal hemodynamic status. This is because systemic vascular resistance index may be increased and contribute to progressive myocardial remodeling even with low systolic blood pressure values of (100–119 mmHg) (11). This is important for antihypertensive therapy selection in this group of patients and indicates a fact of enormous practical significance, namely, that routine blood pressure measurements may be insufficient to rule out hemodynamic dysfunction (11). Thus, the following types of vasodilators would seem to be indicated as first-line therapy in this group of patients: angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and calcium channel blockers; this is consistent with the results of other studies (23, 26, 35).

Moreover, the results of our study indicate that patients with Cushing's disease have lower thoracic fluid content than controls. Thoracic fluid content accurately reflects the amount of intra- and extracellular fluid and is a sensitive indicator of fluid retention and hypervolemia (8). Our study observations regarding this parameter are not entirely consistent with those reported by other authors. Development of arterial hypertension in patients with Cushing's disease may be associated with an increased mineralocorticoid activity of glucocorticoids, enhanced reabsorption of sodium in the renal tubules, and—consequently—increased intravascular volume (23, 24). Some reports emphasize that this does not seem to be the main pathophysiological mechanism responsible for arterial hypertension in patients with Cushing's disease (23). The low thoracic fluid content values observed in our study also support this conclusion. The combination of a low cardiac index (an indirect measure of intravascular volume) and normal left ventricular ejection fraction in patients with Cushing's disease seems to be an argument against the use of diuretics as a first-line antihypertensive therapy.

Our study also showed that patients with Cushing's disease had lower values of cardiac function parameters (stroke index, velocity index, cardiac index, acceleration index) in comparison with controls. This may be explained by rapid remodeling and hypercortisolism-induced fibrosis of the myocardium (17, 36). Patients with Cushing's disease often exhibit evidence of structural remodeling of the myocardium, associated with concentric left ventricular hypertrophy (17). The presence of both arterial hypertension and hypercortisolemia in patients with Cushing's disease considerably worsens the structural and functional status of their myocardium (37, 38). They develop myocardial fibrosis, which is directly related to the effects of cortisol and not merely a result of myocardial hypertrophy due to pressure overload (36). These structural changes may impair left ventricular hemodynamic function, which first manifests as diastolic dysfunction and, subsequently, as systolic dysfunction

and development of symptomatic heart failure (17, 39, 40). One prospective study demonstrated that a successful curative treatment normalized cortisol levels and ultimately led to resolution of myocardial remodeling (41). A study in patients with heart failure showed a significant correlation between changes in the cardiac index value determined *via* impedance cardiography and the left ventricular ejection fraction measured echocardiographically (11). Moreover, a hemodynamic assessment was reported to be of clinical significance in patients with heart failure with preserved left ventricular systolic function (42). There was also a significant correlation between impaired left ventricular systolic function and low values of parameters characterizing blood flow, such as velocity index and acceleration index (43). Other studies suggest that, in patients with arterial hypertension, impedance cardiography may be a useful method for assessing left ventricular dysfunction, whose important predictors are cardiac index ($p=0.005$) and systemic vascular resistance index ($p=0.048$) (9). Earlier studies demonstrated the usefulness of impedance cardiography in assessing left ventricular dysfunction and increased arterial stiffness in middle-aged and elderly patients. Left ventricular diastolic dysfunction was shown to be associated with a lower stroke index, velocity index, acceleration index, and Heather index and a higher systemic vascular resistance index (10). Also, echocardiographic evidence of impaired left ventricular global longitudinal strain was reported to be associated with a hemodynamic profile similar to that found in patients with Cushing's disease (low cardiac index; high systemic vascular resistance index) (44). These correlations were also confirmed in patients with Cushing's disease, in whom an impaired left ventricular global longitudinal strain was associated with left ventricular diastolic dysfunction and were detectable at early stages of pituitary disease (45).

Clinical Implications

This study showed that impedance cardiography can be more sensitive than routine blood pressure measurements and may be a valuable early method of detecting subclinical left ventricular dysfunction in Cushing's disease. Impedance cardiography results indicating early cardiovascular hemodynamic abnormalities despite normal blood pressure values may be an additional argument for initiating early therapeutic intervention, even in cases where the diagnosis of arterial hypertension is uncertain. Moreover, detecting an impaired cardiac function may prompt a more intensive treatment in patients on antihypertensive medications.

Study Limitations

The main limitation of our study was the relatively small sample size. This is a result of low Cushing's disease incidence but also of the study's prospective design. At the time of their diagnosis, many patients with Cushing's disease exhibit signs of significant cardiovascular dysfunction. However, this was an exclusion criterion in our study, which further reduced the study population. Moreover, the patients with Cushing's disease included in this study had no clinically overt cardiovascular dysfunction, and any individuals with severe comorbidities were excluded at the time of recruitment. When interpreting study results, we should consider the potential effects of arterial hypertension (despite its good control) and of antihypertensive treatment. The potential effect of the sex of patients with Cushing's disease on their hemodynamic dysfunction requires further studies.

CONCLUSIONS

1. Hormonal disorders associated with Cushing's disease led to cardiovascular dysfunction manifesting as impaired cardiac function, low indices of myocardial contractility, low thoracic fluid content, and increased systemic vascular resistance.
2. Assessing Cushing's disease patients with the use of impedance cardiography may be useful in detecting early cardiovascular complications and help make decisions as to early introduction of medical treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethics Committee of Military Institute of Medicine in Warsaw. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design, data acquisition and interpretation, and correction of the manuscript: AJ, GG, PK, BU-Ż, PW, GZ, AK, RW, and MB. Data analysis and editing of the manuscript: AJ, PK, GG, BU-Ż, and PW. All authors contributed to the article and approved the submitted version.

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Metformin and Bone Metabolism in Endogenous Glucocorticoid Excess: An Exploratory Study

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Context: Glucocorticoid excess exhibits multiple detrimental effects by its catabolic properties. Metformin was recently suggested to protect from adverse metabolic side-effects of glucocorticoid treatment. Whether metformin is beneficial in patients with endogenous glucocorticoid excess has not been clarified.

Objective: To evaluate the phenotype in patients with endogenous Cushing's syndrome (CS) treated with metformin at the time of diagnosis.

Patients and Methods: As part of the German Cushing's Registry we selected from our prospective cohort of 96 patients all 10 patients who had been on pre-existing metformin treatment at time of diagnosis (CS-MET). These 10 patients were matched for age, sex and BMI with 16 patients without metformin treatment (CS-NOMET). All patients had florid CS at time of diagnosis. We analyzed body composition, metabolic parameters, bone mineral density and bone remodeling markers, muscle function and quality of life.

Results: As expected, diabetes was more prevalent in the CS-MET group, and HbA1c was higher. In terms of comorbidities and the degree of hypercortisolism, the two groups were comparable. We did not observe differences in terms of muscle function or body composition. In contrast, bone mineral density in metformin-treated patients was superior to the CS-NOMET group at time of diagnosis (median T-Score -0.8 *versus* -1.4, $p = 0.030$). CS-MET patients showed decreased β-CTX levels at baseline ($p = 0.041$), suggesting reduced bone resorption under metformin treatment during glucocorticoid excess.

Conclusion: This retrospective cohort study supports potential protective effects of metformin in patients with endogenous glucocorticoid excess, in particular on bone metabolism.

Keywords: metformin, hypercortisolism, glucocorticoids, bone density, osteoporosis, cortisol

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INTRODUCTION

Metabolic side-effects of glucocorticoids (GC) are common and challenging in both endogenous and exogenous GC excess. Patients with endogenous Cushing's syndrome (CS) typically show comorbidities like arterial hypertension, visceral obesity, dyslipidemia, muscle dysfunction, osteoporosis and impaired glucose metabolism (1). CS is associated with poor quality of life,

morbidity and increased mortality, even after successful surgery leading to biochemical remission (2–4). In a recently published study, Pernicova et al. (5) reported that metformin administration improved metabolic profiles of glucocorticoid-treated patients with inflammatory diseases in a randomized, double-blind, placebo-controlled, phase 2 clinical trial. Metformin treatment was associated with favorable effects on lipid profile, liver function, appetite, intima-media thickness and bone mineral density as well as bone turnover (5). However, whether metformin has beneficial effects in patients with endogenous GC excess is largely unknown. In patients with type 2 diabetes mellitus, metformin is the most widely used oral antihyperglycemic agent. Recently, new potential therapeutic applications in non-diabetic patients have been described, such as cardioprotection (6, 7), major depressive disorder (8) and cancer (9–11). The mechanisms of action are still not fully understood. The aim of this retrospective cohort study was to analyze the metabolic profile and bone turnover of patients with and without pre-existing metformin treatment at the time of endogenous GC excess. We hypothesized that standard metformin use is beneficial for bone metabolism in patients with florid CS.

PATIENTS AND METHODS

Patients

This cohort study was performed as part of the German Cushing's Registry. General characteristics of the registry have been described in detail previously (12–14). We screened the prospective registry cohort consisting of 96 patients with endogenous CS for metformin intake at the time of diagnosis. Inclusion criteria for the current study were florid pituitary or adrenal CS, successful surgery leading to biochemical remission; exclusion criteria were subclinical hypercortisolism, ectopic CS, persistent/recurrent CS, adrenostatic or radiation therapy. We identified 10 patients who were taking metformin at the time of diagnosis of CS (CS-MET group). The mean metformin dose at the time of diagnosis was 1670 ± 472 mg per day. Metformin was initiated as routine therapy for diabetes at least 3 months prior evaluation of CS. For comparison, we selected 16 patients without metformin therapy at the time of diagnosis of CS and afterwards (CS-NOMET group). Matching was done according to age, body mass index (BMI), sex and subtype of CS. Patient selection is shown in **Figure 1**. All 26 patients had biochemically confirmed and clinically florid CS, diagnosed between 2012 and 2019 at Ludwig-Maximilian-University Munich. Diagnosis and subtype differentiation of CS were done as reported earlier according to the current guidelines and recommendations (12, 15). One year after successful surgery patients were re-evaluated clinically and biochemically in a standardized fashion. In CS-MET group, 9 out of 10 patients continued metformin therapy until one-year follow-up. For the comparison of bone remodeling markers, a previously described registry control group of patients in whom CS was excluded (NO-CS group, n=95) was used (14). The German Cushing's Registry (NeoExNet, No. 152-10) was approved by the LMU ethics committee, and all patients gave written informed consent.

Laboratory Analysis

In all patients, blood samples were taken in a fasting state at the time of diagnosis and one year after successful surgery in line with the follow-up visit. The analyses of standard laboratory values were performed in the central laboratory of the LMU Klinikum Munich using standard methods. The bone formation marker intact procollagen I-N-propeptide (PINP) and the bone resorption marker β-CTX (CrossLaps) were measured at the Endocrine Laboratory of Department of Medicine IV.

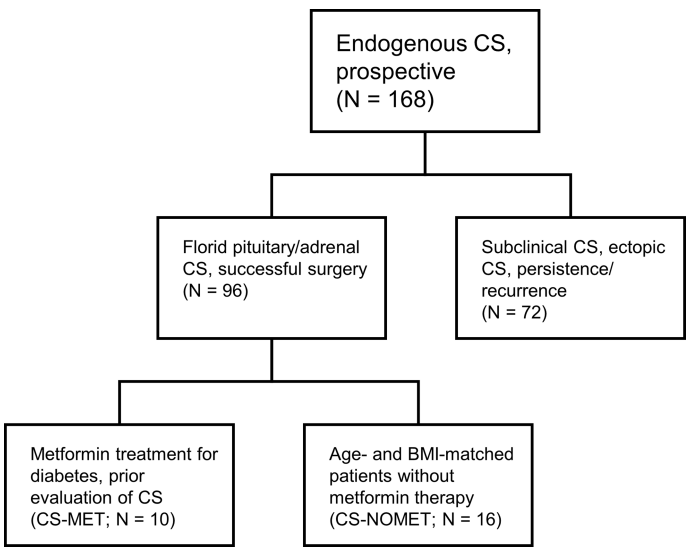

FIGURE 1 | Patient selection. CS, Cushing's syndrome; BMI, body mass index.

The samples were centrifuged within 20 minutes, stored at -80° and then measured on the iSYS automated analyzer (IDS-iSYS, Boldon, UK) by validated assays (16–18).

Bone Density and Muscle Strength Measurements

Bone mineral density (BMD) was measured at the lumbar spine and the femur (GE Lunar Prodigy Advance). Minimal T-Score was determined from both measurements using a gender-specific reference cohort as previously reported (14). BMD data at the time of diagnosis was available in 8 of 10 patients of CS-MET group and 13 of 16 patients of CS-NOMET group. For the assessment of muscle function, hand grip strength was measured three times on both hands per visit in a sitting position. The measurements were performed in a standardized manner with the JAMAR hydraulic hand dynamometer (Patterson Medical, Nottinghamshire, UK), as previously described (19). To adjust for age and gender (normalized grip strength) grip strength was standardized to the manufacturer's information on normative grip strength data (20).

Biometrics and Bio-Impedance Measurements

Bio-impedance and anthropometric measurements like BMI, waist-to-arm-ratio and waist-to-hip-ratio were performed by the same investigator in a standardized manner. Body cell mass and body fat percentage was estimated by using a bio-impedance measuring device at 50 kHz with 400 µA by Data Input (Poecking, Germany), according to the manufacturer's information. Two pairs of current-introducing and voltage-sensing electrodes were attached to the dorsum of hand and foot. All impedance measurements were taken after fasting, the arms relaxed at the sides without touching the body.

Quality of Life

To analyze quality of life in patients with CS, we used the disease-specific questionnaire Cushing's quality of life (CushingQoL) (21). In addition, for quantification of depressive symptoms, Beck's Depression Inventory was evaluated at the time of diagnosis and one year in remission of CS.

Statistical Evaluation

Statistical analysis was performed using SPSS (version 26). Patient characteristics are shown as median and 25th and 75th percentile in brackets. For comparison between baseline and follow-up Wilcoxon signed rank test was used. Differences between the groups were analyzed using Mann-Whitney-U-Test. *P*-values of ≤ 0.05 were considered to indicate statistical significance.

RESULTS

Patient Characteristics

Clinical and biochemical characteristics of the two patient groups are shown in **Table 1**, and anthropometric data is shown in **Table 2**. Cortisol concentrations in urinary free cortisol (UFC), late night salivary cortisol (LNSC) and 1 mg dexamethasone suppression test (DST) did not differ between the two groups at baseline and during follow-up (**Table 1**).

Diabetes, BMI, and Body Composition

At baseline, all 10 patients of CS-MET group had confirmed diabetes, compared to 4 of 16 in the CS-NOMET group and, thus, had higher HbA1c levels (p = 0.001, **Table 1**). No relevant difference between the two groups was present at baseline in terms of BMI, body fat percentage and estimated muscle mass by bio-impedance measurements (**Table 2**). One year after remission, BMI and HbA1c had decreased in both groups. Compared to CS-NOMET group, metformin-treated patients showed a reduction in body fat percentage following remission that was borderline significant (p = 0.050, **Table 2**).

Bone Mineral Density and Muscle Function

At the time of diagnosis, vitamin D serum concentrations were similar between groups, and no patient had bisphosphonate or

TABLE 1 | Baseline and 1-year follow-up characteristics of patients with CS ± metformin.

Patient Characteristics	CS with metformin (CS-MET, n = 10)			CS without metformin (CS-NOMET, n = 16)			
	Baseline	After surgery	*P vs.* BL	Baseline	After surgery	*P vs.* BL	*P**
Sex, female/male, n (%)	8 (80%)/2 (20%)	—	—	14 (87%)/2 (13%)	—	—	—
Diagnosis, pituitary/adrenal, n (%)	6 (60%)/4 (40%)	—	—	9 (56%)/7 (44%)	—	—	—
Age, years	59 [52; 64]	—	—	52 [39; 58]	—	—	0.165
Postmenopausal, n (% of female)	7 (88%)	—	—	10 (71%)	—	—	—
Vitamin D supplementation, n (%)	2 (20%)	—	—	8 (50%)	—	—	—
Metformin dose, mg per day	2000 [1000; 2000]	2000 [963; 2000]	0.458	—	—	—	—
Vitamin D, ng/mL	22 [16; 30]	25 [14; 39]	0.507	25 [18; 34]	32 [27; 42]	**0.017**	0.336
HbA1c, %	7.3 [6.9; 9.3]	6.3 [5.7; 6.7]	**0.005**	6.2 [5.7; 6.6]	5.5 [5.2; 5.9]	**0.002**	**0.001**
UFC, µg/24h	244 [163; 486]	20 [7; 36]	**0.018**	313 [138; 773]	24 [10; 38]	**0.001**	0.660
DST 1 mg	14.7 [7.3; 24.6]	—	—	11.3 [6.5; 18.1]	—	—	0.484
LNSC, ng/mL	6.0 [3.0; 10.0]	0.7 [0.5; 1.1]	**0.018**	4.3 [2.7; 7.2]	1.0 [0.6; 1.2]	**0.001**	0.660
ACTH in pituitary CS, pg/mL	60 [31; 93]	18 [11; 40]	0.173	69 [62; 118]	11 [8; 17]	**0.012**	0.328
ACTH in adrenal CS, pg/mL	4 [2; 5]	20 [13; 31]	0.068	4 [2; 5]	27 [9; 33]	**0.028**	0.927

*Data are given as median and 25th and 75th percentile in brackets. Bold p-values indicates statistical significance. *CS-MET vs CS-NOMET at baseline. Comparisons between baseline and follow-up were performed by a Wilcoxon signed rank test, comparisons between groups at baseline with Mann-Whitney-U-Test.*

CS, Cushing's syndrome; BL, baseline; HbA1c, hemoglobin A1c; UFC, urinary free cortisol; LNSC, late night salivary cortisol; DST, dexamethasone suppression test.

TABLE 2 | Anthropometric and musculoskeletal characteristics at baseline and 1-year follow-up of patients with CS ± metformin.

Patient Characteristics	CS with metformin (CS-MET; n = 10)			CS without metformin (CS-NOMET; n = 16)			
	Baseline	After surgery	*P vs.* BL	Baseline	After surgery	*P vs.* BL	*P**
BMI, kg/m^2	37 [29; 43]	33 [29; 36]	**0.013**	33 [31; 43]	31 [27; 35]	**0.002**	0.586
Waist-to-hip-ratio	1.1 [1.0; 1.2]	1.0 [0.9; 1.1]	0.213	1.0 [0.9; 1.1]	0.9 [0.8; 1.0]	**0.026**	**0.041**
Waist-to-arm-ratio	3.8 [3.2; 4.7]	3.6 [3.3; 3.9]	**0.037**	3.5 [3.2; 3.7]	3.2 [2.8; 3.3]	**0.039**	0.077
BMD lumbar spine (T-Score)	0.2 [-0.5; 2.8]	—	—	-1.1 [-2.0; 0.2]	—	—	**0.037**
BMD femur (T-Score)	-0.8 [-0.9; -0.2]	—	—	-1.3 [-1.8; -0.2]	—	—	0.238
Body fat, %	37 [28; 47]	31 [23; 42]	**0.050**	40 [34; 49]	36 [30; 37]	0.075	0.431
Muscle mass, kg	31 [23; 39]	30 [24; 32]	0.225	29 [22; 32]	29 [26; 32]	0.273	0.639
Grip strength, % of normal controls	95 [77; 113]	67 [54; 93]	0.169	84 [68; 99]	79 [50; 97]	0.079	0.363

*Data are given as median and 25th and 75th percentile in brackets. Bold p-values indicates statistical significance. *CS-MET vs CS-NOMET at baseline. Comparisons between baseline and follow-up were performed by a Wilcoxon signed rank test, comparisons between groups at baseline with Mann-Whitney-U-Test.*
CS, Cushing's syndrome; BL, baseline; BMI, body mass index; BMD, bone mineral density.

denosumab treatment. BMD in metformin-treated patients was higher compared to patients without metformin (median T-Score -0.8 in CS-MET group versus -1.4 in CS-NOMET, p = 0.030, **Figure 2**), and the concentration of the bone resorption marker β-CTX at baseline was lower in the CS-MET group than in the CS-NOMET group (p = 0.041, **Figure 3A**). PINP, a bone formation marker, showed no difference between the two groups (p = 0.201, **Figure 3B**). One year after successful surgery both bone markers strikingly increased, with no difference between CS-MET and CS-NOMET group. Differences in muscle function measured by grip strength did not reach statistical significance. However, patients with metformin had a trend to less muscular impairments during GC excess (**Table 2**).

DISCUSSION

This exploratory cohort study analyzed the metabolic effect of metformin intake at time of diagnosis in patients with endogenous GC excess. Our results suggest that metformin has a beneficial effect on bone metabolism during endogenous hypercortisolism.

In a recently published study we showed that during florid CS bone metabolism is characterized by decreased bone formation and increased bone resorption, followed by a strong activation of bone turnover after successful treatment inducing biochemical remission of CS (14). In the present study, metformin-treated patients had better BMD and lower serum β-CTX concentrations, indicating decreased bone resorption during hypercortisolism compared to patients with florid CS and no metformin therapy. Likewise, Pernicova and colleagues observed in their randomized study decreased bone resorption markers and increased BMD in metformin-treated patients compared to placebo-treated patients, all receiving exogenous glucocorticoids (5). The results of our study are in line with a beneficial effect on bone metabolism during GC excess and can be interpreted that also patients with endogenous CS may benefit from metformin administration. Decreased concentrations of the bone resorption marker β-CTX under metformin treatment

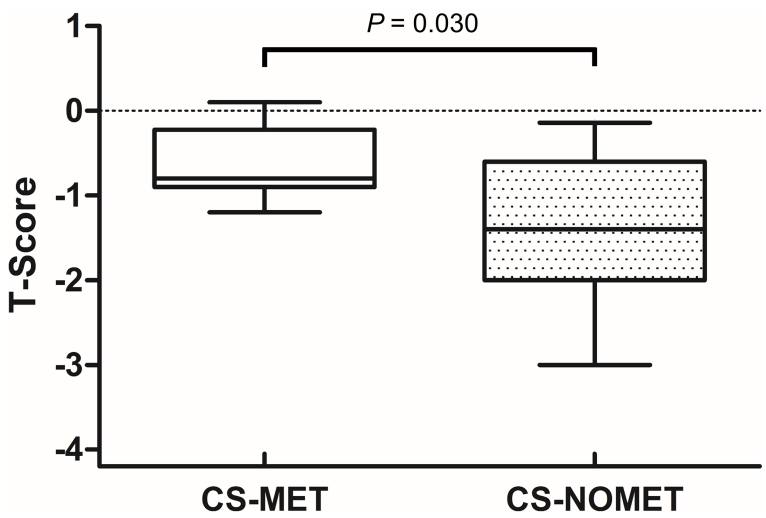

FIGURE 2 | Bone mineral density (T-Scores) in patients with florid Cushing's syndrome (CS) and pre-existing metformin therapy (CS-MET) or without pre-existing metformin (CS-NOMET). Boxplot = median and ranges of T-Scores. CS-MET: n = 8; CS-NOMET: n = 13. Comparison between groups by Mann-Whitney-U-Test; $p \leq 0.05$ was considered statistically significant.

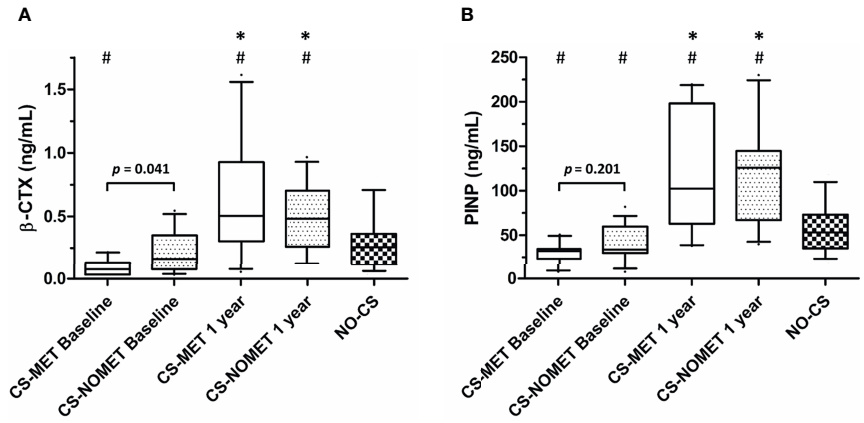

FIGURE 3 | Bone turnover markers during glucocorticoid excess and one year after surgically induced remission in patients with endogenous Cushing's syndrome (CS). Pre-existing metformin treatment (CS-MET): n = 10; no pre-existing metformin treatment (CS-NOMET): n = 16; patients with excluded CS (NO-CS): n = 95. **(A)** β-CTX = β-Crosslaps; **(B)** PINP = procollagen I-N-propeptide. Box and whiskers (10-90 percentile). Comparison between groups was performed by a Mann-Whitney-U-Test, between time points by a Wilcoxon signed rank test; $p \leq 0.05$ was considered statistically significant. $^{*}p \leq 0.05$ *versus* baseline; $^{\#}p \leq 0.05$ *versus* 'NO-CS'.

were already reported in patients with type 2 diabetes (22). Furthermore, metformin was shown to have osteogenic effects *in vitro* and *in vivo via* an increase in the expression of Runx2 and in the phosphorylation/activation of AMP-activated-protein-kinase (AMPK) (23, 24). Metformin was recently reported to improve the osteogenic differentiation potential of bone marrow-derived mesenchymal stem cells from patients with type 2 diabetes (25). However, metformin intake was as well associated with decreased bone formation markers, such as PINP, contrary to animal studies (22). Our study showed slightly lower PINP concentrations in the metformin-treated group, without reaching statistical significance. Whether metformin treatment is associated with a reduced risk of fractures remains controversial (26). Furthermore, metformin was shown to improve liver metabolism during GC excess (5), which in turn could have positive effects on bone health (27, 28).

In a recently conducted study on the long-term outcome of CS associated myopathy we identified age, HbA1c and waist-to-hip-ratio as predictors of myopathy outcome in patients with CS in remission (19). Despite a more pronounced hyperglycemic state (**Supplementary Table 1**), patients with metformin in our study showed a trend to less muscular impairment in grip strength during hypercortisolism (**Table 2**). Whether metformin administration generally blunts or improves exercise training-induced effects on skeletal muscle is controversial (29–31). Quality of life and depressive symptomatology revealed no significant differences between the two study groups (**Supplementary Table 1**). However, metformin showed antidepressant effects in larger cohorts and animal models (8, 32), and was even shown to correct abnormal circadian rhythm on a cellular level *via* an activation of AMPK (33). Mechanistically, the action of metformin is not yet fully understood. AMPK pathway is suggested to be a key mediator of glucocorticoid-metformin interaction (5, 34–36). In addition, metformin could show positive and protective effects during endogenous GC excess *via* an increase of serum insulin-like growth factor-I (37) and fibroblast growth factor 21 (38–40), and/or *via* a suppression of neuroendocrine tumor growth (9, 41, 42). However, the underlying mechanism behind a potential protection against glucocorticoid-associated adverse side-effects remains largely unknown.

So far, a potent agent reducing adverse GC side-effects is lacking. Further studies are required to investigate the effect during hypercortisolism and on long-term outcome and persistent symptoms such as myopathy, cardiovascular risk and cognitive disorders. Clearly, there is a need for a prospective randomized controlled trial on the effect of metformin in patients with endogenous CS.

Strength and Limitations of the Study

Because of the retrospective selection of patients with pre-existing metformin treatment at the time of diagnosis, groups differ regarding hyperglycemia. The interpretation of metabolic profiles should thus be done with caution. Another limitation is the small number of patients that follows from the rarity of the disease, and, due to the retrospective study design, the unknown duration of metformin therapy prior to diagnosis of CS. Although BMI was not significantly different between the two groups, increased truncal obesity in patients with metformin could positively affect BMD. On the other hand, however, diabetes is a known risk factor for osteoporosis *per se*, which further emphasizes the protective effect of metformin. Moreover, a strength of the study is the pre-existing metformin treatment, in the way that metformin intake already existed during the pathogenesis and development of endogenous GC excess.

CONCLUSION

This study supports the concept that metformin has a protective effect on bone metabolism in patients with endogenous glucocorticoid excess.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by LMU ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FV served as the principal investigator in this work and was responsible for the study conception and design, the analysis and interpretation of the data, and the drafting of the manuscript. GR, SZ, and AO contributed to the collection and analysis of the data. KS, RS, and MB substantially contributed to the interpretation of the data and the drafting of the manuscript. MR contributed to the conceptual design of the study, the collection, analysis and interpretation of data, and the drafting of the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.765067/full#supplementary-material

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Ketoconazole- and Metyrapone-Induced Reductions on Urinary Steroid Metabolites Alter the Urinary Free Cortisol Immunoassay Reliability in Cushing Syndrome

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Introduction: Twenty-four-hour urinary free cortisol (24h-UFC) is the most used test for follow-up decision-making in patients with Cushing syndrome (CS) under medical treatment. However, 24h-UFC determinations by immunoassays (IA) are commonly overestimated because of steroid metabolites' cross-reaction. It is still uncertain how ketoconazole (KTZ)- and metyrapone (MTP)-induced changes on the urinary steroid metabolites can alter the 24h-UFC*IA determinations' reliability.

Methods: 24h-UFC was analyzed by IA and gas chromatography-mass spectrometry (GC-MS) in 193 samples (81 before treatment, 73 during KTZ, and 39 during MTP) from 34 CS patients. In addition, urinary steroidome was analyzed by GC-MS on each patient before and during treatment.

Results: Before treatment, 24h-UFC*IA determinations were overestimated by a factor of 1.75 (95% CI 1.60–1.94) compared to those by GC-MS. However, during KTZ treatment, 24h-UFC*IA results were similar (0.98:1) to those by GC-MS (95% CI, 0.83–1.20). In patients taking MTP, IA bias only decreased 0.55, resulting in persistence of an overestimation factor of 1.33:1 (95% CI, 1.09–1.76). High method agreement between GC-MS and IA before treatment (R^2 = 0.954) declined in patients under KTZ (R^2 = 0.632) but not in MTP (R^2 = 0.917). Upper limit normal (ULN) reductions in patients taking KTZ were 27% larger when using 24h-UFC*IA compared to 24h-UFC*GC-MS, which resulted in higher false efficacy and misleading biochemical classification of 15% of patients. Urinary excretion changes of 22 urinary steroid metabolites explained 86% of the 24h-UFC*IA interference. Larger urinary excretion reductions of 6β-hydroxy-cortisol, 20α-dihydrocortisol, and 18-hydroxy-cortisol in patients with KTZ elucidated the higher 24h-UFC*IA bias decrement compared to MTP-treated patients.

Conclusion: KTZ and MTP alter the urinary excretion of IA cross-reactive steroid metabolites, thus decreasing the cross-reactive interference of 24h-UFC*IA determinations present before treatment. Consequently, this interference reduction in 24h-UFC*IA leads to loss of method agreement with GC-MS and high risk of overestimating the biochemical impact of KTZ and MTP in controlling CS because of poor reliability of reference ranges and ULN.

Keywords: cushing syndrome, urinary free cortisol, mass spectrometry, immunoassay, ketoconazole, metyrapone

1 INTRODUCTION

Endogenous Cushing syndrome (CS) is characterized by chronic high levels of circulating cortisol caused by either pituitary, adrenal, or ectopic tumors associated with high cardiometabolic morbimortality (1). The first-line treatment of all forms of endogenous CS is surgical resection of the primary tumor. However, tumor resection is often delayed, unsuccessful, or not feasible, resulting in more than 50% of patients with CS requiring medical therapy at some point during the follow-up of the disease (2, 3). Among medical treatments, steroidogenesis inhibitors (SEI) are the drugs most used in CS, reducing cortisol biosynthesis by inhibiting enzymes of the adrenal steroidogenic pathway. Ketoconazole (KTZ) and metyrapone (MTP) have been the most used SEI for decades (4). While KTZ blocks multiple steps of the adrenal steroidogenic pathway through the inhibition of numerous cytochrome p450 enzymes (5), MTP is a more specific inhibitor of the 11β-hydroxylase enzyme, which also blocks 18-hydroxylase to a minor extent (6).

In patients with CS under medical treatment with SEI, clinical follow-up decisions are primarily based on the biochemical control of the disease, which is defined by the normalization of cortisol levels (7). In this regard, twenty-four-hour urinary free cortisol (24h-UFC) is the biomarker most widely accepted to assess the patient's cortisol levels because it resembles time-integrated tissue exposure to free cortisol over a day; moreover, it is not susceptible to pulsatile secretion and circadian variability as well as changes in cortisol-binding proteins in the serum (8). However, in addition to free cortisol, the urine also contains abundant cortisol metabolites with similar chemical structures. As these metabolites share common antigenic epitopes with cortisol (ring-A cortisol metabolites), cross-reactivity and subsequently overestimated 24h-UFC results are expected when assessed by immunoassay (IA) methods (9). In healthy subjects and patients with active CS, 24h-UFC results obtained by several IA (8, 10–16) have been found to be overestimated by approximately 1.7- to 2.0-fold when compared with those obtained by mass spectrometry (MS), the gold standard method to assess 24h-UFC. Furthermore, serum cortisol determined by IA is also known to be greatly overestimated by MTP treatment due to the accumulation of the circulating steroid 11-deoxycorticosterone (11-DOC) (17–19). Nonetheless, it is unknown if the well-known cross-reactivity interference of 24h-UFC*IA determinations is modified in patients with CS during KTZ or MTP treatment. Moreover, the effects of KTZ and MTP in the urinary excretion of steroid metabolites and their impact on the 24h-UFC*IA bias are also unknown. Therefore, the aim of the present study was to assess how the urinary steroid metabolites changes in patients with CS during medical treatment with KTZ or MTP alter the 24h-UFC determination reliability of IA when compared to GC-MS.

2 METHODS

2.1 Study Design and Participants

The present cohort study included patients with a confirmed diagnosis of *de novo*, persistent, or recurrent endogenous CS (aged >18 years) (20) attended at Hospital Clínic de Barcelona from 2015 to 2019. Consecutive patients fulfilling inclusion criteria were enrolled in the study. Inclusion criteria were at least two adequate (**Supplementary Table 1**) 24-h urine samples before initiation of SEI treatment and at least two adequate 24-h urine samples during the maintenance phase treatment (≥3 months) with therapeutic doses of either KTZ (≥400 mg/day) or MTP (≥500 mg/day). Patients were included in the study and followed up until (1) CS remission was obtained from surgical excision of the tumor, or/and radiotherapy, or bilateral adrenalectomy; (2) death; or (3) December 31, 2020. Patients with CS due to adrenocortical carcinoma were excluded because of possible hypersecretion of multiple adrenal steroid precursors (21). The study design flowchart is shown in **Supplementary Figure 1**. The Institutional Research and Ethics Committees (CEIC) from Hospital Clínic de Barcelona approved the present study (HCB/2019/0179). Written informed consent was obtained from all patients prior to study inclusion. Baseline characteristics of patients are found in **Table 1**.

2.2 24h-Urinary Samples Selection

24h-urinary samples available during follow-up, including those before SEI, during SEI initiation below therapeutic doses, and while on treatment maintenance phase, were included for each patient. Periodicity of 24h-urinary samples depended on each patients' clinical need for a follow-up visit (1 month–6 months). 24h urinary samples were collected in a sterile container with no preservative, initiating after the first-morning void, storing it at 2–8°C during the gathering, and concluding with the first-morning void of the following day. An aliquot of 10 ml was preserved at −80°C until analysis. Ninety-five urinary samples from patients before treatment, 81 during KTZ, and 44 during

TABLE 1 | Baseline characteristics of patients at the start of treatment.

	Ketoconazole (*n* = 23)	Metyrapone (*n* = 11)	*p*-value
Age	46 ± 16	54 ± 15	0.104
Age diagnosis (years)	41 ± 16	54 ± 15	**0.025**
Time symptoms to diagnosis (months)	7 (4–15)	7 (2–12)	0.714
Time from diagnosis to metabolite assessment not using SEI	25 (0–89)	0 (0–24)	0.070
Cushing syndrome etiology			
Cushing disease (%)	17 (74)	6 (54)	0.200
Adrenal Cushing (%)	5 (22)	4 (37)	
Ectopic Cushing (%)	1 (4)	1 (09)	
Sex			
Female (%)	12 (52)	4 (36)	0.283
Male (%)	11 (48)	7 (64)	
Number of corrective surgeries			
None	11 (48)	7 (64)	0.901
1	10 (44)	4 (36)	
2	1 (4)	–	
3	1 (4)	–	
Use of SEI			
Neoadjuvant	12 (52)	7 (64)	0.513
Recurrence post-surgery	9 (39)	3 (27)	
Recurrence post-surgery post radiotherapy	2 (9)	1 (09)	
Basal line tests			
BMI	25 (23–35)	26 (24–27)	1.0
Glomerular filtrate (CKDPI) ml/min	103 ± 24	106 ± 13	0.982
AST (5–40 U/L)	17 (16–21)	21 (18–38)	0.198
ALT (5–40 U/L)	24 (17–33)	30 (24–38)	0.119
GGT (5–40 U/L)	22 ± 11	30 ± 12	0.714
Serum cortisol (10–25 µg/dl)	19 (16–32)	26 (19–33)	0.216
ACTH (10–60 pg/ml)	30 (16–84)	20 (10–35)	0.283
Late-night salivary cortisol (<1.56 µg/L)	4.5 (2.7–9.5)	7.4 (2.7–19.5)	0.659
24h UFC by IM (20–100 µg/day)	284 (191–795)	790 (293–1455)	0.304
24h UFC by MS (13–60 µg/day)	189 (88–510)	492 (182–1113)	0.150

Normal distributed variables are expressed as mean ± standard deviation. Non-parametrical variables are expressed as median (interquartile range). p-values come from independent t-test or Mann–Whitney U when appropriate. SEI, Steroidogenesis inhibitor; BMI, Body mass index; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; GGT, Gamma-glutamyl transferase; ACTH, Adrenocorticotropic hormone; 24h UFC, Twenty-four-hour urinary free cortisol.

The bold p-value indicates statistical significance (p < 0.05).

MTP were initially included. We excluded urinary samples that were not adequate (15), assessed by urine volume, creatinine excretion, and glomerular filtration rate (**Supplementary Table 1**). We also excluded urinary samples if the patient was taking any of the following medications within 1 month of the sample collection: corticosteroids; dopamine agonists; synthetic progestins and estrogens; somatostatin analogues; weight loss medications; strong CYP3A4 inducers (e.g., phenytoin and pioglitazone); strong CYP3A4 inhibitors (e.g., clarithromycin, conivaptan, and itraconazole); absorption interferents of KTZ (e.g., histamine H2 receptor antagonists and high doses of proton-pump inhibitors/sucralfate); drugs with systemic exposure increased by KTZ (e.g., HMG-COA reductase inhibitors); and inducers of QTc prolongation. A total of 193 samples were finally included in the analysis: 81 before SEI treatment, 73 during KTZ, and 39 during MTP.

2.3 Urinary Free Cortisol and Adrenal Steroid Profile Assessment

UFC*IA measurements were performed following routine methods in our hospital using a chemiluminometric (CM) IA (LIAISON, Diasorin, Italy) after a previous extraction of urine with dichloromethane. UFC*MS measurements were performed by GC–MS as previously described (15). Urinary adrenal steroid profile was measured in one 24-h urine specimen at baseline and one during SEI, following a procedure based on Shackleton et al. (22). The reference standards and the internal standards were obtained from Sigma (Steinheim, Germany), Steraloids Inc (Newport, USA), and NMI (Pymble, Australia). Steroids were extracted from urine with Sep-Pak C18 cartridges (Waters, Milford, MA, USA) and hydrolyzed with sulfatase (Sigma, Steinheim, Germany) and β-glucuronidase/arylsulfatase (Roche Diagnostics, Penzberg, Germany) overnight and re-extracted with Sep-Pak C18 cartridge. The extracts were derivatized with methoxyamine hydrochloride and trimethylsilylimidazole (23, 24). GC-MS analyses were performed on a Shimadzu GC-MS-QP2010 Ultra instrument. Steroids were separated on a Sapiens-5MS+ capillary column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness) from Teknokroma (Barcelona, Spain). The oven temperature conditions were as follows: started at 50°C, maintained at this temperature for 3 min, elevated at 80°C/min to 240°C, increased at 2°C/min until 290°C, and maintained for 4 min at 290°C. The ion source and transfer line temperatures were set to 270°C and 280°C, respectively. Extracts were injected splitless into the chromatographic system and the mass detector was operated in synchronous selected ion monitoring mode.

2.4 Statistical Analysis

Linearity, normality, homoscedasticity, and absence of multicollinearity were checked to use the appropriate comparative test. 24h-UFC*IA bias was calculated as 24h-UFC*IA/24h-UFC*MS. (1) 24h-UFC*ULN; (2) control status of the disease (<1.2 ULN = disease control or >1.2 ULN = uncontrolled); (3) 24h-UFC and 24h-UFC*ULN decrease (%) from baseline to maintenance therapy were calculated with 24h-UFC*IA and *MS. Pearson correlation, linear, and non-parametric Passing-Bablok regression analysis were used to compare the performance of IA and MS. Bland–Altman plots were used to test agreement between methods. Independent-samples tests were used to compare baseline characteristics. Pairwise comparisons (Paired *t*-test, Wilcoxon test, McNemar's test) were employed when testing binomial variables. Linear mixed models with unstructured repeated covariance were used to test for the main effects (maximum likelihood) of SEI (KTZ and MTP) and CS etiology as well as its interactions effects on the 24h-UFC*IA bias. Spearman correlations followed by polynomial regressions with stepwise method were performed to identify those metabolites independently associated with the 24h-UFC*IA bias. Fold changes (FC) were employed to assess the change on each metabolite from baseline to treatment.

Post-hoc comparisons were assessed with Bonferroni correction. Pairwise metabolite comparisons employing absolute concentrations during independent tests when appropriated were performed, adjusting each metabolite concentration to each patient's 24h-UFC*MS concentration to characterize the metabolite effect *per se*, independently of the hypercortisolemia severity. All the comparisons stated as different have statistical significance with *p*-value (two-sided adj. $p < 0.05$). Polynomial models were adjusted for age and sex when needed. Statistical analyses were performed using the R environment 4.1 and SPSS software version 27.

3 RESULTS

3.1 24h-UFC Performance by IA and GC-MS Before and During Treatment With SEIs

Before initiation of the medical treatment for hypercortisolism, 24h-UFC*IA determinations of patients were overestimated by 1.76 (95% CI, 1.60–1.94) when compared to 24h-UFC*GC-MS; however, both methods held a high linear relationship ($r = 0.977$, $R^2 = 0.954$) (**Figure 1A**). The 24h-UFC*IA bias was constant despite 24h-UFC concentrations (**Figure 1B**) and the agreement between methods was acceptable (**Figure 1C**). When patients were on treatment with KTZ, the 24h-UFC linear relationship between methods sharply decreased ($r = 0.795$, $R^2 = 0.632$). Moreover, 24h-UFC*IA results were no longer overestimated, giving similar values [0.98 (95% CI, 0.83–1.20)] to those obtained by GC-MS (**Figure 1D**) irrespectively from 24h-UFC concentrations (**Figure 1E**). During KTZ treatment, method agreement between IA and GC-MS was unacceptable as 25% of the 24h-UFC samples lay outside the methods agreement range (**Figure 1F**). These results were independent of CS etiology. Lastly, 24h-UFC*IA and GC-MS determinations from patients under MTP conserved high linear relationship ($r = 0.958$, $R^2 = 0.917$); however, it was significantly lower in patients with Cushing disease ($R^2 = 0.695$) than in patients with ectopic CS ($R^2 = 0.931$) and adrenal CS ($R^2 = 0.948$). 24h-UFC*IA determinations persisted, overestimated by a factor of 1.33 (95% CI, 1.09–1.76) compared with GC-MS results (**Figure 1G**) without differences between CS etiologies and 24h-UFC*IA concentrations (**Figure 1H**). Thirty-five percent of samples were outside the methods agreement range (**Figure 1I**).

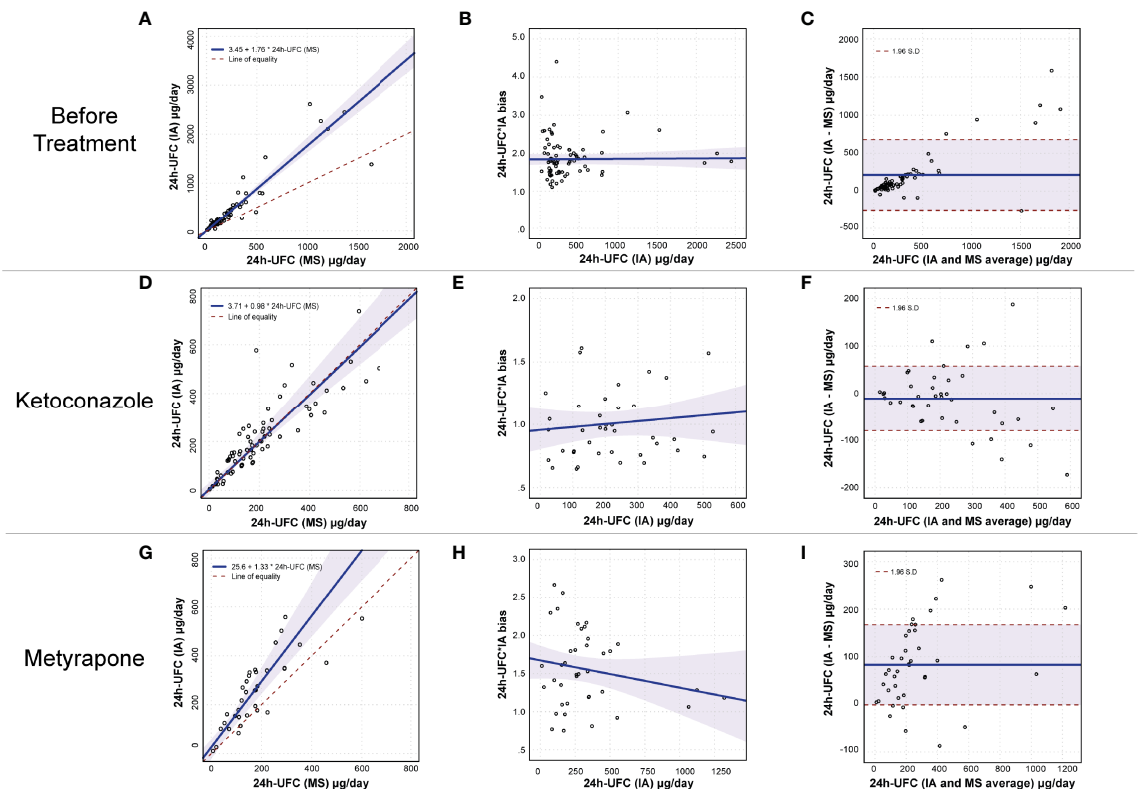

FIGURE 1 | 24h-UFC performance method comparison before and during SEI treatment. **(A, D, G)** Passing Bablok regression fit plot ($n = 81$, 73, and 39) between IA and MS before treatment, and during KTZ and MTP, respectively. The 0.95% confidence bounds are calculated with the bootstrap (quantile) method. **(B, E, H)** Regression fit plot for the 24h-UFC*IA bias (*y*-axis) against 24h-UFC*IA concentration, before treatment, during KTZ and MTP, respectively. 95% confidence bounds are calculated to the linear regression method. **(C, F, I)** Bland–Altman plots assessing method agreement on 24h-UFC measurement. 95% confidence bounds are calculated with the bootstrap (quantile) method. 24h-UFC, 24-hour urinary free cortisol; IA, Immunoassay; MS, Mass spectrometry.

3.2 Clinical Implications of the 24h-UFC Determination Differences Between IA and GC-MS

3.2.1 Before Treatment

Before treatment, mean cohort 24h-UFC concentration assessed by IA was 507.5 µg/day (Min–Max = 137–8343.6), whereas mean cohort concentration by GC-MS was 292.5 (Min–Max = 75.5–4,857.4), resulting in a 24h-UFC*IA bias of 1.84 (1.72–1.97). Within-subject variation of the 24h-UFC*IA bias before treatment was 17% (95% CI, 14–25) (**Figure 2A**). Pairwise comparison of the 24h-UFC*ULN determined by IA vs. GC-MS on each patient revealed no differences between methods (**Table 2**). ULN*MS – ULN*IA difference increased with higher 24h-UFC concentrations (r = 0.666, p =0.000) although no direction of association was found (**Figure 2B**). In all patients, there was congruency of both methods in the 24h-UFC*ULN biochemical status classification (p = 0.508).

3.2.2 During KTZ Treatment

24h-UFC*IA results were equally overestimated in patients taking low doses of KTZ (100–<400 mg/day) as before treatment. However, in patients taking therapeutic doses

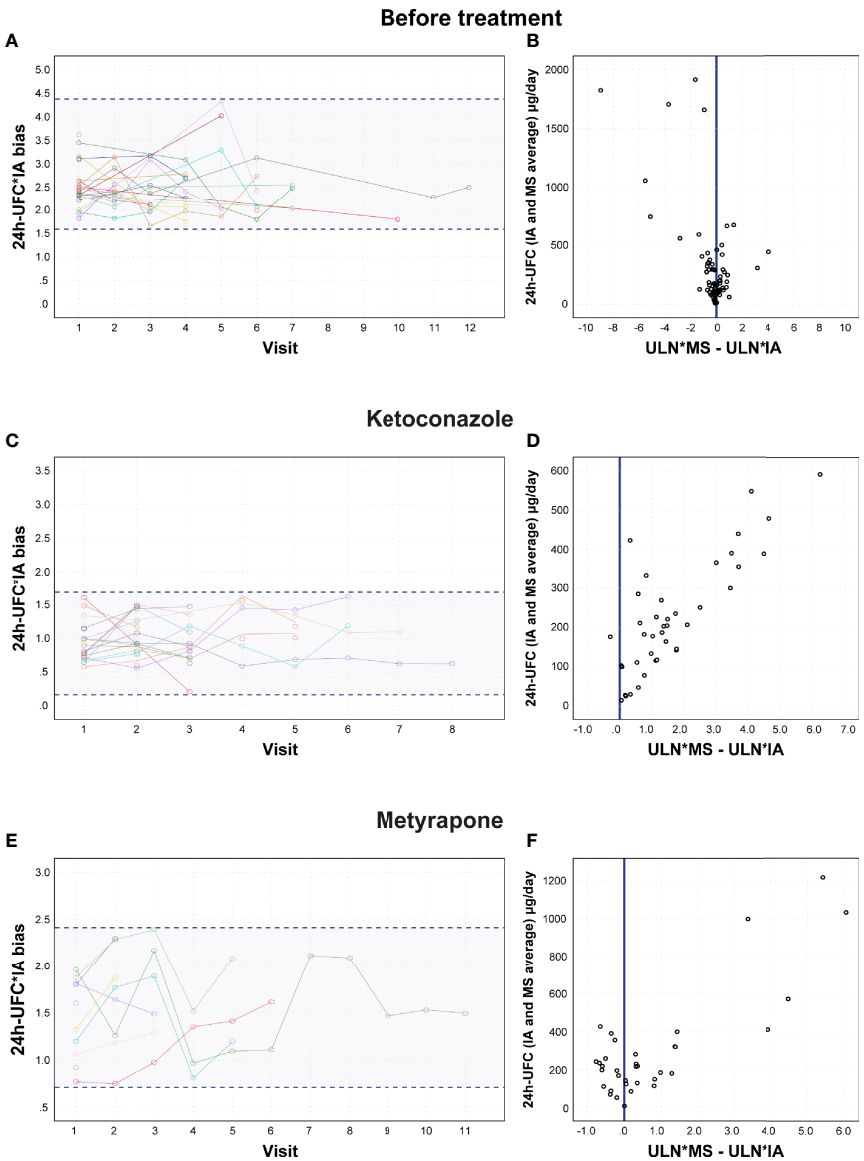

FIGURE 2 | 24h-UFC*IA bias variation and ULN differences between methods. **(A, C, E)** Within-subject 24h-UFC*IA bias variation between visits. Each line represents a patient with each node as the 24h-UFC*IA bias of the visit. **(B, D, F)** 24h-UFC ULN difference between methods association plot to 24h-UFC results. 24h-UFC, 24-hour urinary free cortisol; IA, Immunoassay; MS, Mass spectrometry; ULN, Upper limit normal.

TABLE 2 | Cortisol response differences between IA and MS.

	Before treatment (n = 81)		Ketoconazole^{TD} (n = 41)		Metyrapone^{TD} (n = 35)	
	IA	MS	IA	MS	IA	MS
24h-UFC*ULN	2.16 (1.37–4.48)	2.08 (1.25–4.22)	2.0 (1.13–3.13)	3.26 (2.04–5.29)	2.69 (1.56–3.7)	2.89 (1.84–4.86)
24h-UFC*ULN (MS–IA)	–0.12 (–0.55–0.25)		1.22 (0.57–2.39)*†		0.18 (–0.36–1.28)*†	
24h-UFC reduction %			63.1 (46.7–80.6)	32.7 (10.6–60.2)	40.1 (26.6–94.5)	38.9 (–38.7–93.3)
24h-UFC reduction % (MS–IA)			–27.9 (–41.4––12.2)*†		–0.4 (–50.9–8.8)†	
24h-UFC*ULN reduction			2.43 (1.09–6.29)	1.13 (0.4–3.52)	3.18 (1.08–10.2)	4.05 (–0.56–10.15)
24h-UFC*ULN reduction (MS–IA)			–1.18 (–2.92––0.56)*		–1.12 (–3.87–0.98)	
Creatinine urinary excretion (mg/24 h)	1,154.0 (947–1,308)		1,143.5 (873.5–1,294.5)		1,158 (1,030–1,375)	

*Cohort median (interquartile range) values are displayed. *p-value < 0.05 from two-related sample Wilcoxon test (Baseline–Treatment). †p-value < 0.05 from independent post-hoc intergroup comparison analysis (Bonferroni) between Ketoconazole vs. Metyrapone group. 24h-UFC, Twenty-four-hour urinary free cortisol; MS, Mass spectrometry; IA, Immunoassay; TD (superscript), Therapeutic dose ≥ 400 mg/day.*

(KTZ >400 mg/day) the 24h-UFC*IA bias decreased 0.94 (95% CI [0.69–1.24]) in a dose-dependent way (p = 0.000) (**Supplementary Figure 2**). Thus, 24h-UFC*determinations were similar to those by GC-MS with a minimum residual bias of 1.01 ± 0.30 (p = 0.000). KTZ explained 40% of the variance of the 24h-UFC*IA cross-reactivity interference (p = 0.000) and no interaction effect between KTZ and CS etiology was found (**Table 3**). The decrease in the 24h-UFC*IA bias was independent of treatment duration (p = 0.890). Within-subject variability of the 24h-UFC*IA cross-reactivity interference during the maintenance phase with KTZ was 17.1% (95% CI, 11–23) (**Figure 2C**). Comparison of 24h-UFC*ULN between methods in each patient revealed that ULN*IA was lower (p = 0.000) than ULN*MS (**Table 2**), which caused a discordant classification of the biochemical control status in 6 patients (15.0%, p = 0.035). ULN*MS – ULN*IA difference in patients taking KTZ (1.22 [0.57–2.39]) was larger than the one found in patients before treatment (p = 0.000) and kept incrementing as 24h-UFC concentrations increased (r = 0.791, p = 0.000) (**Figure 2D**). Improvements in hypercortisolism evaluated as the 24h-UFC reduction were falsely higher by 27.9% (12.2%–41.4%) when assessed by IA vs. GC-MS (**Table 2** and **Figure 3A**). These differences resulted in 10 patients (29%) having a >50% ULN reduction from baseline only when calculated with IA (p = 0.002). In patients with KTZ treatment, ULN reduction differences between methods were independent from baseline 24h-UFC concentrations (p = 0.812). Previous parameters during KTZ did not differ among CS etiologies.

3.2.3 During MTP Treatment

In patients taking MTP, the 24h-UFC*IA bias did not decrease in patients with ectopic CS and differently decreased in patients with Cushing disease than in those with adrenal CS (**Table 3**). 24h-UFC*IA determinations during MTP follow-up in patients with Cushing disease were overestimated by a factor of 1.64 (1.20–1.81), while in patients with adrenal CS, by 1.35 (1.09–1.61), both differing from those found on patients with ectopic CS, which were overestimated by 2.35 (1.97–2.61) (p < 0.01). Neither MTP dose (250 mg–3 g) nor treatment duration was associated with the 24h-UFC*IA bias decrease rate. Within-subject variability of the 24h-UFC*IA cross-reactivity interference during MTP treatment was 19% (95% CI [10–27]) (**Figure 2E**). Post-hoc analysis showed that MTP lowered the 24h-UFC*IA bias 0.55 less (95% CI [0.30–0.80]) than KTZ > 400 mg/day (p = 0.000). 24h-UFC*ULN comparison between methods in each patient revealed that ULN*MS was 0.93 higher (95% CI, 0.32–1.54]) than ULN*IA. ULN*MS – ULN*IA differences were larger than when not treated (p = 0.016) but not distinct than in patients with KTZ (p = 0.364). ULN*MS – ULN*IA differences were also larger with higher 24h-UFC concentrations (r = 0.626, p = 0.000) (**Figure 2F**). When comparing MTP efficacy between methods, ULN baseline

TABLE 3 | 24h-UFC*IA bias changes from baseline to maintenance phase of KTZ or MTP treatment.

Fixed effects	Estimate (95% CI)	Std. Error	t	Sig.
Ketoconazole				
KTZ	–0.969 (–1.248 – –0.691)	0.097	–8.632	0.000
KTZ * Cushing disease	0.02 (–0.539 – 0.58)	0.282	0.072	0.943
KTZ * adrenal CS	–0.07 (–0.829 – 0.688)	0.383	–0.184	0.855
KTZ*ectopic CS	–0.243 (–0.517 – 0.032)	0.139	–1.747	0.083
Metyrapone				
MTP	–0.555 (–1.211 – 0.141)	0.331	–1.678	0.096
MTP * Cushing disease	–0.716 (–1.23 – –0.201)	0.26	–2.756	0.007
MTP * adrenal CS	–0.946 (–1.432 – –0.459)	0.246	–3.852	0.000
MTP* ectopic CS	–0.230 (–0.575 – 0.015)	0.174	–1.322	0.189

*Estimates were calculated based on the maximum likelihood change from baseline 24h-UFC*IA bias. All results come from a generalized linear mixed model (LMM) with repeated measurements adjusted for age. Main effects were calculated for metyrapone and ketoconazole. Interaction effects between CS etiology and each medical treatment are also shown. 95% CI for the mean change. t and Sig. values from LMM. Std. Error, Standard error for estimate; Sig, Significance; KTZ, Ketoconazole; MTP, Metyrapone; CS, Cushing syndrome.*

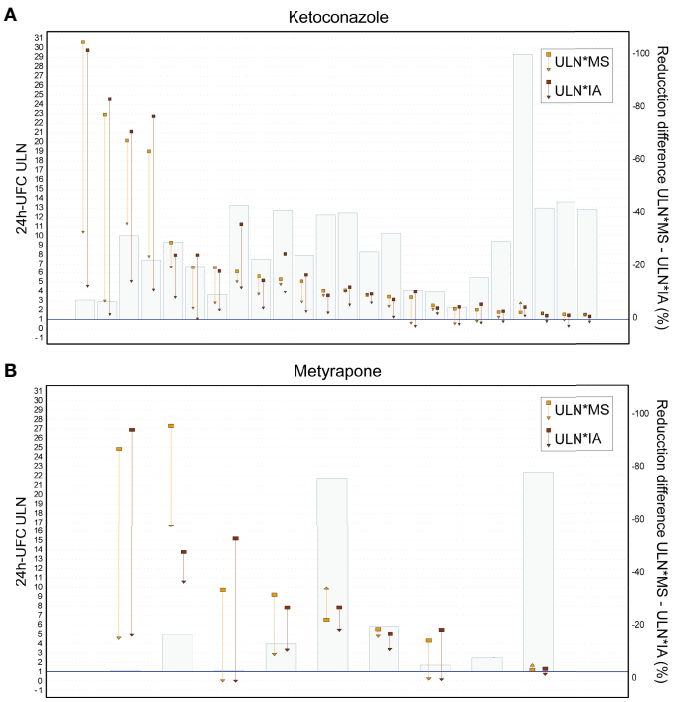

FIGURE 3 | 24h-UFC ULN reduction differences between methods. **(A)** Patients with Ketoconazole. **(B)** Patients with Metyrapone. Each patient's 24h-UFC ULN change is displayed by a pair of vertical arrows (IA and MS). Squares are baseline 24h-UFC ULN to triangles on end of maintenance phase. Bars are the difference % of the 24h-UFC ULN*MS–24hUFC ULN*IA. 24h-UFC, 24-hour urinary free cortisol; IA, Immunoassay; MS, Mass spectrometry; ULN, Upper limit normal.

reductions were >50% different between IA and GC-MS in some patients, while in others, similar reductions were found (**Figure 3B**). Because of the large variance between subjects treated with MTP, no statistical difference was found when performing pairwise comparison of each patient ULN and 24h-UFC decrement assessed by IA vs. GC-MS (**Table 2**).

3.3 SEI Treatment Induced-Changes on Urinary Adrenal Steroid Profile and Its Association With 24h-UFC Determination Differences Between Methods

Regardless of the CS etiology or medical treatment status, the urinary abundance of 22 metabolites explains the 86% of the 24h-UFC*IA cross-reactivity interference fluctuation (p = 0.000) (**Table 4** and **Figure 4**). 6β-Hydroxy-cortisol (6β-OH-cortisol) was the metabolite determining for most of the 24h-UFC*IA bias variability (R^2 = 48.3%, p = 0.000) followed by 20α-dihydrocortisol (20α-DHF) (R^2 = 24.4%, p = 0.000) (**Supplementary Figure 3**). The FC of 14 metabolites was associated with the decrease of the 24h-UFC*IA bias found in patients during treatment with SEI (**Table 4**). As 24h-UFC*IA determinations were less overestimated in patients taking KTZ than in those with MTP, we then searched for FC differences in the cross-reactive metabolites among treated groups. Baseline metabolites' concentration and during treatment with KTZ or MTP as well as mean FC are displayed in **Table 5**. Among GC cross-reactive metabolites, 18-OH-Cortisol and 20α-DHF concentrations decreased more than 50% in patients with KTZ, while no significant reductions were observed in those with MTP. Conjugated cortisol and corticosterone also declined in KTZ (FC = 0.72 and 0.63 respectively, $p < 0.01$) but not in patients under MTP. Moreover, 6β-OH-cortisol urinary excretion was reduced by 80% in patients under KTZ ($p < 0.001$) while no statistical change was found in patients with MTP. Higher KTZ doses were linearly associated with larger reductions of the GC cross-reactive metabolites 6β-OH-cortisol, cortisone, conjugated cortisol, 11-Oxo-etiocholanolone, and 11β-Hydroxy-androsterone; however, no association with MTP dose was found (**Supplementary Figure 4**). On the other hand, β-cortol and 5αTHF were the only GC metabolites that reduced in MTP (FC = 0.71 and 0.64) and not in KTZ (FC = 1.2 and 0.80). Moreover, a 90%–130% increase in concentration of GC precursors 17-OH-pregnanolone (17HP), pregnanediol (PD), and pregnanetriol (PT) was observed in patients after taking KTZ, while no difference was seen in patients after MTP (**Table 5**). However, mean increase from baseline concentrations in tetrahydro-11-deoxycortisol (THS) was over 400% in patients with MTP, while only a 95% increase was found in those under KTZ (**Table 5**). 5α-tetra-hydro-deoxycorticosterone was the only mineralocorticoid that increased as a result in patients with MTP with a mean concentration increment >300%. However, 5α-tetra-11-dehydrocorticosterone had a 50% augment in those with KTZ (**Table 5**). Portrayal of the urinary adrenal steroid changes by KTZ and MTP is displayed in **Figure 4**.

TABLE 4 | Adrenal steroid metabolites determining the 24h-UFC*IA bias.

	Linear regression model					FC correlations	
	β	95% CI		Std. Error	*p*	Correlation coefficient	*p*
		LL	UL				
Androgens							
Androsterone	–0.109	–0.144	–0.075	0.017	0.000	0.427 [8]	0.021
Etiocholanolone	0.092	0.059	0.124	0.016	0.000	0.357 [12]	0.042
Androgen precursor							
Pregnenediol	–0.693	–1.223	–0.163	0.259	0.012	0.096	0.615
16-Hydroxy-Dehydroepiandrosterone	–0.071	–0.117	–0.026	0.022	0.003	0.353 [13]	0.040
Glucocorticoid precursor							
Pregnanediol	0.116	0.065	0.166	0.025	0.000	0.305 [14]	0.046
Glucocorticoids							
11-Oxo-etiocholanolone	–0.039	–0.076	–0.002	0.018	0.041	0.450 [6]	0.013
11β-Hydroxy-androsterone	0.047	–0.008	0.101	0.027	0.092	0.288	0.122
Tetrahydrocortisone	0.008	0.002	0.014	0.003	0.012	0.193	0.306
Cortisone	–0.239	–0.359	–0.119	0.059	0.000	0.438 [7]	0.016
5α-Tetrahydrocortisol	0.037	0.021	0.052	0.008	0.000	0.381 [10]	0.042
β-Cortol	–0.035	–0.053	–0.017	0.009	0.000	0.231	0.220
β-cortolone	–0.054	–0.080	–0.029	0.013	0.000	0.567 [4]	0.002
α-cortol	0.041	0.015	0.067	0.013	0.003	0.366 [11]	0.047
6β-Hydroxy-cortisol	0.144	0.005	0.283	0.068	0.043	0.681 [1]	0.000
18-Hydroxy-cortisol	–0.089	–0.132	–0.046	0.021	0.000	0.471 [5]	0.011
20α-dihydrocortisol	0.013	0.006	0.019	0.003	0.001	0.594 [2]	0.001
20β-dihydrocortisol	0.083	0.034	0.131	0.024	0.001	0.570 [3]	0.002
Mineralocorticoids							
5α-Tetrahydrodeoxycorticosterone	0.349	0.196	0.502	0.075	0.000	0.395 [9]	0.034
Tetrahydrocorticosterone	–0.004	–0.009	0.000	0.002	0.076	0.176	0.362
5α-Tetra-11-dehydrocorticosterone	–0.487	–0.787	–0.186	0.147	0.002	0.144	0.456
5α-Tetrahydrocorticosterone	–0.129	–0.256	–0.003	0.062	0.045	0.080	0.682
Tetrahydroaldosterone	–0.104	–0.173	–0.034	0.034	0.005	0.046	0.313

*Multivariate linear regression model was adjusted for age and sex ($F = 8.138$, $p = 0.000$, $R^2 = 0.861$). Dependent variable of the model is the 24h-UFC*IA bias. β coefficient represents the degree of change in the metabolite concentration for every 1-unit of change in 24h-UFC*IA bias. 95% CI, confidence intervals for coefficient β. LL, Lower level. UL, Upper level. p-value and Std. Error for calculated β in the model.*

*Superscript numbers on the correlation coefficient of each metabolite indicate order of significance of association with 24h-UFC*Bias*

4 DISCUSSION

In the present study, we described how the unique changes induced by KTZ or MTP on the urinary steroid profile differently altered the performance of the 24h-UFC*IA in a cohort of patients with CS. We identified that IA bias overestimation of 75% found in patients before treatment decreased to almost 0% in patients taking KTZ, while it decreased to 33% in those with MTP therapy. Reductions of ULN were magnified by 27% when using IA compared to GC-MS. These results led to the false categorization of 15% of patients taking KTZ as biochemically controlled when using ULN*IA. Furthermore, 24h-UFC*ULN differences between methods were more significant in patients taking KTZ than MTP. Finally, we demonstrated that 86% of the extent of the 24h-UFC*IA cross-reactivity interference was explained by the abundancy variation of 22 steroid metabolites and that the different degrees of 24h-UFC*IA bias found in patients with KTZ vs. MTP were caused by distinct reductions of urinary metabolites like 6β-OH-cortisol.

Though our results are only based on the IA cortisol LIAISON Diasorin kit, studies employing other IA [Siemens ADVIA Centaur XP (17); Gamma Coat CA 1529, kit A and Spectria cortisol RIA, kit B (18), Roche CM cortisol IA (19), Access Cortisol IA (12)] have also found cross-reactivity interference of steroid metabolites in the cortisol determination. A recent letter by Perrin et al. (10) mentions that a CM microparticle IA using reagent ARCHITECT Cortisol (Abbott Diagnostics) is reliable for 24h-UFC follow-up in CS patients on SEI if the same technique is used before and during treatment. This statement should be taken carefully, as Abbot IA overestimated 24h-UFC by a factor of almost 2:1 in healthy controls and CS patients without any SEI treatment. In contrast, in patients taking KTZ or MTP, IA cross-reactivity interference was entirely abolished, giving similar 24h-UFC values to those obtained by LC-MS. These results suggest the same pattern of bias decrement in the IA during treatment with SEI as observed in our IA, which could lead to a similar false magnification of KTZ or MTP efficacy as observed in our patient. Establishing specific ranges of normality for patients taking SEI would not solve IA poor performance as we observed a non-linear decrease in the cross-reactivity interference that depended on KTZ dose. This would make it necessary to establish several limits of normal values. The wide intraindividual variation in the IA bias found even before treatment could be associated with day-to-day variability in the steroid secretion, as it occurs with cortisol (25, 26). In fact, like Wood et al. (16) using Coat-A-Count Cortisol and ADVIA

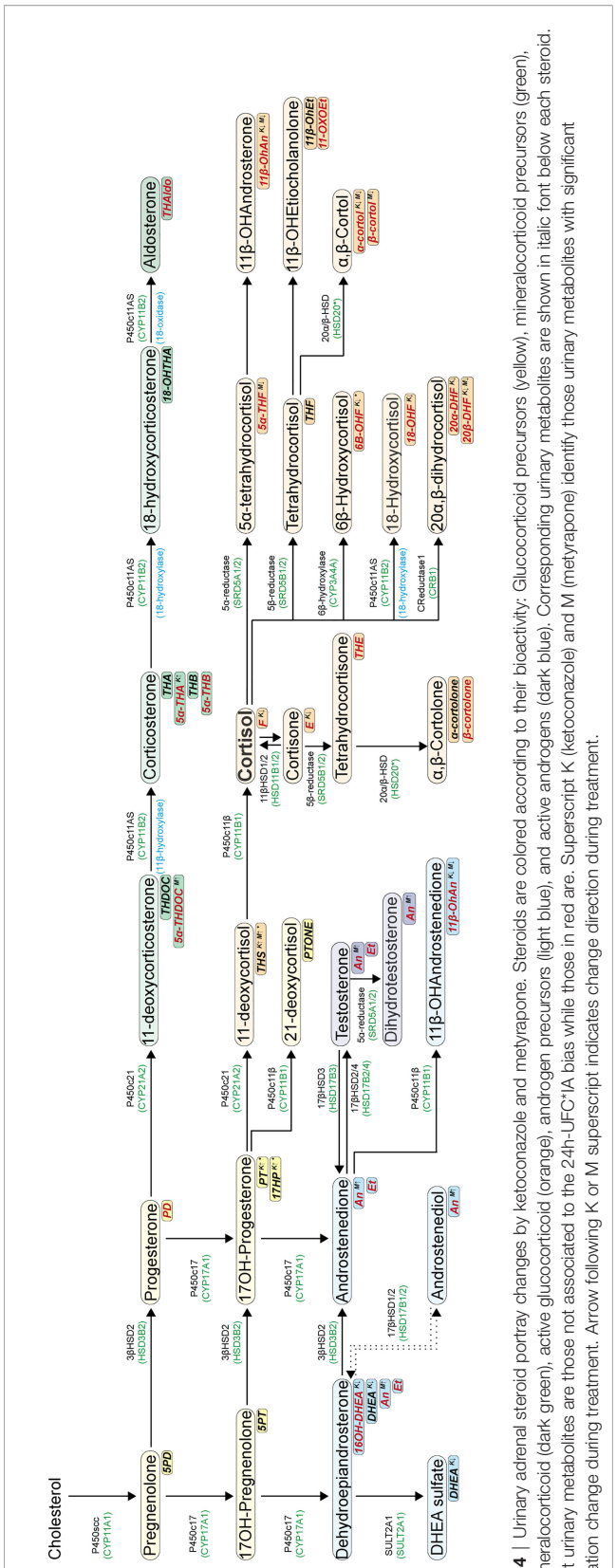

FIGURE 4 | Urinary adrenal steroid portray changes by ketoconazole and metyrapone. Steroids are colored according to their bioactivity: Glucocorticoid precursors (yellow), mineralocorticoid precursors (green), active mineralocorticoid (dark green), active glucocorticoid (orange), androgen precursors (light blue), and active androgens (dark blue). Corresponding urinary metabolites are shown in italic font below each steroid. Black font urinary metabolites are those not associated to the 24h-UFC*IA bias while those in red are. Superscript K (ketoconazole) and M (metyrapone) identify those urinary metabolites with significant concentration change during treatment. Arrow following K or M superscript indicates change direction during treatment.

Centaur IA, we found that the 24h-UFC*IA bias was not associated with the 24h-UFC concentrations but with the change in the urinary abundance of several metabolites. Nonetheless, Bianchi et al. (12) found higher interference with increasing 24h-UFC concentrations using the Access Cortisol IA, which corroborate performance variability among IA.

24h-UFC has been the most used biomarker to assess the normalization of cortisol secretion and, therefore, to study the efficacy of SEI (6, 27–32). Clinical trials and studies evaluating MTP efficacy (6, 29, 30) have employed MS to measure 24h-UFC. However, comparisons with studies that determined MTP efficacy by 24h-UFC*IA (31–33) and with centers following patients with IA would lead to false conclusions, as we found that ULN*MS tend to be 0.96 higher than ULN*IA, increasing with higher 24h-UFC concentrations. Contrary to date, no study (31, 34–41) has assessed KTZ efficacy employing MS in the 24h-UFC determinations. In our patients with KTZ, 24h-UFC reductions by IA were 27.9% larger than by GC-MS, resulting in 29% of patients having >50% reduction of the ULN and in 15% being classified as biochemically controlled, different to GC-MS results. Therefore, parameters resulting from 24h-UFC determinations should be carefully considered because even when ULN are normalized to each kit reference range of normality, the change in the bias that KTZ causes in each IA could lead to different results. However, we previously published that in terms of CS diagnosis, both methods present a very similar diagnostic value (15). However, a detailed analysis of the receiver operating characteristic curves pointed out that, at the same sensitivity, low levels of UFC are more specific when measured by IA; on the contrary, high levels of UFC, at the same specificity, are less sensitive when measured by IA. This suggests that 24h-UFC*IA might be more useful for CS screening and that 24h-UFC* might be more valuable for excluding CS.

Studies assessing serum cortisol cross-reactions (17, 19, 42) have led to the recommendation of using IA with high antibody specificity (10) without interference for adrenal precursors known to accumulate during KTZ (17-OHP) or MTP (11-DOC and 21-DOC) treatment (43, 44). However, we found that, in urine, the abundance of 22 adrenal metabolites determined 86% of the degree of IA interference, pointing out that cortisol IA determinations are interfered by several metabolites (45) rather than only by those stated in current practice guidelines for the use of SEI (3–5, 46, 47). In our group of patients, 6β-OH-cortisol, 20α-DHF, 20β-DHF, β-cortolone, and 18-OH-cortisol were the metabolites with more IA interference. Inhibition of 6β-hydroxylase by KTZ (48) produced an 80% decrease of 6β-OH-cortisol, whereas changes by MTP were not found. 20α-DHF and 18-OH-cortisol were also significantly reduced only in patients with KTZ in accordance with the inhibitory activity against 11β-HSD1 and P450c11AS (49). These unique changes explained most of the reduction difference in 24h-UFC*IA bias between KTZ and MTP, though other changes of precursor's metabolites were also associated. KTZ induced changes in the urinary excretion of 44% of the adrenal steroids, while it was 23% for MTP. As expected, accumulation over 100% of 17-OH-pregnanolone 17HP, PD, and PT was found in patients with KTZ (42, 44). 11-DOC

TABLE 5 | Urinary metabolites abundance changes during treatment with SEIs.

	Before treatment Concentration (µg/day)	Ketoconazole Concentration (µg/day)	Fold Change	Metyrapone Concentration (µg/day)	Fold Change
Androgen precursor					
16–Hydroxy–Dehydroepiandrosterone	55.4 (23–90.6)	26.5 (14.9–64.5)	0.56*	47.9 (28.8–93.3)	0.97
5–Pregnenetriol	314.6 (110.1–738.3)	512.7 (177.8–1191.2)	1.42	214 (77.7–750.1)	0.92
Dehydroepiandrosterone	58.2 (18–571.1)	30.4 (16–65)	0.62**	129.3 (32.8–1033.4)	0.99
Pregnenediol (5–pregnene–3b20a–diol)	58.1 (30.1–94.7)	63 (39.5–92.6)	1.04	47.7 (38–137.4)	0.97
Androgens					
Androsterone	1048.2 (612–1440.5)	986.4 (771.2–1621.5)	0.99	1456.7 (600–3191.7)	1.51*
Etiocholanolone	1290.1 (845.6–2163.7)	1810.3 (1269.4–2437.4)	1.02	740.4 (369.1–3265.3)	0.99
Glucocorticoid precursor					
17–OH–pregnanolone	374.5 (194.6–583.8)	875.6 (318.5–1469.3)	1.94**	361.6 (173.6–823.9)	1.06
Pregnanediol	327.1 (158.7–438.6)	627.5 (285.3–1010.6)	2.18***†	427 (90.4–769.9)	1.12
Pregnanetriol (PT)	507.3 (303.9–868.7)	1352.8 (645.4–2350.2)	2.35***†	430.8 (318.8–1396.4)	1.11
Pregnanetriolone	91.2 (46.9–184.7)	71.6 (41.1–169.9)	1.12	69.8 (33.1–349.9)	1.37
Tetrahydro–11–deoxycortisol	3187.7 (1013.1–7197)	7200.4 (2388–12352.8)	1.95*†	28993.3 (4405–100941)	4.57**
Glucocorticoids					
11–Oxo–etiocholanolone	784 (252.8–922.7)	856.5 (297.8–1144.6)	0.99	510.8 (116–1683.7)	0.87
11β–Hydroxy–androsterone	1046.4 (527.3–1853.8)	521.4 (362.3–864)	0.53**	496.3 (397.8–1113)	0.59*
18–Hydroxy–cortisol	602.2 (176.9–1487.8)	242.3 (134.2–410.1)	0.38**	203.7 (94.2–1054.2)	0.29
1β–Hydroxy–etiocholanolone	572.5 (240.2–916.3)	547.5 (397.7–1061.8)	0.92	446.2 (52.8–1324.5)	0.76
20α–dihydrocortisol	15993.8 (4917.5–32167.8)	4974.6 (3896.5–8183.1)	0.48***	6716 (2066.2–23829.3)	0.8
20β–dihydrocortisol	1976.1 (930.1–3565.9)	1053.4 (840.9–1721.8)	0.73**	1085.6 (513.2–2327.4)	0.59*
5α–Tetrahydrocortisol	3226.4 (1789.9–9248.7)	2626 (1215.4–4172.1)	0.8	4061.7 (1487.6–5269.6)	0.64*
6β–Hydroxy–cortisol	283.5 (125.6–740.3)	62.1 (31.3–109.8)	0.21***†	121.2 (42.9–297.1)	0.42
Cortisol	534.2 (301.2–1031.1)	405.5 (272.1–584.2)	0.72**	319.7 (243.7–867.1)	0.79
Cortisone	422.2 (222.8–974.4)	383.5 (197.6–476.7)	0.63**	389.2 (156.6–897.8)	0.81
Tetrahydrocortisol	8730.4 (4211.9–11508.9)	7900.8 (4106.4–11467.7)	0.8	6847.9 (2063–16499.7)	0.81
Tetrahydrocortisone	10023.2 (6042–18295.6)	12089.5 (6314.6–14920.3)	0.98	11672.8 (5140.2–17625.2)	0.81
α–cortol	4504.4 (2746.2–6551.9)	3326.8 (2555.5–5809.9)	0.72*	3146 (2137.7–10115.1)	0.81*
α–Cortolone	4048.1 (2805.5–5348.8)	3798.3 (2912.5–5032.4)	0.97	3707.6 (2521.8–7992.3)	0.96
β–Cortol	2051.7 (1094.3–3957.6)	2431.6 (1438.5–4362.9)	1.22	1588.9 (832.9–3668.3)	0.71*
β–cortolone	4015.6 (2786.1–5303.6)	3767.4 (2885.5–5014.8)	0.97	3675.6 (2499.6–7929.5)	0.95
Mineralocorticoids					
5α–Tetra–11–dehydrocorticosterone	55.8 (31.3–68.5)	88.5 (65.5–131)	1.5**	66.1 (46–114.8)	1.38
5α–Tetrahydrocorticosterone	601.3 (215.1–1198.7)	700.4 (399.1–1034.4)	0.92	498.1 (326.5–1224.3)	0.85
5α–Tetrahydrodeoxycorticosterone	76.5 (46.4–113.6)	106.2 (48.7–187.9)	1.38	133.6 (84.6–517.4)	3.52*
Tetrahydro–11–dehydrocorticosterone	167.5 (67–282.8)	153.2 (111.9–227.4)	1.34	121.8 (59–700.8)	0.89
Tetrahydroaldosterone	19.5 (8.3–56.9)	22.4 (9.3–53)	0.68	18.8 (7–45.1)	1.11
Tetrahydrocorticosterone	2500.7 (885.1–5867.4)	3894.8 (2129.9–14887.3)	1.47	2389.5 (649.4–4704.6)	0.72
Tetrahydrodeoxycorticosterone	85.1 (53.2–147.9)	120.8 (74.9–207.6)	1.55	122.9 (92.1–218.4)	1.4

*Before treatment concentrations are displayed as a unique group because no differences were found in any metabolite between other groups according to its treatment. *p < 0.05; **p < 0.01; ***p < 0.001 (Hochberg adjustment applied to p-values to control type 1 error at 0·05) from Wilcoxon or paired t-test were applied accordingly to distribution of the data. †p < 0.05 from Mann–Whitney U, comparing FC from patients under ketoconazole vs. metyrapone.*

metabolite THS was also augmented in patients with KTZ by 100%. Nonetheless, patients with MTP had urinary increments of over 300% of THS and 11-DOC metabolite 5α-THDOC, resembling the potent inhibition on the 11β-hydroxylase enzyme (7). MTP-treated patients also had an accumulation of androsterone with an excretion increase of 50%, supporting the documented accumulation of mineralocorticoid and androgens by MTP (28).

Limitations of the study arose from the differences in pre-analytical treatments, transition modes, and calibration of MS that introduce sources of variability in the cortisol measurement between centers. However, the use of standardized methodology across laboratories should tackle these inconveniences (9). On the other hand, the consistent results from a large number of samples of different medical treatment scenarios of patients with CS under KTZ or MTP are strengthened and point out the loss of reliability of IA in patients under SEI treatment when compared to the gold standard MS.

5 CONCLUSION

Different degrees of cross-reaction interference on 24h-UFC*IA determinations before treatment and during KTZ or MTP make IA less suitable for cortisol evaluation. The loss of bias brings overestimated reductions of 24h-UFC, magnifying the efficacy of the medical SEI. We encourage authors to be aware of the 24h-UFC method when comparing SEI efficacy results, as biased conclusions could occur when evaluating efficacy with IA. Moreover, clinicians should take into consideration that 24h-UFC*IA determinations are no longer overestimated in patients taking KTZ and that 24h-UFC*ULN are not reliable for patients

taking KTZ or MTP because IA reference range values take into consideration the cross-reactivity interference not present in patients under SEI. Finally, it is noteworthy to know that the IA cross-reaction interference can come from most urinary adrenal steroid metabolites rather than only by specific precursors and that MTP- and KTZ-induced specific changes in their excretion would distinctly affect IA methods.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Research and Ethics Committees (CEIC) from Hospital Clínic de Barcelona. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: FH, GC, and AV-B. Data curation: AV-B, FH, GC, DD-C, JL-M, LB, MM, and IH. Formal analysis: AV-B, GC, FH, DD-C, JL-M, and LB. Funding acquisition: FH. Investigation: All authors participated. Supervision: FH and GC. Writing—review and editing: All authors participated. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.833644/full#supplementary-material

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Metyrapone Versus Osilodrostat in the Short-Term Therapy of Endogenous Cushing's Syndrome: Results From a Single Center Cohort Study

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Background: Although surgery is considered the first-line treatment for patients with endogenous Cushing's syndrome (CS), medical therapy is often required to control severe hypercortisolism. Metyrapone and osilodrostat are both steroidogenic inhibitors targeting the 11β-hydroxylase, however, their therapeutic effectiveness has not yet been directly compared. This study aimed to evaluate metyrapone and osilodrostat in the short-term therapy of CS.

Methods: Retrospective analysis of patients with endogenous CS treated with metyrapone or osilodrostat as monotherapy for at least 4 weeks. Main outcome measures were serum cortisol and 24h urinary free cortisol (UFC) at baseline (T0) and after 2 (T1), 4 (T2), and 12 weeks (T3) of therapy.

Results: 16 patients with endogenous CS were identified (pituitary n=7, adrenal n=4, ectopic CS n=5). Each 8 patients were treated with metyrapone and osilodrostat. Despite heterogeneity, both groups showed comparable mean UFC levels at T0 (metyrapone: 758 µg/24h *vs* osilodrostat: 817 µg/24h; p=0.93). From T0 to T1, the decrease of UFC was less pronounced under metyrapone than osilodrostat (-21.3% *vs* -68.4%; median daily drug dose: 1000 mg vs 4 mg). This tendency persisted at T2 (-37.3% vs -50.1%; median drug dose: 1250 mg *vs* 6 mg) while at T3 a decrease in UFC from T0 was more pronounced in the metyrapone group (-71.5% *vs* -51.5%; median dose 1250 mg *vs* 7 mg). Under osilodrostat, a QTc-interval prolongation was identified at T3 (mean 432 ms vs 455 ms). From T0 to T2, the number of antihypertensive drugs remained comparable under metyrapone and decreased under osilodrostat (n= -0.3 *vs* n= -1.0).

Conclusion: Although both drugs show comparable therapeutic efficacy, osilodrostat seems to reduce cortisol levels and to control blood pressure faster.

Keywords: metyrapone, osilodrostat, Cushing's syndrome, hypercortisolism, medical therapy, blood pressure, isturisa, efficacy

1 INTRODUCTION

Endogenous Cushing's syndrome (CS) is a rare disorder with an incidence of 0.2–5.0 per million people per year (1). If the underlying glucocorticoid excess is not properly diagnosed and rapidly treated, it may lead to several comorbidities and increased mortality (2–4).

Surgery is considered the first-line treatment for patients with endogenous CS (2), e.g. transsphenoidal adenomectomy in Cushing's disease (CD) or adrenalectomy in case of cortisol-producing adrenal adenomas (CPA) or adrenocortical carcinomas (ACC). However, medical therapy is often required, e.g. to reduce perioperative risk, to control persistent hypercortisolism after surgery, or in case of advanced disease due to ectopic CS or ACC (5–7). Drugs that are typically used for this purpose are inhibitors of the adrenal steroidogenesis, glucocorticoid receptor blockers, and (in case of CD) somatostatin receptor ligands or dopamine receptor agonists (2). Among the adrenal steroidogenesis inhibitors, metyrapone and osilodrostat selectively inhibit the last enzyme of the cortisol biosynthesis, 11β-hydroxylase (CYP11B1), preventing the conversion of 11-deoxycortisol into cortisol. Metyrapone was first described in the 1950s and is still widely used today (8–11). Osilodrostat was approved by the European Medicines Agency (EMA) only recently, explaining why studies on its therapeutic efficacy are limited (6, 12–17). Furthermore, a direct comparison between metyrapone and osilodrostat has not yet been described.

The primary aim of this retrospective monocentric study was to compare the short-term efficacy of metyrapone and osilodrostat on cortisol levels in patients with endogenous CS.

2 SUBJECTS AND METHODS

2.1 Subjects

Patients with endogenous CS admitted to the University Hospital Würzburg were retrospectively reviewed. Those who were treated with metyrapone or osilodrostat as monotherapy for at least four weeks between December 2017 and December 2021 were considered eligible. CS was diagnosed according to established criteria (2, 18, 19). The investigated time points are visualized in **Figure 1**. Hormonal workup with basal serum cortisol (taken from 08:00 and 10:00 a.m.), serum cortisol after an overnight 1 mg dexamethasone suppression test (DST), and 24h urinary free cortisol (UFC) was performed in all patients before any medical treatment (baseline, T0). Furthermore, biochemical routine parameters (sodium, potassium, transaminases, creatinine, cholesterol, lipoproteins, triglycerides, leukocytes), blood pressure, and electrocardiogram were also evaluated at T0. Follow-up visits were carried out after 2 weeks (T1), 4 weeks (T2), and 12 weeks (T3) of therapy. Analysis of hormonal and biochemical routine parameters (electrolytes, transaminases, creatinine, and leukocytes) was performed at T1, T2, and T3. Cholesterol, triglycerides, and blood pressure were analyzed at T2. Electrocardiography was repeated at T2 and T3 (**Figure 1**).

All patients provided written informed consent to at least one of two disease-specific clinical registries, which were approved by the local ethics committee of the University Hospital of Würzburg (approval number 88/11 for the European Network for the Study of Adrenal Tumors registry and approval number 85/12 for the Network of Excellence for Neuroendocrine Tumors registry).

2.2 Methods

2.2.1 Hormonal Analysis

As previously performed (20, 21), commercially available analytical procedures were used for measurement of serum and salivary cortisol (the Immulite 2000 Xpi from Siemens), and for the analysis of UFC (a manual radioimmunoassay from Immuntech).

2.2.2 Electrocardiogram Analysis

For the analysis of the QTc-interval the Bazzett formula [QTc=QT/√(RR/1seconds)] was used.

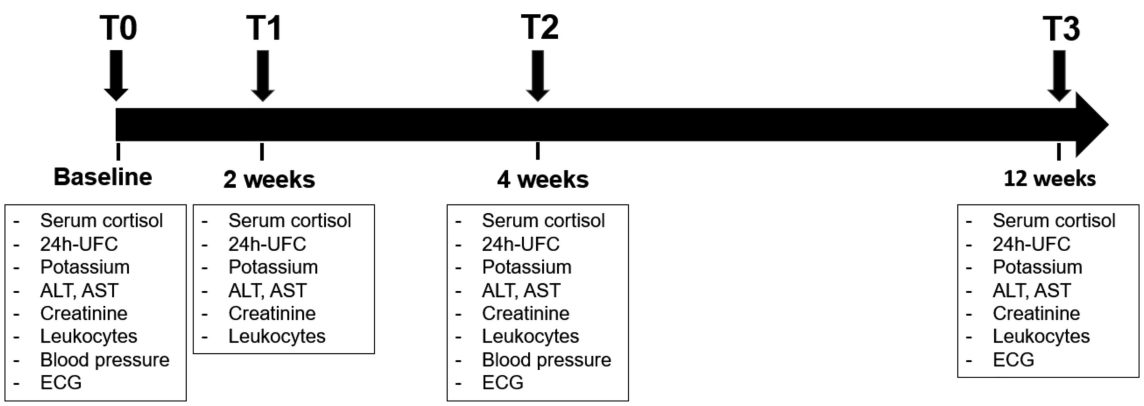

FIGURE 1 | Timeline of the study with description of follow-up visit. The data analysis was performed at baseline (T0), after 2 weeks (T1), after 4 weeks (T2) and after 12 weeks (T3). ALT, alanine transaminase; AST, aspartate aminotransferase; Crea, creatinine; ECG, electrocardiography; GGT, gamma-glutamyltransferase; K, potassium; LDL, low density lipoprotein; UFC, urinary free cortisol.

2.2.3 Statistical Analysis

Continuous variables were reported as mean ± standard error of mean (SEM) or as median with range, whereas categorical variables were provided as numbers and percentages. Data distribution was evaluated with the Shapiro-Wilk test. Parametric and non-parametric data were analyzed with Student´s T-tests and Mann-Whitney U test, as appropriate. Dichotomic variables were analyzed with the Fisher's exact test or the Chi-square ($\chi 2$) test. To compare the effect of metyrapone and osilodrostat on hormonal and biochemical parameters, a two-way repeated measures ANOVA was used. The delta (change) percentage from T0 to a subsequent study time point was calculated to evaluate the alteration of a parameter during the course of medical treatment with metyrapone or osilodrostat. A p-value < 0.05 was considered statistically significant.

Statistical Analysis was performed with SPSS version 26 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

3 RESULTS

3.1 Study Population

In total, 7 patients with CD, 5 patients with ECS, and each 2 patients with CPA and ACC were analyzed. The metyrapone population consisted of 2 patients with CD, 4 patients with ECS, 1 patient with CPA, and 1 with ACC. The osilodrostat population included 5 patients with CD, 1 patient with ECS, 1 patient with CPA, and 1 patient with ACC (**Table 1**). Except for the ACC patients who were previously treated with a platinum-based chemotherapy (etoposide, doxorubicin, cisplatin) along with mitotane, none of these patients had previous or concomitant drug therapy for hypercortisolism. In both ACC patients mitotane was suspended at least 2 months before starting with metyrapone or osilodrostat. Previous surgery was performed in 2 patients under metyrapone (each 1 with CD and ACC) and in 3 patients under osilodrostat (2 with CD and 1 with ACC). Prior radiotherapy was performed in 1 patient with CD under metyrapone. No significant differences were observed between the 2 groups considering sex, age, basal serum morning cortisol, serum cortisol after DST, UFC, and ACTH (**Table 1**). Clinical characteristics of the entire cohort of patients at T0 are summarized in **Table 1** and reported in **Supplementary Table 1**.

Mean time of therapy was 17.0 ± 3.4 weeks for the metyrapone group and 9.5± 1.1 weeks in the osilodrostat group ($p < 0.0001$).

Median drug dose in the metyrapone group was 1000 mg at T1 (number of patients, n = 7), 1250 mg at T2 (n = 8), and 1250 mg at T3 (n = 5). For osilodrostat, median dose was 4 mg at T1 (n = 6), 6 mg at T2 (n = 8), and 7 mg at T3 (n = 4).

3.2 Hormonal Values

In the metyrapone group, mean serum cortisol was 27.8 ± 5.5 μg/dL at T0 (normal range 5-25 μg/dl). During follow-up, mean serum cortisol was 21.0 ± 3.8 μg/dL at T1 ($p = 0.61$ compared with T0), 22.3 ± 2.0 μg/dL at T2 ($p = 0.67$), and 8.3 ± 2.5 μg/dL at T3 ($p = 0.007$) (**Figure 2A**). In the osilodrostat group, mean serum cortisol was 22.8 ± 3.5 μg/dL at T0, 21.1 ± 6.4 μg/dL at T1 ($p = 0.99$ compared with T0), 18.7 ± 4.3 μg/dL at T2 ($p = 0.82$) and 13.0 ± 1.6 μg/dL at T3 ($p = 0.44$) (**Figure 2A**).

Compared to T0, at T1 serum cortisol decreased by 4.9% in patients treated with metyrapone, and by 14.4% in patients

TABLE 1 | Clinical characteristics of the two study groups.

	Metyrapone (n=8)	Osilodrostat (n=8)	p value, χ^2
Females (%)	3 (37.5%)	7 (87.5%)	0.25, $\chi^2 = 1.33$
Age at therapy initiation	52.1 ± 3.8	50.1 ± 4.1	0.72
Cushing subtype			
Cushing's disease	2 (25%)	5 (62.5%)	0.38, $\chi^2 = 3.09$
Ectopic Cushing's syndrome	4 (50%)	1 (12.5%)	
Cortisol-producing adrenal adenoma	1 (12.5%)	1 (12.5%)	
Adrenocortical carcinoma	1 (12.5%)	1 (12.5%)	
Biochemical analysis			
Basal serum cortisol (μg/dl)	27.8 ± 5.5	22.8 ± 3.5	0.45
Serum cortisol after 1-mg dexamethasone suppression test (μg/dl)	27.3 ± 8.1	12.6 ± 5.7	0.24
ACTH (ng/l)	83.9 ± 28.3	38.8 ± 12.2	0.15
Urinary free cortisol (μg/d)	758 ± 309	817 ± 644	0.93
Late-night salivary cortisol	2.3 ± 1.1	0.9 ± 0.5	0.32
Potassium (mmol/l)	3.9 ± 0.3	4.2 ± 0.2	0.53
Blood pressure			
Systolic blood pressure (mmHg)	142.0 ± 7.6	139.4 ± 4.8	0.77
Diastolic blood pressure (mmHg)	83.5 ± 4.6	83.7 ± 4.1	0.97
Drug therapy			
Therapy duration (weeks)	17.0 ± 3.4	9.5 ± 1.1	0.07
Median dose at T1 -mg (range)	1000 (500-2000)	4.0 (3.0-7.0)	–
Median dose at T2 -mg (range)	1250 (500-2000)	6.0 (4.0-20.0)	–
Median dose at T3 -mg (range)	1250 (1000-2000)	7.0 (6.0-10.0)	–

Data are reported as total number and percentage, as mean value and SEM or as median value and range. ACTH, adrenocorticotropic hormone; SEM, standard error of the mean; T1, after 2 weeks of treatment; T2, after 4 weeks of treatment; T3, after 12 weeks of treatment.

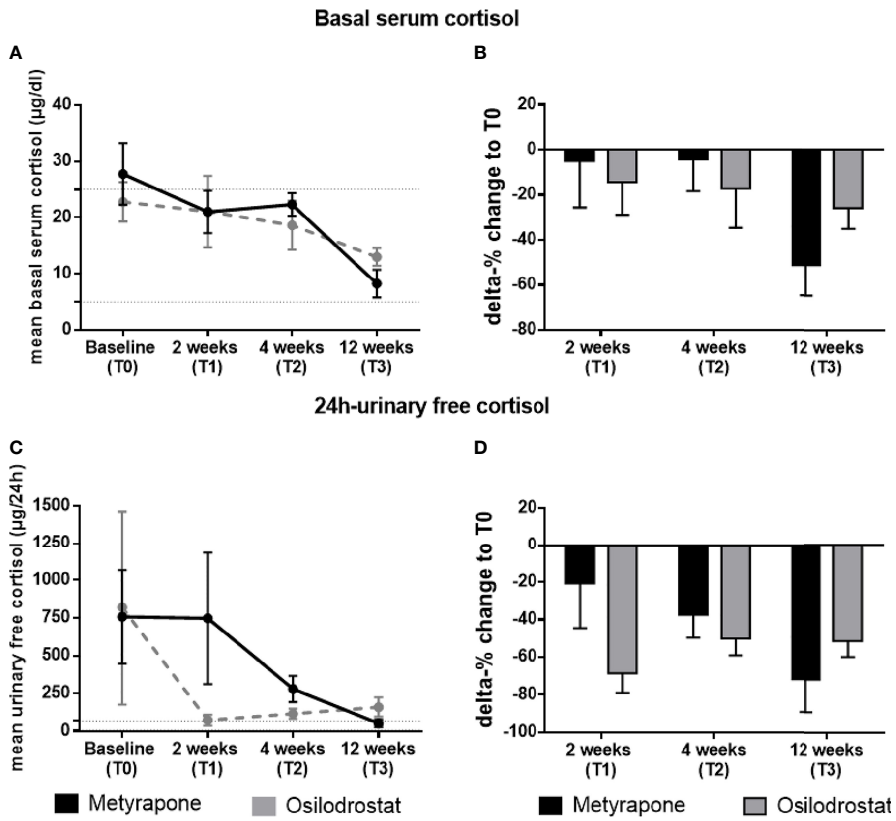

FIGURE 2 | Changes of basal serum cortisol and 24h-urinary free cortisol during the follow-up in patients treated with metyrapone or osilodrostat. Changes in absolute values **(A)** and delta percentage **(B)** of morning basal serum cortisol during metyrapone or osilodrostad treatment from T0 (baseline) to 2 weeks (T1), 4 weeks (T2) and 12 weeks (T3) of therapy. Changes in absolute values **(C)** and delta percentage **(D)** of 24h-urinary free cortisol during metyrapone or osilodrostad treatment from T0 (baseline) to 2 weeks (T1), 4 weeks (T2) and 12 weeks (T3) of therapy. Absolute values are reported with mean and standard error of mean (SEM). Normal range of serum cortisol and 24h-urinary free cortisol is reported within the dotted lines in **(A, B)**.

treated with osilodrostat ($p = 0.63$) (**Figure 2B**). This difference was comparable to the results at T2 (-4.2% for metyrapone and -17.2% for osilodrostat, always compared to T0, $p = 0.57$). At T2, serum cortisol levels were below 25 µg/dL in 5/8 (62.5%) patients under metyrapone and in 5/8 (62.5%) patients under osilodrostat. At T3, a more pronounced decrease of cortisol was found in the metyrapone than in the osilodrostat group (-51.1% *vs* -25.8%, always compared to T0, $p = 0.23$; **Figure 2B**). At this last time point, all patients under metyrapone (5/5) and under osilodrostat (4/4) presented with a morning serum cortisol below 25 µg/dl.

Mean UFC (normal range 0-70 µg/d) in the metyrapone group was 758 ± 309 µg/d at T0. At T1, T2 and T3 was 748 ± 434 µg/d ($p = 0.99$ compared to T0), 281 ± 87 µg/d ($p = 0.73$) and 53 ± 25 µg/d ($p = 0.62$). On the other hand, mean UFC under osilodrostat at T0 was 817 ± 644 µg/d. During follow-up, UFC levels were 74 ± 36 µg/d at T1 ($p = 0.44$), 117 ± 34 µg/d at T2 ($p = 0.41$) and 131 ± 55 µg/d at T3 ($p = 0.55$) (**Figure 2C**). From T0 to T1, UFC decreased more, but not significantly, in the osilodrostat group than in the metyrapone group (-21.3% *vs* -68.4%, $p = 0.15$) (**Figure 2D**). Comparing the two groups directly from T0 to T2, both groups showed a more comparable decrease of UFC (-37.3% under metyrapone *vs* -50.1% under osilodrostat, $p = 0.59$). At this time point, 0/6 patients treated with metyrapone and 3/7 patients (42.9%) under osilodrostat had a normalized UFC. At T3, the delta change of UFC from baseline was more pronounced in the metyrapone group (-71.5% *vs* -51.5%, $p = 0.40$) (**Figure 2D**). Moreover, 2/3 patients (66.7%) under metyrapone had a normalized UFC, compared to 2/4 patients (50%) of the osilodrostat group.

In order to prevent adrenal insufficiency, a "block and replace" therapy with hydrocortisone was initiated in a subgroup of patients. In the osilodrostat group, 2/8 patients received hydrocortisone at T2, facing 3/8 patients at T3. None of the patients under metyrapone received hydrocortisone at T2, whereas 3/5 patients had hydrocortisone at T3. Of note, no difference in UFC levels was identified by including or excluding patients with block and replace therapy in the analysis.

3.3 Blood Pressure and Antihypertensive Drugs

A reduction of both systolic and diastolic blood pressure was observed at T2 compared to T0 in the osilodrostat group at T2

(systolic -3.7%, mean 134.2 ± 5.7 mmHg, p = 0.07; diastolic -16.2%, 69.2 ± 6.6 mmHg, p = 0.07), whereas in the metyrapone group the systolic pressure did not change relevantly and the diastolic slightly increased (systolic +0.5%, 142.5 ± 6.9 mmHg, p = 0.12; diastolic +4.9%, 88.7 ± 4.2 mmHg, p = 0.35) (**Figures 3A, B**).

The effect of osilodrostat on the blood pressure allowed a mean reduction of one antihypertensive drug at T1 (**Figure 3C**).

3.4 Adverse Events

Metyrapone was discontinued after 4 weeks in 2 patients (both ECS) because of adverse events (asthenia and dizziness). 1 patient under metyrapone was lost to follow-up before T3. In the osilodrostat group, the therapy was discontinued in 1 patient with CD at T2 because of adverse events (depression, asthenia, and nausea), and in 2 additional patients after tumor resection (1 CD and 1 ECS). Another patient under osilodrostat did not show up at T3 for unknown reasons and was lost to follow-up.

At T1, 1 patient under metyrapone and one under osilodrostat required potassium replacement therapy. At T2 and T3, two patients under metyrapone and 3 patients under osilodrostat required potassium replacement therapy. Analysis of potassium levels was performed only in patients without potassium replacement therapy. No significant differences in potassium levels were identified at T1 and T2. However, at T3, no substantial changes in potassium levels was identified in patients under metyrapone (-1.5% from T0, 4.3 ± 0.4 mmol/L, p = 0.99), while in the osilodrostat group an increase (+9.6% from T0, 4.7 ± 0.1 mmol/L, p = 0.43) was detected.

As reported in **Figure 4**, a progressive increase of the QTc-interval was identified in the osilodrostat group, but not in the metyrapone one (455 ± 23 ms vs 432 ± 3 ms). Of note, in 1 patient under osilodrostat it was necessary to interrupt the therapy at T3 because of a QTc of 503ms.

Regarding aspartate aminotransferase (AST) and alanine aminotransferase (ALT), no substantial differences between the metyrapone and osilodrostat group were observed at T1, T2 and T3 (**Supplementary Figures 1A, B**).

We observed a not clinically relevant increase in creatinine levels in both groups (at T3 from T0, +2.4% under metyrapone, *vs* +15.3% under osilodrostat; **Supplementary Figure 1C**).

4 DISCUSSION

We performed a retrospective analysis of patients with CS comparing the short-term effects of metyrapone and osilodrostat on hypercortisolism. Our data suggest that osilodrostat could reduce cortisol levels more rapidly than metyrapone, thereby allowing a better blood pressure control. Nevertheless, adverse effects like QTc prolongation under osilodrostat need to be carefully evaluated during therapy.

The efficacy of osilodrostat in different forms of CS was highlighted in previous studies, with a cortisol-normalization achieved 15 to 44 days after treatment initiation (6, 12–14, 16). In few of these cases, however, patients were previously or concomitantly treated with other drugs for hypercortisolism (13, 17). In our cohort, a reduction in UFC was obtained already after 2 weeks of therapy with osilodrostat, with a normalization of cortisol levels after 4 weeks in 42.9% of patients. This was achieved with a relative low dose of osilodrostat (6 mg/day), considering that a mean dose of 10 mg/day was reported in more than half of the patients in the phase III LINC3 trial (6). On the contrary, the metyrapone-dose was relatively high compared with a previous prospective study, in which mean doses of 750 mg and 1000 mg were reported after 1 and 3 months of treatment (8). Although the applied drug dosages of osilodrostat and metyrapone could not be directly compared, with this study we demonstrated that cortisol could be normalized with a relatively low dose of osilodrostat. Moreover, a faster decrease of UFC was achieved with osilodrostat, indicating that osilodrostat might have a superior short-time efficacy compared to metyrapone. However, this result needs to be further validated in larger (ideally prospective) studies.

The current study illustrated that metyrapone has an increasing efficacy over time. In detail, after a mild decrease of cortisol levels after 2 weeks and 4 weeks, an impressive reduction

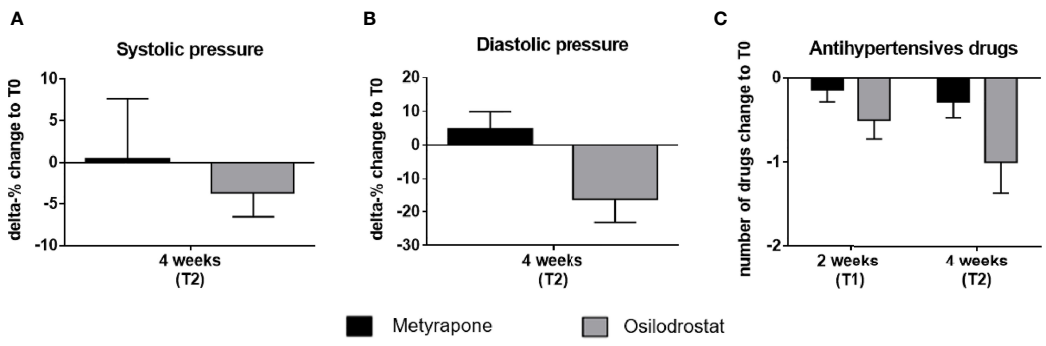

FIGURE 3 | Delta percentage of systolic and diastolic blood pressure and change in number of antihypertensive drugs under metyrapone or osilodrostat treatment during follow-up compared with baseline. Changes in percentage from T0 (baseline) of **(A)** systolic, **(B)** diastolic blood pressure after 4 weeks (T2) of metyrapone or osilodrostat therapy compared with baseline (T0). **(C)** Changes in number of anti-hypertensive drugs after two weeks (T1) and 4 weeks (T2) of treatment in comparison to baseline.

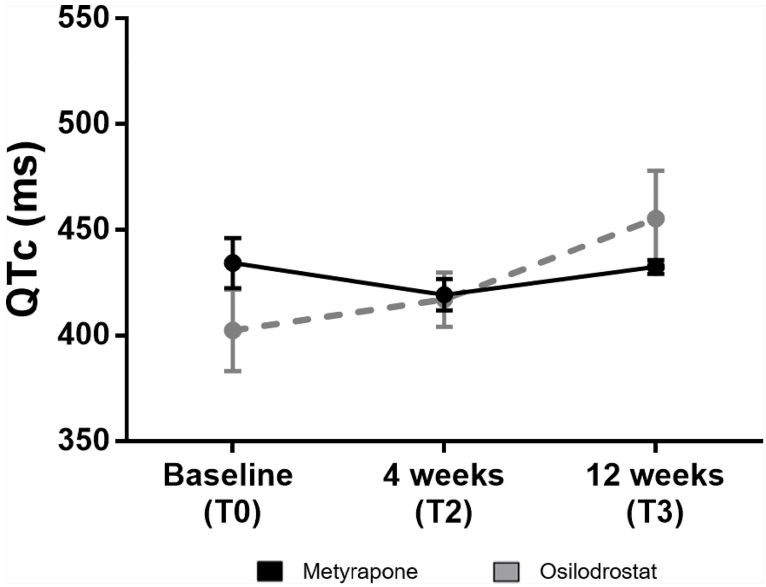

FIGURE 4 | QTc-interval at baseline and during follow-up under metyrapone or osilodrostat treatment. Changes in absolute values of QTc-interval in electrocardiography under metyrapone or osilodrostat treatment. Electrocardiogram and QTc-interval analysis was performed at 4 weeks (T2) and at 12 weeks (T3). Values are reported with mean and standard error of mean (SEM).

in UFC and a normalization of cortisol levels in 66.7% of the patients were identified after 12 weeks of therapy. This is in accordance with a previous report, where a 70% cortisol normalization rate was observed after 3 months (8).

During follow-up, we found a more pronounced decrease in both systolic and diastolic blood pressure under osilodrostat compared to metyrapone. A lower number of antihypertensive drugs under osilodrostat was observed after 4 weeks of treatment. In line with a previous study (8), metyrapone did not show a significant impact on blood pressure.

Hypokalemia is a well-known adverse effect of both metyrapone and osilodrostat. In fact, the inhibition of CYP11B1 indirectly causes an increase in steroid precursors with mineralocorticoid activity (6, 8). Aware of this adverse effect, we routinely performed potassium controls, and 2 patients under metyrapone and 3 patients under osilodrostat received an oral potassium replacement therapy. However, none of the patients presented severe hypokalemia (potassium <2.5 mmol/L). In patients receiving oral supplementation, potassium levels increased to the normal range.

A QTc-interval prolongation was described to be a relevant adverse event of osilodrostat, affecting 4% of the patients (6). In the present analysis, the QTc-interval increased over time as well. After 12 weeks of therapy, a mean QTc-interval of 455 ms was identified; in 1 patient, drug discontinuation was necessary due to a QTc interval of 503 ms. Accordingly, periodical controls with ECG under osilodrostat are recommended to identify relevant QTc prolongations. Of note, no significant QTc-interval prolongation was observed under metyrapone.

In the LINC 3 study, 4% of the patients showed an increase in ALT or AST under osilodrostat treatment (6). In our small study, no increase of transaminases during osilodrostat therapy was detected. This discrepancy could be due to the relatively lower dosage of osilodrostat that was used in our patients. Although hepatically metabolized, metyrapone it is not known to induce hepatic injury (2).

The current analysis has certainly relevant limitations. First, due to the rareness of CS and the very recent approval of osilodrostat by the EMA, the number of patients in both treatment groups is still very low and, therefore, the power for statistical comparisons is limited. Additionally, some patients were lost to follow-up or interrupted the therapy so at T3 only a reduced amount of patients was analyzed. Second, a retrospective design is always prone to bias and, obviously, no standardized management (e.g. regarding the dosage and follow-up visits) was implemented. This approach might have underestimated the adverse events. Third, both groups were inhomogeneous in terms of CS subtypes and clinical characteristics (although serum cortisol and UFC were comparable).

Nevertheless, to our knowledge, this is the first study that directly compared metyrapone and osilodrostat as short-term therapy of endogenous CS. The present analysis is, therefore, relevant for daily clinical practice, facilitating the choice of a certain steroidogenesis inhibitor when a prompt decrease of cortisol levels is indicated. Osilodrostat might be superior in rapidly reducing cortisol excess, but also clinical parameters like blood pressure. However, careful monitoring of the QTc intervals is required.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University Hospital of Würzburg (approval number 88/11 for the European Network for the Study of Adrenal Tumors registry and approval number 85/12 for the Network of Excellence for Neuroendocrine Tumors registry). The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MD and BA designed the research. MD, BA and UD performed the statistical analyses and drafted the manuscript. All authors collected samples and clinical data from patients, contributed to writing the manuscript, and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.903545/full#supplementary-material

Supplementary Figure 1 | Delta percentage of transaminase and creatinine under metyrapone or osilodrostat treatment during follow-up compared with baseline. Changes in percentage from T0 (baseline) of transaminases and creatinine throughout the study. Follow-up was performed at 2 weeks (T1), 4 weeks (T2) and 12 weeks (T3). ALT, alanine transaminase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase.

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PERSPECTIVE
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Cushing's Disease Management: Glimpse Into 2051

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Major advancements are expected in medicine and healthcare in the 21st century- "Digital Age", mainly due to the application of data technologies and artificial intelligence into healthcare. In this perspective article we share a short story depicting the future Cushings' Disease patient and the postulated diagnostic and management approaches. In the discussion, we explain the advances in recent times which makes this future state plausible. We postulate that endocrinology care will be completely reinvented in the Digital Age.

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Keywords: Cushings disease, circadian rhythms, digital, future, technology development

SHORT STORY: THE MIDNIGHT SUN

23 June 2051
00:00:
Sound exposure: limited to the breathing sounds & background noise
Light exposure: no blue light exposure.
Heart Rate: Normal
Systolic Blood Pressure: Trending higher at night
Diastolic Blood Pressure: Trending higher at night
Glucose: Trending higher
Hypnograph: Stage 3/Stage 4 sleep.
Stress level: "High"
Cortisol Level: "High" from 00:00 to 24:00
23 June 2051
06:00:
The alarm gently rings and plays "Good morning".
Sunlight creeps through the curtains and fills the room with warmth and light.

Claire awakens, rubbing her eyes seeing the analytics of the previous day affirming good productivity and excellent sleep patterns. A red notification blares on the side indicating high stress levels throughout the night, tense muscles around the head and forecasting a feeling of headache that may prop up in the day, suggesting a dose of painkiller before work. The weather outside is reported as very good with no rains and work schedule is displayed after that.

The living room and kitchen is spick and span; the bath water exactly 37 degrees Celsius, the day's clothes laid out- creaseless and ironed. Breakfast table is laid with 2 eggs, sunny side up.

The self-driving ride is on time with the first meeting on the way to work. The planned day runs smoothly. The bank account at 15:00 hours shows that the amount has increased as expected. The watch detects an exercise pattern in the evening, a slow walk for 60 minutes with increase in heart rate to warm up level. The 10,000 steps goal for the day was achieved.

Bright red – the notification remains visible on the side and beams again indicating nocturnal trend of high stress in the evening. A reminder to watch the trend is added to her digital notes.

30 June 2051

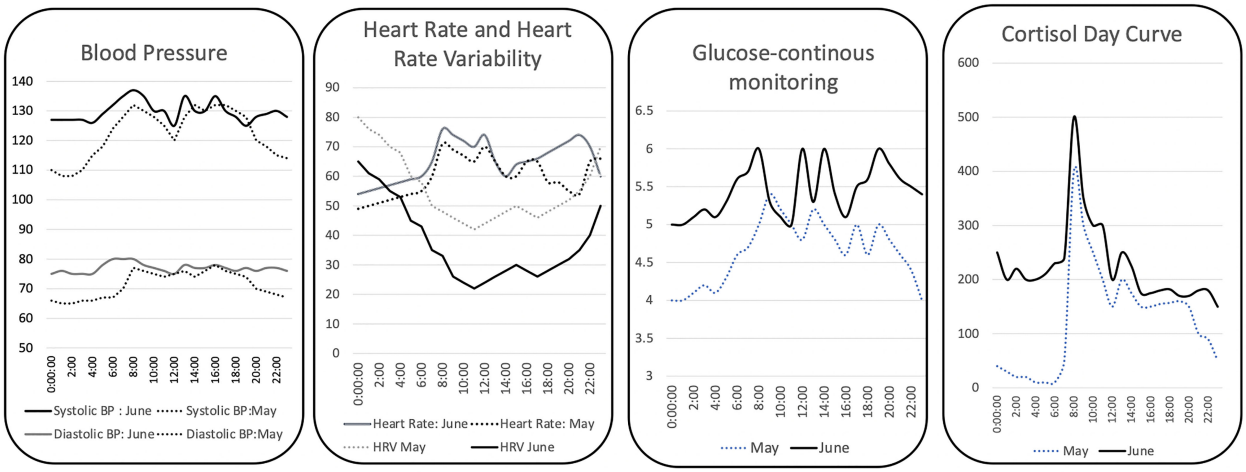
06:00

Alarm beeps: Reminder: check the detailed analytics (**Figure 1**).

Heart Rate: Trending higher
Heart Rate Variability: Trending Lower
More information: see detailed analytics
Systolic Blood Pressure: Abnormal
More information: see detailed analytics
Diastolic Blood Pressure: Abnormal
More information: see detailed analytics
Glucose: Abnormal
More information: Baseline trend higher; see detailed analytics
Hypnograph: Awake.
Stress level: "Very High"
Cortisol Level: "High" from 00:00 to 24:00
More information: No Dip of cortisol levels at night; see detailed analytics

1 July 2051

06:00



Blood Pressure: Systolic and Diastolic blood pressure has a diurnal circadian rhythm with a nocturnal dip in the month of May; this dip is decreased in the month of June with an overall increase.

Heart Rate is trending overall higher in June when compared to May. Heart Rate Variability assumed to be time interval (RR interval) is normally highest at night and lower in the early morning with subsequent increase as seen in May. This pattern is attenuated with an overall decrease in June.

Glucose monitoring shows an overall baseline increase in glucose and the nocturnal dips are attenuated with post-prandial surge.

Cortisol day curve shows attenuation of the circadian rhythm and overall very high levels.

FIGURE 1 | Deep Analytics interface showing the difference in daily patterns. Blood Pressure: Systolic and Diastolic blood pressure has a diurnal circadian rhythm with a nocturnal dip in the month of May; this dip is decreased in the month of June with an overall increase. Heart Rate is trending overall higher in June when compared to May. Heart Rate Variability assumed to be time interval (RR interval) is normally highest at night and lower in the early morning with subsequent increase as seen in May. This pattern is attenuated with an overall decrease in June. Glucose monitoring shows an overall baseline increase in glucose and the nocturnal dips are attenuated with post-prandial surge. Cortisol day curve shows attenuation of the circadian rhythm and overall very high levels.

Alarm beeps: **The stress hormones are in dangerous high levels, please visit the nearest healthcare facility for deeper analysis**.

The morning ride slows at the entrance of a healthcare facility and signals -disembarkation. Claire alights and enters the reception grumbling about the 'obvious glitch in the watch'. They take the wearable and download all the data to the nearest workstation. Indeed, very high cortisol levels with a loss of the usual circadian rhythm in all parameters is observed. A higher order specialist workstation (termed Endocrinology) is assigned to Claire. She waits for her turn; foot tapping impatiently and enters the room. An endocrinologist is seated at the computer table. She smiles serenely and asks about the day, general feelings, and emotions. The Heart rate monitor reflects an increase, as Claire feels visibly uncomfortable sharing deep thoughts with a stranger. A new watch is given to her with an additional chip which would measure the levels at increased intervals, more precisely, throughout the day for next week and upload results to her doctor's database. The situation would be reviewed in one week to decide upon the next course of action.

The week passes routinely. The only reminder is on the day of the appointment when the ride slows again in front of the healthcare facility. This time, the lady is whisked directly into her endocrinologist's room. The data, uploaded live and pathways studied, shows high hormones -steroids with an active pituitary to adrenal pathway suggesting a pathology in the pituitary gland. The proposed diagnosis is explained, and a small chip is inserted underneath her collar bone. After the chip insertion, she is made to pass through a scanner and remain there for 5 minutes.

The screen confirms the diagnosis: Cushing's disease: microscopic hot spot in pituitary. The next screen recommends an available specific targeted treatment to target this region deliverable through a small nano-based therapeutic implant on the forearm.

Claire is free to go after that. Daily circadian patterns, monitored through the watch, slowly returns to normal within a month. Every time a similar increase in cortisol levels over a threshold is seen, an additional dose is delivered remotely through the same implant.

No further alarms are heard as life continues as usual.

DISCUSSION

In the 20th century, practice of medicine and healthcare benefited from significant scientific breakthroughs. We are at inflection point for another incredible breakthrough in healthcare – in the sense that digitization will enable the application of data technologies and artificial intelligence into healthcare. The term 'digital biomarker' has been introduced. FDA defines a digital biomarker as "characteristic or set of characteristics, collected from digital health technologies, that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" (1). This ability to derive biomarkers from daily patterns can potentially provide context to enrich normal values for the population, derive individual person-centric baseline values, and assess changes in health status over time to make clinical diagnosis. Modern-day wearables can be in the form of headbands, sociometric badges, camera clips, smart watches or sensors embedded in clothing and have the ability to monitor vital physiological measurements such as heart rate, electrocardiogram, heart rate variability, respiratory rate, oxygen saturation, temperature, pressure sensors, activity levels, sleep patterns, environmental sound and light exposure etc (2). The requirements for authorisation by the U.S. FDA or regulatory CE-marking, remuneration, and privacy/data security depend on the specificities of the product, its purpose, the technology, the risks and benefits, and the data it processes.

Devices with capability to measure blood pressure, in the form of multi-parameter, miniaturized solutions for home environments are currently being pursued with great interest (3). Correlations of ambulatory blood pressure, especially high nocturnal blood pressure with cardiovascular risk has been observed and automated methods of blood pressure monitoring are being encouraged (4). Various techniques are being exploited for these measurements including miniaturization of cuff oscillometry, tonometry, pulse propagation techniques and pulse wave analysis (3, 5). Pulse propagation techniques include using the PTT (pulse transit time) or the PAT (pulse arrival time) (time required for the pulse to travel between 2 arterial sites) is directly proportional to the blood pressure. Photoplethysmography (PPG) uses optical and inertial sensors to detect blood flow patterns. The technology indirectly measures the blood flow rate through the amount of light absorbed or reflected by blood vessels. Since the relationship between PPG and blood pressure is non-linear, a machine-learning algorithm is used to convert blood flow information to blood pressure measurement. As more data is collected, the algorithm will get more precise (3, 5). Protocols for validation of these ambulatory blood pressure measurements are being developed and some of these devices will likely receive regulatory approvals in the near future. Please see **Table 1** for a summary of devices with FDA approval or CE mark.

Currently, non-invasive methods for glucose measurements in a simple wearable like a watch, are under development. Methods using a subcutaneous wired enzyme glucose sensor inserted in the body which transmits data to a smart phone are available and approved by the FDA (6–8) (**Table 2**). These systems can be applied by self (6, 7) or need to be implanted by a healthcare professional.

Cortisol rises early in morning and is highest before awakening, it falls naturally throughout the day and can spike in response to meals and to stress. Current methods for measuring cortisol concentrations is a laboratory-based blood test and is time consuming. Increasingly more rapid and direct plasma assays are being developed (9, 10). Salivary and sweat cortisol concentrations reflect the systemic steroid concentrations (11, 12). In terms of development, several independent researchers across the globe are working on systems which can be used to measure cortisol concentrations

TABLE 1 | Blood pressure monitoring devices with FDA approval or European CE Mark.

Device	Technology	Calibration	Regulatory Approval
1. Omron Heartguide (OMRON Corporation, Japan): wrist watch 2. Caretaker 4 (Caretaker Medical, US): wrist mounted with inflatable finger cuff	Cuff Oscillometric Method: Integration of miniature cuff into a smart watch	Self-calibration	1. FDA approval 2. FDA approval
3. BPro (Healthstats, Singapore)	Wrist watch radial artery -Tonometry	Requires calibration	FDA Approval
4. Biobeat	Pulse Arrival Time (PAT)	Requires calibration	FDA Approval
5. Aktiia	Photoplethysmography (PPG) and Pulse Wave Analysis	Requires calibration	CE Mark

TABLE 2 | FDA approved methods of non-invasive continuous glucose monitoring.

	Method	Frequency	Application	Duration	Calibration
1. Abbot Freestyle Libre systems	Subcutaneous wired enzyme glucose sensing technology	1 minute	Self	14 days	Factory-calibrated
2. Dexcom G6 system	Subcutaneous wired enzyme glucose sensing technology	5 minutes	Self	10 days	Factory-calibrated
3. Ever sense CGM systems	Fluorescent sensor	5 minutes	Healthcare provider	90-180 days	User calibrate 1-2 times a day

in body fluids and can thus be estimated on a superficial patch or wearable. A cortisol sensor has been formulated using extended gate-field-effect transistor (13–17). This has been developed as wearable contact lenses which can detect cortisol concentration in tears (13). This cortisol sensor is integrated with transparent antennas and wireless communication circuits to link with the smartphone (14). A similar sensing system applied on the wrist with capability to measure sweat cortisol levels has been developed and tested which shows promise (14–17) (**Table 3**). It is very likely that such a device will be developed and integrated into the traditional wearable watch as cortisol levels have applications in measurement of daily stress or allostatic load.

With regards the percentage of population using a wearable, whether the utopian type of order written in this short story can be true, is also highly probable. Insurance companies or other healthcare payers are likely going to mandate wearing of a daily wearable, so to enable preventive care. It is likely that the premium rates may be higher in individuals refusing to comply in the beginning but in the long run when the population adopts this technology, it will become a mainstay.

This lady above has ACTH-dependent Cushing's syndrome secondary to a pituitary adenoma also called Cushing's Disease (CD). Cushing's disease was first described in a landmark monograph more than a century ago, in 1910 by Dr. Harvey Cushing. He described his first patient, Minnie G. to have "… syndrome of painful obesity, hypertrichosis, and amenorrhea, with overdevelopment of secondary sexual characteristics accompanying a low grade of hydrocephalus and increased cerebral tension. Pituitary, adrenal, pineal or ovary?" (18–21).

Diagnosis and management of CD has evolved significantly in the last century. Despite the advances, significant pitfalls and challenges remain. The typical patient presents 5-10 years into the illness, when the high cortisol hormones lead to downstream multi-organ problems. They present to healthcare when frank symptoms and signs are visible which includes significant change in appearance (moon shaped facies, central obesity) and change in metabolic status (hypertension, diabetes mellitus) and body composition (central visceral obesity and osteoporosis). After clinical suspicion, multiple tests (1mg dexamethasone suppression test, 24 hrs urine free cortisol, midnight salivary cortisol, ACTH, cortisol assays) are required to confirm the diagnosis. Once diagnosis is confirmed, then localisation is extremely challenging and pituitary adenomas secondary to Cushings' is detected on magnetic resonance imaging with sensitivity ranging from 42% to 85%. Early, small lesions <4 mm in size are even more difficult to localise. Functional imaging, in the form of 11c-methionine PET, is still under

TABLE 3 | Current and upcoming methods of cortisol assessment.

Test Principle	Sample	Time
1.EIA (competitive, chemiluminescence)	Serum/Plasma	18-40 min
2.ECLIA (Competitive electrochemiluminescence immunoassay)	Serum/Plasma/urine	18-40 min
3.CMIA (Competitive Chimiluminescence Microparticle Immunoassay)	Serum/Plasma/saliva	30 min
4.EIA (competitive, dry technology chemiluminesence)	Serum/Plasma/saliva/urine	10 min
5.LC-MS/MS	Serum/Plasma/saliva/urine	Varies depending on lab; direct measurement shortens time
6. Wireless immunosensing of cortisol through contact lenses	Tears	Instant
7. Graphene based wireless Wearable device	Sweat	Instant mobile technology

research development. The invasive inferior petrosal sinus sampling needs to be performed which can localise the lesion at best to the pituitary gland only (21). Many of the tumours are sent for surgery without localisation and are localised intraoperatively (22). Surgical treatment is the mainstay for pituitary adenomas but remains challenging and only a handful of patients go into remission (at best 60-70%) (23). Medical treatment has evolved with 2 FDA approved therapeutics (pasireotide and mifepristone). However, even these are not superior to curative excision (24).

Early diagnosis in CD can be made through changes in heart rate and blood pressure dynamics (25, 26). The hypothalamic-pituitary-adrenal axis (HPA), responsible for the circadian rhythm of endogenous cortisol secretion, contributes to the circadian rhythm of blood pressure (26). In CD, the typical dip in nocturnal blood pressure (lower by 10% from baseline) is absent and the daytime heart rate is higher (25). Heart rate variability shows a characteristic pattern in terms of circadian differences and the typical pattern of highest between 10-2 PM at night is attenuated in Cushings disease (27). Corticosteroids also affects insulin signalling pathways directly and through an increase in growth hormone and results in higher post prandial glucose and blunted circadian pattern (28). A characteristic pattern has also been reported in patients with acromegaly, a pituitary condition with high growth hormones even before it affects glucose tolerance (29).

Differential diagnosis includes: Phaeochromocytoma, and primary aldosteronism. Periodic patterns would suggest phaeochromocytoma and similar pattern as CD with normal steroid concentrations suggest primary aldosteronism.

Adrenal and pituitary incidentalomas are commonly detected during screening for non- related medical concerns. These may represent subclinical hypercortisolism (30, 31) in otherwise clinically asymptomatic patients. The wearables can potentially be used to ascertain subclinical disease and to differentiate from pseudo-Cushings' syndrome (occurs in obesity, alcoholism etc). Conversely, with the advent of regular wearables incorporating cortisol, it is possible that such subclinical glucocorticoid excess will be detected more frequently and may even be the causative mechanism in some patients with metabolic abnormalities. We envision that initially, the wearables will be useful in patients with clinical suspicion like above. However, with time as more long term longitudinal data is collected in the population (over 10-20 years), big data analytics is set to uncover digital biomarkers (patterns) that can be used to make an early diagnosis before definite clinical signs appear. We envision that by 2051, preventive care with remote digital monitoring is highly probable at a population scale level.

Next steps for localisation require the characterisation of CRH-ACTH-cortisol pathway (32). Cushings disease has a unique metabolomic signature (33) and with advancement in omics platforms (34), and in artificial intelligence predictive analytics it is highly probable that the pathway can be used to identify the active areas.

Pituitary lesions in Cushing's syndrome are only detected by MRI in <60% of cases. Hybrid imaging combining PET and MRI such as 11C-methionine PET co-registered with volumetric MRI will likely improve the sensitivity and specificity in the near future (35). As novel data reveals more information on exact gene and protein expressions in these tumours, it will become possible to design advanced functional imaging methods which targets these areas to show "hotspots".

Molecular targeted therapies such as ACTH antagonists (36) or melanocortin type 2 receptor (MC_2R) (37), EGFR, retinoic acid receptors, CDK with specific inhibitors for CD, and cyclin E-Mediated Human Proopiomelanocortin pathway (38–42) are being developed. Efficiency in targeted delivery can be achieved with the conjugation of drugs with target cell surface-targeting moieties and encapsulation of unique nanocarriers/nanoparticles (43). Studies evaluating the clinical efficacy of these therapeutics will bring some of these into clinical practice.

While the above case vignette, appears to be a sci-fi fantasy and significant challenges in each area of diagnostics and therapeutics remain; the wearables and the massive data that will be accrued, will likely transform healthcare through predictive modelling and implementation of personalised care. One of the key factors for successful implementation is defining specific problems for targeted wearable solutions in specific disease states and establishing partnerships with clinician champions (44). We envision that these methods are set to bring about a major paradigm shift in the management of most endocrine related conditions. The practice of endocrinology is set to evolve significantly in the coming decades.

At the turn of the 20th century, Dr. William Osler said:

"Listen to your patient; he is telling you the diagnosis,"

In the 21st century:

"Look and analyse the digital physiological and behavioural trends; therein lies the diagnosis".

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RD and BB conceptualized the short story, performed literature review, critically reviewed and wrote the final draft. SB reviewed and critically evaluated the final draft. All authors reviewed the final manuscript.

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Prevalence and clinical characteristics of Crooke's cell adenomas in 101 patients with T-PIT-positive pituitary adenomas: Case series and literature review

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Purpose: We aimed to perform a retrospective analysis of a rare subtype of corticotroph adenoma, Crooke's cell adenoma, to better understand its clinical features.

Methods: We collected *T-PIT*-positive pituitary adenomas and screened Crooke's cell adenomas from January 2020 to December 2021 in our center. Case reports of such tumors were also collected through a literature search. Clinical data such as biochemical tests, imaging examinations, and pathological data of the above cases were analyzed.

Results: A total of 101 *T-PIT*-positive patients were treated in our center in the last 2 years, and 4 were finally pathologically diagnosed with Crooke's cell adenomas. All of these patients were male with elevated adrenocorticotropic hormone levels, and 50.0% presented with hypercortisolemia, Cushing's syndrome, visual impairment, and headache. The tumor diameter was significantly larger in these 4 patients (37.0 mm) than in the other patients (26.0 mm), and their tumor invasive behavior was more pronounced. Cases reported in the literature were mainly female (72.8%), and the clinical presentation was also dominated by Cushing's syndrome (65.1%) and hormonal dysfunction. Tumors were more common as macroadenomas (33.2 mm) and suprasellar growths (63.8%). The tumor recurrence rate was as high as 55.6%, with 6 cases progressing to pituitary carcinomas and 7.7% of tumor-related deaths. Our further integrated analysis of our center and reported cases revealed that gender, Cushing's syndrome, visual dysfunction, hormonal disorders, and tumor growth characteristics were statistically different in different tumor categories.

Conclusion: Crooke's cell adenoma is a tumor subtype with obvious clinical aggressive behavior, and an in-depth analysis of its clinical characteristics may assist in developing a comprehensive treatment plan.

KEYWORDS

Crooke's cell, pituitary adenoma, ACTH, Cushing disease, hyalinization

Introduction

Pituitary Crooke's cell adenomas (CCAs) are a rare subtype of corticotroph adenomas typically associated with Cushing's disease, accounting for less than 1% of pituitary adenomas (1). These tumors, first described by Arthur Carleton Crooke in 1935 (2), represent a distinct clinicopathological subtype of pituitary adenomas. They were remarkably aggressive and showed the characteristics of Crooke's cell - cytokeratin (CK) filaments accumulating heavily around the nucleus, making them appear distinctly hyaline in hematoxylin and eosin (HE) staining. The neoplastic Crooke's cells are strongly immunoreactive *T-PIT* and *CK8/18* and exhibit variable adrenocorticotropic hormone (*ACTH*) immunoreactivity. It has been suggested that CCA should meet the diagnostic criteria of at least 50% of neoplastic Crooke's cells in corticotroph adenomas. The presence of hyaline change is reported to be the result of respondence of excess glucocorticoids, but the mechanisms have not been well understood. CCAs usually take the form of invasive macroadenomas with a high rate of recurrence after standard resection. We reviewed 4 cases of CCA, with clinical, radiological, and histopathological features. We are seeking a better understanding of their clinical-pathological characteristics, as well as assessing their immunophenotype and prognosis.

Materials and methods

Patient information

We performed a retrospective analysis of patients with pituitary adenoma who underwent surgery at The First Affiliated Hospital of Sun Yat-sen University from January 2020 to December 2021. All masses were removed by the same surgical team, and their pathological findings supported the diagnosis of *T-PIT*-positive pituitary adenoma. We identified CCAs with a multidisciplinary synergistic diagnosis of neurosurgery, pathology, endocrinology, etc.

Biochemical and imaging data

During the perioperative period, hormonal and imaging studies are performed in our center on patients with pituitary adenomas. The reference ranges for hormones are as follows: 8 AM Cortisol, Urinary cortisol, 24h urinary cortisol, *ACTH*, *PRL*, *GH*, *TSH*, *LH*, and *FSH*. Above or below these ranges are considered abnormal hormone secretion. We used 3.0T Magnetic Resonance Imaging (MRI) for routine tumor screening. For the pituitary gland, thin cuts in coronal and sagittal positions are used to facilitate visualization of the cavernous sinus and optic chiasma. For microadenomas, dynamic MRI scans have been used to increase the sensitivity; while for macroadenomas, conventional coronal and sagittal pituitary MRI with contrast is generally adequate for treatment planning. The direction of tumor growth was classified as supra-sella, para-sella (cavernous sinus), clivus and infra-sella (sphenoidal sinus).

Pathological data

We used HE staining and reticulin fiber staining for the initial diagnosis of tumorigenic lesions in the sella area masses, based on which *T-PIT* (AM0486), *PIT-1* (AM0451), and *SF-1* (AM0443) immunostaining was used to distinguish the tumor cell spectrum origin. The transcription factor antibodies are all purchased from Xiamen Talent Biomedical Technology Co., Ltd. (Fujian Province, China). *ACTH*, *PRL*, *GH*, *TSH*, *LH*, *FSH*, and *CK8/18* immunostaining was used for further classification of tumor categories. The *Ki-67* index was used to determine the proliferative activity of the tumor. The final pathology report was issued by two experienced pathologists after discussion.

Review of the literature

We searched the PubMed database using the terms "Crooke's cell", "Crooke's cell hyaline deposition", and "Crooke's cell adenoma", etc. We reviewed all relevant English-language literature published before December 2021 and performed a summary analysis of the case reports.

Statistical analysis

Statistical analysis was performed using SPSS software (version 19.0, IBM Corp.). Continuous variables with normal distribution were presented as mean ± standard deviation.

Comparing two sets of quantitative data following a normal distribution using the Student's t test. The frequencies of categorical variables were compared using a chi-square test. A value of $p<0.05$ was considered statistically significant.

Results

Prevalence of CCAs

Over the past 2 years, we treated a total of 418 patients with pituitary-related disorders, of whom 391 completed pathological testing for transcription factors and hormones, while a total of 101 patients (25.8%) were positive for *T-PIT*. 4 patients eventually received a pathological diagnosis of CCA, the prevalence of which was approximately 1.0%.

Characteristics of CCAs

Of the 391 patients, there were 244 clinically silent adenomas, of which 66 (27.0%) were *T-PIT* positive cases. As shown in Table 1, of the 97 non-CCA cases recorded in our center, their average age was 45.5 ± 14.0 years, and 17.5% (17/97) were male; whereas no female patients were found in CCA cases and the mean age was 45.0 ± 8.0 years. Clinically, a total of 18 (18.6%) non-CCA patients with significant clinical symptoms associated with Cushing's Syndrome, and 50.0% (2/4) CCA patients exhibited similar symptoms. Visual dysfunction was observed in 50.5% (49/97) of non-CCA patients and 50.0% (2/4) of CCA patients. 25.8% (25/97) of non-CCA patients and 50.0% (2/4) of CCA patients presented with headache. For hormone secretion, 8.3% (8/97) of non-CCA patients had hypercortisolism, while half of the CCA patients had significantly elevated cortisol levels. All CCA cases had abnormal secretion of *ACTH*, while non-CCA patients presented more often with non-functioning adenomas (NFPAs), with only 18.6% (18/97) having this hematological feature. The overlap with clinical and hormone immunohistochemistry in 101 cases was shown in Supplemental Table 1. The mean tumor diameter was 26.0 mm in non-CCA patients and 37.0 mm in CCA patients, the latter being significantly larger than the former. Tumors in CCA patients showed significant invasive behavior to surrounding structures such as the cavernous sinus (100.0%, 4/4), sphenoidal sinus (100.0%, 4/4), suprasellar region (75.0%, 3/4), and posterior cranial fossa (75.0%, 3/4). Non-CCA tumor invaded mainly towards the suprasellar region (59.8%, 58/97) and the cavernous sinus (50.5%, 49/97). The recurrence rate of CCA (75.0%, 3/4) is significantly higher than that of non-CCA (39.2%, 38/97). One CCA patient (25.0%, 1/4) died due to tumor complications.

Cases presentation

Case 1

A patient aged 32 suddenly developed symptoms such as right eyelid ptosis and visual impairment in 2016, and cranial MRI suggested a 18×38×30 mm (Knosp grading 4) lesion in the sella area. Transsphenoidal resection was performed in November of the same year, and postoperative pathology

TABLE 1 Characteristics of our cases.

Classification			Ratio (%)	
			CCA (n=4)	non-CCA (n=97)
Gender	Male		100.0 (4/4)	17.5 (17/97)
	Female		0	82.5 (80/97)
Age (y)			45.0 ± 8.0	45.5 ± 14.0
Clinical manifestation	Cushing's syndrome		50.0 (2/4)	18.6 (18/97)
	Visual defect		50.0 (2/4)	50.5 (49/97)
	Headache		50.0 (2/4)	25.8 (25/97)
Hormonal dysfunction	Hypercortisolemia		50.0 (2/4)	8.3 (8/97)
	Increased ACTH		100.0 (4/4)	18.6 (18/97)
Biological characteristics of tumor	Diameter (mm)		37.0 ± 16.5	26.0 ± 12.7
	Growth direction	Suprasellar	75.0 (3/4)	59.8 (58/97)
		Cavernous sinus	100.0 (4/4)	50.5 (49/97)
		Sphenoid sinus	100.0 (4/4)	35.1 (34/97)
		Posterior fossa	75.0 (3/4)	16.5 (16/97)
	Recurrence		75.0 (3/4)	39.2 (38/97)
	Tumor Related Death		25.0 (1/4)	0

confirmed the *ACTH* adenoma. Clinical symptoms related to the occupying effect of the tumor were alleviated, but the MRI suggested tumor recurrence in the second month after the operation. Transsphenoidal surgery was performed again, followed by gamma knife radiotherapy while the MRI showed a residual tumor in the left cavernous sinus area. In February 2018, the patient presented with weakness, memory loss, abdominal purple striae, and central obesity. Cranial MRI suggested a 18×19×9 mm lesion in the sella area. In September 2018, after intolerable side effects from mifepristone, he underwent bilateral adrenalectomy in our hospital and gradually developed postoperative hyperpigmentation of the face and limbs. In December, nasal bleeding was appeared and endoscopy revealed a neoplasm at the olfactory fissure protruding into the common nasal tract with a maximum diameter of about 34 mm, and tumor recurrence is considered. In January 2019, a surgical resection was performed and the pathology reported an aggressive *ACTH* adenoma with *Ki-67* of about 20%. A residual lesion was found and radiotherapy was re-performed postoperatively. In July 2019, symptoms of nasal bleeding re-emerged and *ACTH* was significantly elevated (Table 2). A nasal endoscopic biopsy was performed and the pathology suggested CCA (Figure 1). In April 2020, the pituitary MRI showed a recurrent mass of 37×43×34 mm in the operated area encapsulating the left internal carotid artery (Figure 2). In August 2020, the mass had invaded into the cerebellopontine angle region, and the headache was relieved after surgery, but vision loss and left-sided facial hypoesthesia occurred. In November, MRI scanning showed that the mass size increased to 53×39×26 mm, compressing the left temporal lobe and brainstem. After considering the risk of surgery, the patient and his family decided to take "Temozolomide 100 mg/day combined with Anlotinib 8 mg/day". At the same time, symptomatic treatments such as hormone replacement, intracranial pressure reduction, and maintenance of water-electrolyte balance were administered. The patient was discharged from the hospital with occasional symptoms such as choking on water, difficulty urinating, chest tightness, dizziness and headache, which were treated symptomatically. MRI was repeated in December 2020, and the lesion further expanded to 65×47×50 mm, involving the temporal lobe and brainstem, with supratentorial hydrocephalus. Unfortunately, the patient died in 2021 due to severe complications.

Case 2

A 54 years old male patient gradually developed symptoms such as hyperphagia, full moon face and buffalo back 7 years ago, without accompanying purple striae, subcutaneous bleeding spots or petechiae. Starting in 2017, he developed generalized skin pigmentation, which was evident in the axillae, buttocks, neck, and joints of the extremities, accompanied by swelling of both lower extremities. In June 2020, the patient was found to have elevated blood pressure, significantly elevated blood cortisol and *ACTH*. MRI of the pituitary gland suggested an occupying lesion of about 25×16×24 mm (Knosp grading 2) in the sella area, with compression of structures like optical chiasma and cavernous sinus. Initial consideration was given to pituitary macroadenoma. Abdominal CT showed multiple nodular hypodense shadows in the left adrenal gland. After admission to our hospital, *ACTH*, cortisol and 24-hour urinary

TABLE 2 Hormonal changes in displayed cases.

Hormone	Case 1		Case 2		Case 3		Case 4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Cortisol (8 AM, 2.90-19.40μg/dL)	1.00↓	15.70	28.60↑	11.80	19.70↑	13.40	12.30	19.20
Urinary cortisol (0.43-11.70μg/dL)	82.50↑		29.10↑	38.20↑	4.00			
24h urinary cortisol (4.30-176.00μg)	3300.00↑		742.05↑	1356.10↑	112.00			
ACTH (1.60-13.90poml/L)	>440.40↑	159.2↑	41.91↑	7.81	22.27↑	9.67	17.72↑	9.58
PRL (1.61-18.77ng/mL for male)	14.49	<0.60↓	17.68	4.31	12.67	6.72	19.25↑	7.69
GH (0-10ug/L)	0.11	0.11	0.31		0.18	0.72	0.69	
TSH (0.56-5.91uIU/mL)	1.85	0.4↓	0.28↓	0.37↓	1.75	0.91	2.20	0.61
LH (2-12IU/L)	2.57	1.95↓	2.36	3.27	1.55↓	5.90	5.71	4.69
FSH (1-8IU/L)	4.51	3.14	7.12	5.07	5.57	8.15↑	9.49↑	9.98↑

Pre, pre-operative; Post, post-operative; ↑ means elevated; ↓ means reduced.

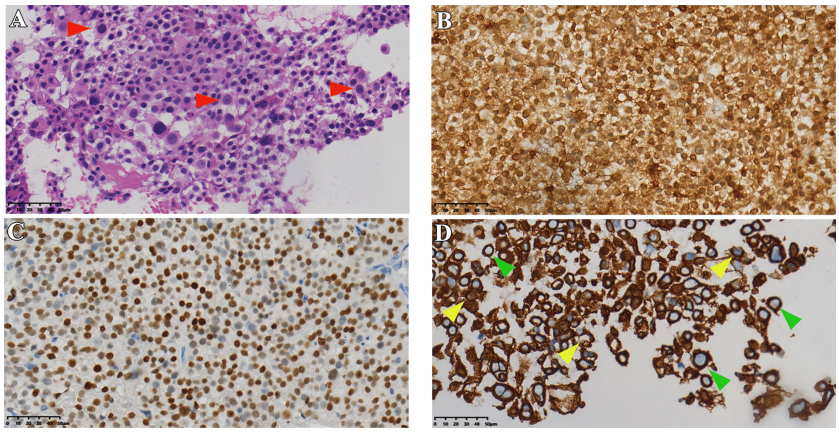

FIGURE 1
Histopathological data of case 1. **(A)** HE staining of tumor tissue (red arrows indicate typical Crooke's hyaline change). **(B, C)** Immunohistochemical staining for *ACTH* and *T-PIT*. **(D)** Immunohistochemical staining of cells keratin showing Crooke hyaline changes (green and yellow arrows show a round and semi-circular immunostaining of *CK8/18*, respectively). Original magnification, 40×.

cortisol were found to be elevated (Table 2). The patient underwent transsphenoidal surgery in July 2020, and the postoperative pathology suggested a CCA with *CK8/18* > 50% (Figure 3), *T-PIT* (+), and *Ki-67* about 3%. No significant postoperative urinary cortisol relief was seen, and no evident residual mass was found in the second day postoperative follow-up CT. However, MRI after 4 months suggested a mass in the operative area, measuring approximately 15×18×14 mm (Figure 4). Cortisol concentration was still high at 34 ug/dL at 8 months after the intervention. Twenty-one months after surgery, blood cortisol was 32.1 ug/dL, ACTH was 59.79 pmol/L, and MRI showed a 22*29*23 mm mass in the sellar area. The patient underwent a second surgery in our hospital in April 2022. Intraoperatively, the tumor was seen to be separated by the pseudocapsule, with a tough texture, and pathological staining showed Ki-67 8%. Cortisol and ACTH did not return to normal on repeat examination, and CT suggested residual tumor.

Case 3

One man presented with dizziness, occasional nausea and vomiting at the age of 42 in 2018. Starting the following year, the above symptoms got worse, so he went to our outpatient clinic in 2021. Pituitary MRI suggested an enlarged pituitary fossa and an abnormal signal occupying the lesion in the sella, the size was about 24×20×17 mm (Knosp grading 3), the initial consideration was a pituitary macroadenoma. 1 month later, the head CT revealed that the mass diameters were about 25mm, 23mm, and 18mm. The tumor was aggressive and the bone of the sella base was damaged, and the cavernous sinus was also compressed. The patient underwent microsurgery in October 2021, following which the elevated *ACTH* was relieved (Table 2), and the

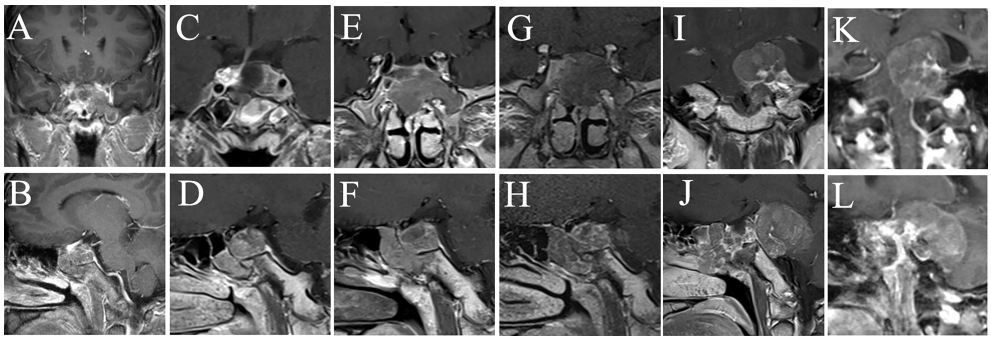

FIGURE 2
MRI examination of case 1. The images show the patient's coronal and sagittal MRI of the head before **(A, B)**, one week after **(C, D)**, six months after **(E, F)**, eight months after **(G, H)**, sixteen months after **(I, J)**, and seventeen months after **(K, L)** the fourth surgery, respectively.

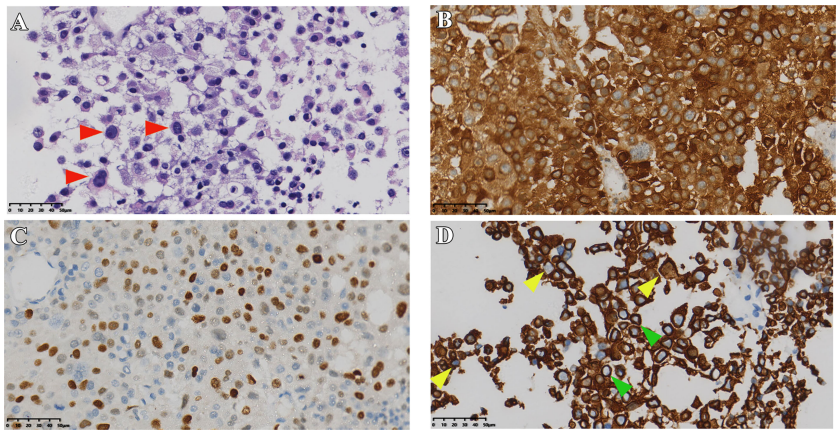

FIGURE 3
Pathological sections of case 2. **(A)** HE staining of tumor (red arrows indicate typical Crooke's hyaline change). **(B, C)** Immunostaining of *ACTH*, *T-PIT*, and *CK8/18* **(D)** green and yellow arrows show a round and semi-circular immunostaining of keratin, respectively). Original magnification, 40×.

pathology suggested cyclic *CK8/18* staining of about 80% (Figure 5), *Ki-67* of about 10%, and infiltration of Crooke cells were observed in bone trabeculae. Hematology and MRI in January 2022 showed no evidence of recurrence (Figure 6).

Case 4

Case 4 is a 50-year-old man with a headache following a "resection of left vocal cord squamous cell carcinoma" in December 2021. The headache was mainly in the right temporal region, with paroxysmal traction pain, with double vision, blurred vision and drooping right eyelid, and elevated *ACTH* on blood examination (Table 2). The cranial MRI showed an occupancy in the sella area with a size of about 21×17 mm (Knosp grading 3), and a pituitary macroadenoma was considered. The head CT showed an irregular mass in the sella and supra-sella area, about 33×22 mm in size, with uniform density. The mass wrapped around the siphon segment of the right internal carotid artery and part of the left posterior communicating artery. Also, the neoplasm was close to the left internal carotid artery, and invaded the dorsum of the sella and part of the slope bone downward. The magnetic resonance DTI sequence showed that part of the right optic nerve fiber bundle was interrupted and significantly reduced compared to the other side. Transsphenoidal surgery was performed, and the pathology suggested that the tumor tissue was *T-PIT* (+), *ACTH*

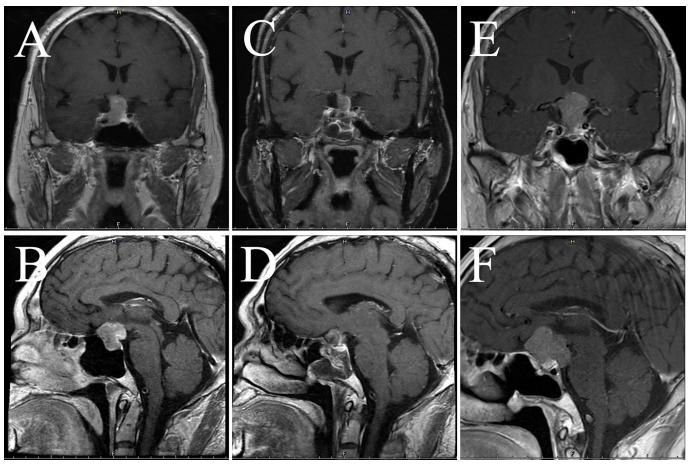

FIGURE 4
Imaging data of case 2. The images show coronal and sagittal MRI of the patient's head preoperatively **(A, B)**, three months postoperatively **(C, D)**, and twenty-one months postoperatively **(E, F)**, respectively.

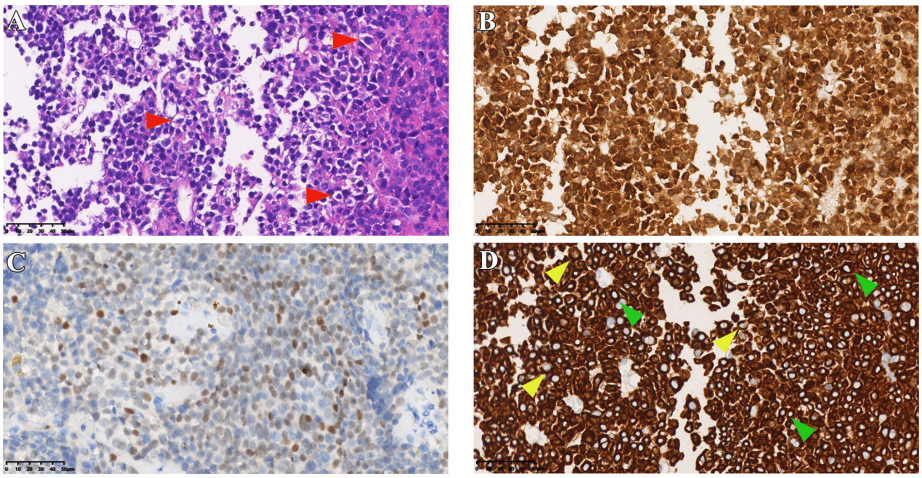

FIGURE 5
Immunostaining results of case 3. **(A)** HE staining of tumor tissue (red arrows indicate typical Crooke's hyaline change). **(B, C)** Immunohistochemical staining for *ACTH* and *T-PIT*. **(D)** Immunohistochemical staining of cells keratin (green and yellow arrows show a round and semi-circular immunostaining of *CK8/18*, respectively). Original magnification, 40×.

positive >95%, Crooke cells accounted for about 80% (Figure 7), and *Ki-67* about 1% (+). Normalized *ACTH* was found in a postoperative reexamination. However, recent imaging data suggested a suspicious residual tumor (Figure 8).

Literature review and statistical analysis

To date, over 100 CCA cases have been published (Table 3) (3–35). Since the hyalinization of corticotropin cells was proposed by Crooke in 1935 (2), Felix et al. (3) first reported 3 cases of adrenocorticotropin adenoma with a large amount of Crook's hyaline deposition in 1981. Since then, cases of a variety of sizes have come forward. In 2003, George et al. reported the largest number of 36 cases to date (13). The authors described their clinical manifestations, pathological manifestations, and therapeutic strategies, and underlined their invasive clinical features. Early symptoms in some cases were consistent with asymptomatic NFPAs. With the development of the disease, they are transformed into functional tumors of hypercortisolism, and finally into pituitary cancer. CCA is mainly macroadenoma with obvious clinical invasiveness. In comparison to

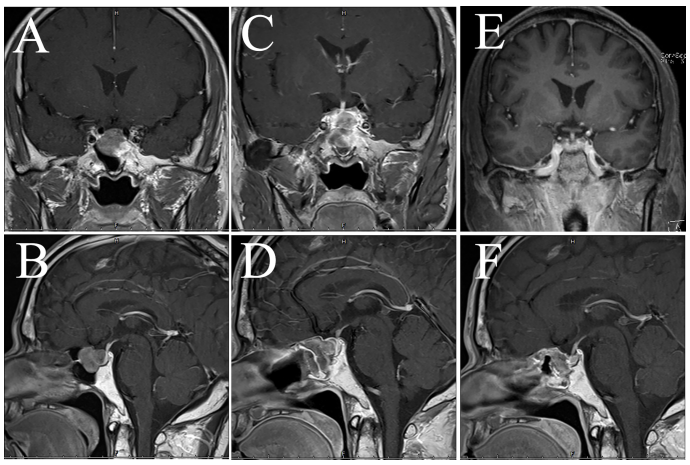

FIGURE 6
MRI examination of case 3. The images show coronal and sagittal MRI of the patient's head preoperatively **(A, B)**, one week postoperatively **(C, D)**, and three months postoperatively **(E, F)**, respectively.

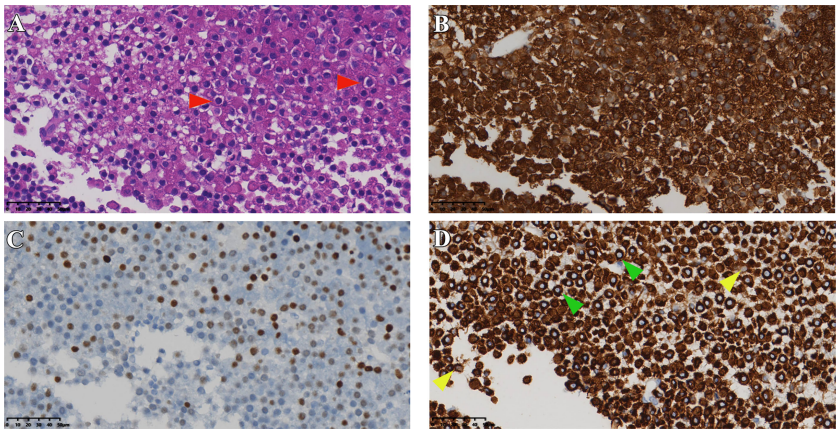

FIGURE 7
Histopathological staining of case 4. **(A)** HE staining of tumor tissue (red arrows indicate typical Crooke's hyaline change). **(B, C)** Immunohistochemical staining for *ACTH* and *T-PIT*. **(D)** Immunostaining of *CK8/18* (green and yellow arrows show a round and semi-circular pattern, respectively). Original magnification, 40×.

microadenomas, the macroadenomas that cause Cushing's disease have different biochemical characteristics, invasive nature, initial remission rate, and post-operative recurrence rate.

We made a retrospective summary of the published cases (Table 4) to better understand the clinical features of CCA. Overall, their mean age was 47.8 ± 13.4 years. There were more female patients (72.8%, 59/81) than men (27.2%, 22/81) in CCA patients. The major symptoms in most patients were Cushing's syndrome (65.1%, 56/86), optic nerve disorder (33.7%, 29/86), and headache (29.1%, 25/86). Notably, up to 71.4% (45/63) of patients were accompanied by an increase in ACTH, while more than half had hypercortisolism. The average diameter of the tumor was 33.2 ± 12.8 mm, and we found that the tumor mainly grew toward the suprasellar (63.8%, 44/69), which coincided with the symptoms of visual impairment in most patients. Additionally, the number of patients with tumor recurrence reached 55.6% (35/63), 6 patients with progression to pituitary carcinoma were reported (9.7%, 6/62), and tumor-related deaths accounted for 7.7% (5/65).

To better understand the clinical characteristics of CCA, we integrated our cases with the CCA cases previously reported for analysis. As illustrated in Table 5, the age composition of the two groups was similar and the data were relatively comparable. Regarding gender, CCA was clearly dominated by female

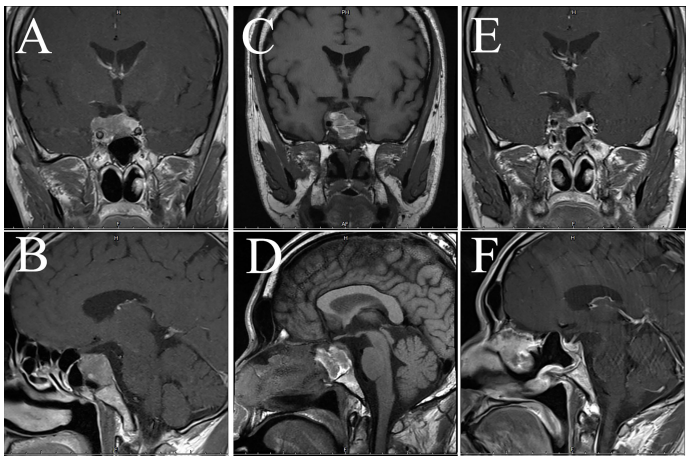

FIGURE 8
Imaging examination of case 4. The images show coronal and sagittal MRI of the patient's head preoperatively **(A, B)**, one week postoperatively **(C, D)**, and three months postoperatively **(E, F)**, respectively.

TABLE 3 Literature summary of Crooke's cell adenomas.

Cases no.	Gender	Age (y)	Clinical Manifestation	Hormonal Dysfunction	Mean Diameter (mm)	Invasive Direction	Refractory Cases	Metastases	Management	Tumor Related Death	Ref.
3	3F	36	100% (3/3) CS	100% (3/3) HC and EA	NM	NM	NM	NM	ST	NM	Felix et al. (1981) (3)
7	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	Martin et al. (1982) (4)
1	F	56	CS, HA	HC	17	SS	no	no	ST	no	Horvath et al. (1983) (5)
10	NM	NM	CS	NM	NM	NM	NM	NM	NM	NM	Robert et al. (1986) (6)
1	F	63	CS	HC	NM	SS and CAS	NM	NM	ST, RT	no	Franscella et al. (1991) (7)
1	F	51	pigmentation, hypertension	HC, EA	NM	SpS and PF	no	no	ST	no	Kamijo et al. (1991) (8)
2	NM	NM	50% (1/2) CS, osteoporosis, hypertension	50% (1/2) HC, 100% (2/2) EA	37	SS and CAS	NM	NM	ST, RT	NM	Ikeda et al. (1997) (9)
1	F	38	CS, hypertension	HC	NM	NM	NM	NM	ST	no	Coire et al. (1997) (10)
1	F	17	HA, secondary amenorrhoea	normal	NM	SS and CAS	yes	sacrum	ST × 4, RT	no	Holthouse et al. (2001) (11)
2	1F, 1M	56.5	50% (1/2) HA, diplopia, tiredness, constipation	50% (1/2) EA, hypopituitarism	NM	SS	no	no	ST	no	Roncaroli et al. (2002) (12)
36	27F, 9M	46	71% (24/34) CS, 76% (13/17) HA, 45% (13/29) VD	94% (15/16) EA, 44% (7/16) HC	NM	67% (18/27) SS	60% (15/25)	8% (2/25)	ST, RT, CT	12% (3/25)	George et al. (2003) (13)
1	F	48	VD, left retro-orbital eye pain	normal	NM	NM	NM	NM	ST	no	Lopez et al. (2004) (14)
1	F	43	CS, VD, HA, fatigue, tiredness	HC, EA	NM	CAS	yes	no	ST × 3	no	Kovacs et al. (2005) (15)
4	4F	44.8	50% (2/4) HA and VD, menstrual irregularities	50% (2/4) HC, hyperprolactinemia	29.8	SS and CAS	25% (1/4)	no	ST, RT	no	Sahli et al. (2006) (16)
2	1F, 1M	51.5	100% (2/2) CS and VD, 50% (1/2) HA	100% (2/2) HC and EA	NM	CAS	100% (2/2)	50% (1/2) spinal	ST, RT, CT	no	Mohammed et al. (2009) (17)
7	5F, 2M	42.9	NM	43% (3/7) HC, 86% (6/7) EA	NM	CAS	57% (4/7)	14% (1/7) liver	ST, RT, CT	no	Takeshita et al. (2009) (18)
1	F	49	CS, VD	HC	15	SS and CAS	yes	no	ST × 4, RT, CT	no	Rotondo et al. (2012) (19)
1	M	52	CS, VD	EA	NM	SS and CAS	yes	no	ST	no	Atkinson et al. (2012) (20)
1	F	16	VD, galactorrhea, amenorrhoea	HC	50	SS	yes	parotid, liver	ST × 8, RT × 3	yes	Kovacs et al. (2013) (21)
1	F	55	CS	HC, EA	30	SS and CAS	yes	no	ST, CT	no	Asimakopoulou et al. (2014) (22)
1	F	58	CS	EA	NM	SS	NM	NM	ST	no	Sathiyabama et al. (2014) (23)
1	M	54	CS	HC, EA	39	SS and CAS	yes	no	ST, CT	no	Kurowska et al. (2016) (24)

(Continued)

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TABLE 3 Continued

Cases no.	Gender	Age (y)	Clinical Manifestation	Hormonal Dysfunction	Mean Diameter (mm)	Invasive Direction	Refractory Cases	Metastases	Management	Tumor Related Death	Ref.
1	M	15	delayed puberty	normal	20	no	no	no	ST	no	Glrl et al. (2017) (25)
1	M	61	CS, HA, VD, diabetes mellitus, hypertension	HC, EA	54	SS and CAS	yes	no	ST × 4, RT, CT	yes	Januszewska et al. (2018) (26)
1	F	55	HA, hypertension, hyperlipidemia, dizziness	normal	25	SS	no	no	ST	no	Todnem et al. (2018) (27)
1	M	64	HA, VD, tinnitus	EA	32	CAS	yes	no	ST × 2, RT	no	Khatri et al. (2019) (28)
1	M	45	HA, VD, dizziness, diplopia	EA, hypogonadism	20	SpS, CAS and PF	no	no	ST	no	Randall G et al. (2019) (29)
1	F	56	hypertension, leg edema	HC, EA	57	CAS	yes	no	ST, CT	no	Tanaka et al. (2019) (30)
2	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	Sema et al. (2019) (31)
1	M	71	HA, impotence, decreased libido	normal	41	SS	no	no	ST, RT, CT	no	Schwann et al. (2020) (32)
1	M	56	post-surgical panhypopituitarism	normal	47	PF	yes	no	ST × 3, RT × 2	no	Cortez et al. (2020) (33)
1	F	39	CS, VD, galactorrhea, hypertension	HC, EA, hyperprolactinemia	30.5	SS and CAS	yes	NM	ST, RT	NM	Ridhi et al. (2021) (34)
5	4F, 1M	46.2	100% (5/5) CS, 60% (3/5) VD	100% (5/5) EA and HC	20.6	SS and CAS	20% (1/5)	no	ST, RT	no	Erica et al. (2021) (35)

CS, Cushing's syndrome; HA, headache; VD, visual dysfunction; HC, hypercortisolemia; EA, elevated ACTH; SS, suprasellar; CAS, cavernous sinus; SpS, sphenoid sinus; PF, posterior fossa; ST, surgical therapy; RT, radiotherapy; CT, chemotherapy; NM, not mentioned.

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TABLE 4 Characteristics of Reported Literatures.

Classification			Ratio (%)
Gender	Male		27.2 (22/81)
	Female		72.8 (59/81)
Age (y)			47.8 ± 13.4
Clinical manifestation	Cushing's syndrome		65.1 (56/86)
	Visual defect		33.7 (29/86)
	Headache		29.1 (25/86)
Hormonal dysfunction	Hypercortisolemia		55.6 (35/63)
	Increased ACTH		71.4 (45/63)
Biological characteristics of tumor	Diameter (mm)		33.2 ± 12.8
	Growth direction	Suprasellar	63.8 (44/69)
		Cavernous sinus	46.4 (32/69)
		Sphenoid sinus	2.9 (2/69)
		Posterior fossa	4.3 (3/69)
	Recurrence		55.6 (35/63)
	Metastasis		9.7 (6/62)
	Tumor Related Death		7.7 (5/65)

patients. Although this is contrary to the characteristics of our center, it is similar to the overall reporting. In addition, CCA was predominantly a functional tumor with a significantly higher proportion of concomitant Cushing's syndrome, hypercortisolism and elevated ACTH than non-CCA, supporting the notion that cortisol disorders promote the formation of CCA. The relatively moderate hormone secretion in the non-CCA group resulted in a higher proportion of visual dysfunction than in the CCA group. No significant difference was observed between the two groups with respect to headache. The tumor diameter was significantly larger in CCA than in the control group, but the tendency for tumor invasion into the sphenoid sinus was lower than in non-CCA. In terms of tumor recurrence, the therapeutic effect of CCA was much worse than that of the control group.

Discussion

Pituitary adenomas are the most common masses of the sellar region arising from adenohypophyseal cells. The pituitary-specific transcription factors, including *PIT-1*, *T-PIT*, *SF-1*, and *GATA-2*, involved in adenohypophysial cell differentiation and maturation are now regarded as key diagnostic tools for the further characterization of pituitary adenomas. Based on the specific transcription factor tested by IHC, the new WHO classification of pituitary tumors has provided an integrated approach for the diagnosis and classification of pituitary adenomas (36). *T-PIT*-driving corticotroph adenomas represent 10 to 15 percent of all pituitary adenomas and are divided into three recognized variants: densely granulated adenomas, sparsely granulated adenomas, and Crooke's cell adenomas. Crooke's cell adenoma is an uncommon variant of *ACTH*-immunoreactive adenoma in which the cells recapitulate Crooke's hyaline change observed in the non-neoplastic pituitary gland under the influence of elevated cortisol levels. In 1935, Crooke et al. first reported the hyaline change of basophils in the anterior pituitary (2). Subsequent studies found intermediate filament-rich Crooke's cells, with ring-like cytoplasmic filaments accumulating, causing dispersal of secretory granules (and both PAS and *ACTH* reactivity) to the peripheral submembrane region or displaced internally next to the nucleus. Porcile and Racodot (37) confirmed that the core material of the hyalinization was made up of 7nm filaments arranged in parallel circles surrounding the nucleus by the ultrastructural method. The remaining secretory granules and other organelles were divided into perinuclear and perimembrane groups. Subsequently, Newman et al. (38) revealed that Crook's hyaline deposition was immunohistochemically composed of intermediate filament keratin. Yet, until now, there is no clear mechanism to explain the phenomenon of increased keratin. Some studies suggest that Crooke-like hyalinization is a cellular inhibitory response to cortisol stimulation, such as the fact that Crooke's cells are commonly accompanied by hypercortisolemia, and the phenomenon that organelles like the Golgi apparatus are squeezed around the cell membrane is considered a manifestation of inhibition of cell function (5, 9). Our results also confirm the above point by analyzing about 200 cases (Table 5). Irregular processing of precorticotropin (*POMC*), a precursor of corticotropin, is thought to be the cause of clinical silence in these tumors (27). Crooke-like change is generally a change in the response of normal corticotropin cells, which is not often involved in *ACTH* tumor cells. This is due to the expression of the glucocorticoid receptors in normal corticotropin cells, while the low or no expression of the glucocorticoid receptors in tumor cells leads to their tolerance

TABLE 5 Relationship between tumor type and clinical information.

Categories			CCA	non-CCA	total	χ^2	t	*p*
Gender		Male	26	17	43	4.284		0.038*
		Female	59	80	139			
Age (y)			47.5	45.5			0.7046	0.4823
Clinical Manifestation	Cushing's Syndrome	yes	58	18	76	40.749		<0.0001*
		no	32	79	111			
	Visual Defect	yes	31	49	80	4.926		0.026*
		no	59	48	107			
	Headache	yes	28	25	53	0.655		0.418
		no	62	72	134			
Hormonal Dysfunction	Hypercortisolemia	yes	37	8	45	43.923		<0.0001*
		no	30	89	119			
	Elevated ACTH	yes	49	18	67	48.851		<0.0001*
		no	18	79	97			
Biological Characteristics of Tumor	Suprasellar Extension	yes	47	58	105	0.372		0.542
		no	26	39	65			
	Cavernous Sinus Extension	yes	36	49	85	0.024		0.877
		no	37	48	85			
	Sphenoid Sinus Extension	yes	6	34	40	16.667		<0.0001*
		no	67	63	130			
	Posterior Fossa Extension	yes	6	16	22	2.532		0.112
		no	67	81	148			
	Relapsed	yes	38	38	76	4.904		0.027*
		no	29	59	88			
	Diameter (mm)		33.9	26.0			2.55	0.0121*

*, implies a statistically difference.

to hypercortisolemia (12). Studies have confirmed that the keratin filaments accumulated in Crooke-like *ACTH* cells are *CK8* and *CK18* (39). Some studies have shown that *CK20* is also involved in the formation of Crooke-like deposition. According to 2022 WHO classification guidelines (36) and previous case reports, *CK8/18* immunostaining is described as a "perinuclear and ring-shaped" change. In 2003, George et al. (13) published a study with the largest number of cases to date. They took the positive rate of *CK8/18* circular immunostaining > 50% as a diagnostic criterion for CCAs, which is still in use today.

Pathological diagnosis of CCA depends on the results of typical histology and immunostaining. In a typical Crooke's cell adenoma, more than 50% of tumor cells exhibit obvious intracytoplasmic circular hyalines stained by HE and *CK8/18* immunostaining, lack of intact reticulin scaffold, and positive *T-PIT* immunostaining (13). In addition, there is an eccentric rhabdoid keratin staining with neoplastic Crooke's cells, although circular enhancement is the earliest and most characteristic change (21). Our medical records also show that semi-circular and strip-shaped keratin staining co-exists with circular enhancement in variable proportions. We note that the circular characteristic of *CK8/18* staining is not the only way of expression, and the types of semi-cyclic and strip staining should attract the attention of researchers as well. It is not an unusual phenomenon that our cases contain various semi-ringlike *CAM 5.2* positive cells (Figures 1D, 3D, 5D, 7D). The *Ki-67* is even as high as 40% in case1, reminding us that despite the existence of atypical Crooke-like cells, the invasiveness of the tumor still needs the close attention of researchers. In addition, the proportion of Crooke-like positive cells is worthy of further study. Conversion of adrenocorticotropic hormone cells to Crooke's cells is reported to account for up to 25% of 52% of *ACTH* adenomas (40). Another study found that in 177 patients with neuroendocrine tumors with positive corticotropin staining confirmed by histology and 213 patients with Cushing's syndrome diagnosed by pituitary surgery, about 74% of the tumor samples experienced Crooke-like changes (41). Also, it has been suggested that as long as the anterior lobe biopsy measures more than 25% of Crook's hyaline deposition, it may indicate that the functional recovery of the HPA axis is slower after the operation (25, 42). A recent study pointed out that due to local infiltration and growth, non-tumor tissue is very common in *ACTH* tumor specimens, and how to identify normal tissue in pathological diagnosis is very important (39). It is not known whether different percentages of Crooke's cells indirectly indicate the degree of tumor invasion, but it can be predicted that once Crooke-like hyaline change occurs in

tumor tissues, its invasive behavior is also stronger than that of general *ACTH* adenomas (9, 43), which deserves the continuous attention of clinicians. Combined with previous literature reports, considering the formation of Crooke's cells is a long-term outcome of hypercortisolemia, which makes us wonder: is "50%" in the previous diagnostic criteria appropriate? After all, Crooke's cell tumor is a highly invasive tumor, and its early diagnosis has a positive sign for the clinical prognosis of the disease.

Treatment of CCAs remains challenging due to its aggressive nature and high recurrence rate. According to the case summaries in our center and the literatures, the diameter of the CCAs is significantly larger than that of non-CCAs' (Tables 1 and 5). Unlike the proportion reported, all CCAs from our center were male (Table 1). In general, the surgical treatment goal of CCAs is still gross-total resection. However, despite the effectiveness of surgical resection, its recurrence rate remains as high as 56.7% (38/67) and the success rate of reoperation is low: case 1 died of severe complications despite multiple treatments, while global tumor-related mortality reached 8.7% (6/69). Radiotherapy may be considered for patients with postoperative recurrence, postoperative residues, or strong invasiveness. A recent study (44) reported that 40% of the patients who received radiotherapy had a 30% reduction in tumor volume, and there was no tumor recurrence or growth during the 12-month follow-up, no complications of radiotherapy, and no patients experienced a second dose of radiotherapy. In this study, radiotherapy is used as an adjuvant for surgery rather than as an independent treatment, since all patients have received at least one surgical treatment. Temozolomide (TMZ), a first-line drug against glioma, is also effective in treating refractory pituitary adenomas. One study revealed that the overall effective rate of TMZ in the treatment of refractory pituitary adenomas was 45%, and 27% of people were in stable condition (26). *ACTH* adenomas, particularly invasive tumors, have a low level of *MGMT* (18), which is an indication for TMZ treatment. However, some studies have shown that as the malignant degree of CCA increases, its *MGMT* level increases, and the effectiveness of TMZ treatment decreases. The effectiveness of TMZ has received positive signals in clinical practice. At least 18-33 months (22, 24) after discontinuation of TMZ treatment, the disease is still in remission, and these data demonstrate the safety and effectiveness of discontinuing temozolomide treatment. A recent study confirmed that no tumor recurrence was found in the seven years after TMZ treatment (30). In addition to using the level of *MGMT* as a reference for tumor treatment, genetic mutations and small molecule RNA also provide potential entry points for tumor treatment. Kyohei Hayashi et al. (45) reviewed 60 cases of *ACTH* adenoma (including 15 cases of Crooke's cell adenoma). They found that the *USP8* mutation rate was high and the downstream *POMC* content was also increased in microadenomas, while macroadenomas such as CCAs had a low *USP8* mutation rate accompanied by decreased expression of *POMC* and *MGMT*, suggesting the suitability of CCAs for TMZ therapy. Garbicz et al. (46) found that *miR-106b-25* and its host gene *MCM7* are potential novel biomarkers of invasive corticotropin immunopositive pituitary adenomas. We have a number of *T-PIT* cases in our NFPAs, and the previously reported anti-oncogene *MEG3* (47) may also serve as a breakthrough in treatment. The percentage of *T-PIT*-positive in NFPAs reported in our center is close to the results of a Japanese study (26.9%) (48), but higher than the prevalence reported in previous literature (5.0-19.0%) (49). The reasons for this result were considered to be caution in diagnoses making and a decrease in the number of asymptomatic patients seeking medical care.

Conclusion

CCA is a rare pituitary adenoma that has received significant attention because of its aggressive nature and high recurrence rate. It should be assisted by a multidisciplinary consultation to deal with this particular type of tumor from the initial stages rather than once the recurrence has already occurred. However, the percentage of tumors immunopositive for keratin and their presentation status are confronted with clinical diversity, and very few studies involving the exploration of their pathogenesis have been performed. Therefore, more cases need to be investigated to further reveal the clinical features of CCA and its underlying mechanisms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DZ: literature review and draft writing; ZW: clinical data analysis; TT and XW: clinical data collection; DH and YZ: manuscript revision; DL and HW: pathological sections analysis and research design. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.947085/full#supplementary-material

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Outcome of CRH stimulation test and overnight 8 mg dexamethasone suppression test in 469 patients with ACTH-dependent Cushing's syndrome

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Objective: To evaluate diagnostic accuracy of the corticotropin-releasing hormone (CRH) stimulation test and the overnight 8 mg dexamethasone suppression test (DST) for the differentiation of Cushing's disease (CD) and ectopic Cushing's syndrome (ECS).

Methods: Retrospective study in 6 European centers. Inclusion criteria: patients with a) overt adrenocorticotropin (ACTH)-dependent Cushing's syndrome at the time of dynamic testing, b) histopathological confirmed tumors and/or c) postoperative biochemical remission and/or adrenal insufficiency. Optimal cut-offs were calculated via receiver operating characteristic (ROC) analysis using CD as reference.

Results: 469 patients were analyzed [78% females; median age 43 years (IQR 19)]. CRH test and overnight 8 mg DST were performed in 420 [CD, n=394 (94%); ECS, n=26 (6%)] and 237 patients [228 CD (96%), 9 ECS (4%)]. Both tests were performed in 205 patients (44%). The post-CRH %-increase at 30 minutes of both ACTH (cut-off ≥31%, sensitivity 83%, specificity 85%, AUC 0.81) and cortisol (cut-off ≥12%, sensitivity 82%, specificity 89%, AUC 0.86) discriminated best between CD and ECS. A test duration of >60 minutes did not improve diagnostic performance of the CRH test. The optimal cortisol cut-off for the %-suppression during the 8 mg DST was ≥55% (sensitivity 80%, specificity 78%, AUC 0.75).

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Conclusion: The CRH test has equivalent sensitivity but higher specificity than the 8 mg DST and is therefore the test of first choice. The diagnostic outcome of ACTH and cortisol is well comparable, however, sampling beyond 60 minutes post-CRH does not provide diagnostic benefits.

KEYWORDS

ACTH, Cushing's disease, Cushing's syndrome, CRH stimulation test, diagnosis, ectopic, endogenous hypercortisolism, high dose dexamethasone suppression test

Introduction

Adrenocorticotropin (ACTH) dependent glucocorticoid excess is the most frequent cause of endogenous Cushing's syndrome. The underlying ACTH source can be located either in the pituitary (so called Cushing´s disease, CD) or - less likely - extra-sellar, with most tumors being found in the lungs (so called ectopic Cushing's syndrome, ECS) (1, 2).

Appropriate tumor localization is crucial for adequate treatment. The major limitation of imaging is that the respective tumoral lesions are usually small and therefore difficult to detect. For instance, in 30-50% of patients with CD, pituitary adenomas are initially not identified via sellar magnetic resonance imaging (MRI) (3, 4). Similarly, ectopic tumors are initially overseen in about 50% of cases (5). Furthermore, approximately 10% of the general population (6, 7) and more than 20% of patients with ECS (8) are reported to carry pituitary 'incidentalomas' (with the consequence of false-positive MRI results).

A thorough biochemical workup is mandatory to establish the source of ACTH hypersecretion. The baseline ACTH concentration is relatively easy to obtain and is usually remarkably higher in ECS than in CD patients (9, 10). Nevertheless, this parameter alone does not allow for a reliable differential diagnosis (10, 11). In contrast, bilateral inferior petrosal sinus sampling (BIPSS), the gold-standard for the differentiation of ACTH-dependent Cushing's syndrome (12, 13), is a challenging and invasive procedure potentially leading to severe complications and a high radiation exposure (14, 15).

Accordingly, a step-by-step differential diagnosis is suggested (1, 16). After initial confirmation of ACTH-dependent Cushing's syndrome, dynamic function tests like the corticotropin-releasing hormone (CRH) stimulation test and variants of the high-dose dexamethasone suppression test (DST) such as the overnight 8 mg DST are suggested to identify persistent pharmacodynamic effects that are typical for CD (i.e., stimulation of ACTH and cortisol by CRH, and suppression of cortisol by high doses of dexamethasone) (11, 17). Although both dynamic function tests are well established, some substantial discrepancies, especially regarding the cut-offs and test protocols applied, were described (9, 18–25). Furthermore, the number of reported patient with CD (ranging from 49 to 288) and ECS (ranging from 7 to 27) was limited.

The aim of this study was to evaluate the diagnostic performance of the CRH stimulation test and the overnight 8 mg DST (either alone or in combination) in a large series of patients with confirmed ACTH-dependent Cushing's syndrome.

Subjects and methods

Participating centers and ethical considerations

This multicenter study was conducted in accordance with the local ethical committees of the participating centers (local ethics committee approval numbers 85/12 in Wurzburg and Berlin, NCH-01-21 in Milan, 152-10 in Munich, 353/2013BO2 in Tubingen, and 1457/2016 in Vienna).

Subjects

Patients with ACTH-dependent Cushing's syndrome who were diagnosed between 1984 and 2020 according to established criteria (26) were retrospectively reviewed. Those who underwent a stimulation test with administration of human CRH and/or an overnight 8 mg DST [with a single dose of 8 mg dexamethasone administered p.o. at 11.00 p.m. (27)] were considered eligible for the current evaluation. A subset of 96 patients (CD, n=78; ECS, n=18) from Munich was already published elsewhere (23).

Standard operating procedures for the dynamic testing procedures

Only dynamic testing procedures that were performed according to standardized protocols were taken into account. CRH stimulation tests had to be carried out in the morning, with

blood sampling for serum cortisol and plasma ACTH at -15 and 0 minutes, and 15, 30, 45, 60, 90, and 120 minutes after injection of 100 µg of synthetic human CRH (as shown in Supplementary Table 1, the distinct time points slightly differed from center to center). With respect to the overnight 8 mg DST, a baseline sample for measurement of serum cortisol was obtained between 8.00 and 9.00 a.m. Afterwards, 8 mg dexamethasone were administered as a single dose p.o. at 11.00 p.m., followed by blood sampling for serum cortisol measurement between 8.00 and 9.00 a.m. the next morning.

Biochemical analysis

Plasma ACTH was measured by Siemens Immulite 2000 XPi (in Berlin, Tübingen, and Würzburg), Nichols Advantage ACTH assay (in Milan), DiaSorin Liaison (in Munich), and Roche Cobas (in Vienna). Serum cortisol was determined by Siemens Immulite 2000 XPi (in Berlin and Würzburg), DiaSorin Liaison (in Munich), Siemens ADVIA Centaur XPT (in Tübingen), and Roche Cobas (in Vienna). In Milan, the Tosoh Bioscience AIA-PACK CORT immunoassay was used for cortisol analysis until 2016; afterwards, the Roche Elecsys was applied.

Interpretation of the biochemical baseline assessment and the two dynamic testing procedures

The biochemical results were interpreted as follows: a) analysis of ACTH and cortisol at baseline; b) post-CRH %-increase of ACTH and cortisol over baseline; c) post-CRH peak of ACTH and cortisol; d) post-dexamethasone %-suppression of cortisol. For this, newly generated cut-offs were applied; their diagnostic accuracy was compared to already published cut-offs for the CRH stimulation test and the overnight 8 mg DST.

Statistical analysis

Statistical analyses were performed with SPSS version 26 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 8 (GraphPad, San Diego, CA, USA). Data are presented as median and interquartile range (IQR). Comparisons between CD and ECS were performed with Mann-Whitney-U-test for non-normally distributed metrically scaled variables and Pearson Chi-Square for dichotomous categorical variables. For comparisons of the different study centers, Kruskal-Wallis-test for non-normally distributed metrically variables were carried out. To calculate optimal cut-offs and the associated sensitivities, specificities, and areas under the curve (AUC), receiver operator characteristic (ROC) analyses were performed, using CD as reference. In addition, the diagnostic outcome was evaluated with the Youden's index (J = sensitivity + specificity-1).

Results

Clinical characteristics of the study cohort

Out of the entire retrospective cohort of 616 patients with ACTH-dependent Cushing's syndrome (Tübingen, n=167 (27%); Munich, n=149 (24%); Vienna, n=118 (19%); Würzburg, n=108 (18%); Milan, n=47 (8%); Berlin, n=27 (4%)), 556 (90%) underwent a CRH stimulation test and/or an overnight 8 mg DST. In 469 (84%) of these patients, diagnostic confirmation was achieved either by histopathology or by the clinical outcome after surgery (i.e., biochemical remission according to common screening tests for Cushing's syndrome and/or temporary adrenal insufficiency). Of note, only this 'gold standard' cohort of 469 patients was taken into account for the calculation of cut-offs and further analyses (clinical characteristics are provided in Table 1).

Basal screening parameters

As outlined in Table 1, all biochemical screening parameters were significantly higher in ECS than in CD patients. However, there were a remarkable overlap, and neither single screening parameters nor a combination of several screening parameters was able to differentiate well between CD and ECS (data not shown).

CRH stimulation test

A CRH stimulation test was performed in 420 patients (CD, n=394 (94%); ECS, n=26 (6%)). Of note, the sampling time points -15 and 45 minutes were excluded from further analyses because only few samples were collected at these time points. As shown in Supplementary Figures 1, 2, all six centers demonstrated a well-comparable test pattern (significant differences between the centers were only observed for ACTH at 90 minutes).

As shown in Figure 1, CD patients demonstrated substantial post-CRH responses of both ACTH and cortisol, with peak levels for ACTH at 15 minutes (median %-increase from baseline 120%, IQR 169%; Figure 1A) and for cortisol at 30 minutes (median %-increase from baseline 44%, IQR 55%; Figure 1B). In contrast, ECS patients demonstrated no relevant post-CRH changes of ACTH and cortisol. Supplemental Table 2 provides the individual responses of ACTH and cortisol during the CRH-stimulation test for all ECS patients.

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TABLE 1 Clinical characteristics of patients with histologically or post-surgically confirmed diagnosis of Cushing's disease or ectopic Cushing's syndrome.

	CD	ECS	p-value
Clinical characteristics			
Subjects [n (%)]	440 (94%)	29 (6%)	–
Females [n (%)]	348 (79%)	17 (59%)	<0.05
Age (years) [median (IQR)]	43 (19)	41 (33)	n.s.
Body mass index (kg/m²) [median (IQR)]	28 (8)	28 (8)	n.s.
Source of ACTH-dependent Cushing's syndrome*			
Pituitary gland [n (%)]	440 (100%)	–	–
Lung [n (%)]	–	22 (76%)	–
Pancreas [n (%)]	–	4 (14%)	–
Others ** [n (%)]	–	3 (10%)	–
Confirmatory diagnostics			
Histology [n (%)]	359 (82%)	26 (90%)	n.s.
Post-operative remission and/or post-operative adrenal insufficiency [n (%)]	81 (18%)	3 (10%)	n.s.
Biochemical screening tests			
ACTH (pg/ml) *** [median (IQR)]	61 (46)	116 (111)	<0.001
Serum cortisol after 1 mg DST (nmol/l) [median (IQR)]	400 (375)	800 (761)	<0.001
24-hour urinary free cortisol (µg/d) **** [median (IQR)]	344 (454)	1634 (1906)	<0.001
Late-night salivary cortisol (nmol/l) [median (IQR)]	19 (22.0)	117 (139)	<0.001
Late-night serum cortisol (nmol/l) [median (IQR)]	477 (287)	811 (681)	<0.01

Data regarding biochemical screening tests were available from: ACTH, n = 469 (100%); 1 mg DST, n = 404 (86%); 24-hour urinary free cortisol, n = 402 (86%); late-night salivary cortisol, n = 161 (34%); late-night serum cortisol, n = 129 (28%).
ACTH, adrenocorticotropin; CD, Cushing's disease; CRH, corticotropin-releasing hormone; DST, dexamethasone suppression test; ECS, ectopic Cushing's syndrome; IQR, interquartile range; n.s., not significant.
*data on individual tumor grade not systematically assessed. ** each one case with a thymus carcinoma, a pheochromocytoma, and an esthesioneuroblastoma; *** collected outside the CRH stimulation test; **** despite indisputable between-center variation in biochemical analysis, 24-hour urinary free cortisol is provided for transparency.

Firstly, baseline levels of ACTH and cortisol were evaluated (i.e., before CRH administration). ROC analysis revealed an optimal cut-off of 110 pg/ml for baseline ACTH (sensitivity 89%, specificity 58%; AUC 0.70) and of 883 nmol/l for baseline cortisol (sensitivity 87%, specificity 58%; AUC 0.72) (Table 2).

Secondly, the CRH-responses of ACTH and cortisol were analyzed. Figures 2, 3 show the individual post-CRH %-increases of ACTH and cortisol throughout the test, along with the corresponding optimal cut-offs and ROC curves. Furthermore, Table 2 provides the diagnostic outcome of the optimal cut-offs for the post-CRH %-increase of ACTH and cortisol. For CRH-stimulated ACTH, the cut-off with the highest Youden's index was ≥31% at 30 minutes (sensitivity 83%, specificity 85%, AUC 0.81) (Table 2). The optimal cut-off for the post-CRH %-increase of cortisol was calculated as ≥12% at 30 minutes (sensitivity 82%, specificity 89%, AUC 0.86) (Table 3).

Thirdly, the diagnostic outcome of different test durations was assessed (taking the post-CRH levels of ACTH and cortisol from the 5 sampling time points 15, 30, 60, 90 and 120 minutes into account). For ACTH levels, AUC values gradually decreased from 0.82 at 15 minutes to 0.58 at 120 minutes (emphasizing a lower discriminatory power at later time points). Although less pronounced, AUC values for cortisol also decreased over time (Tables 2, 3). Samples taken beyond 60 minutes allowed identification of 5 additional CD patients (2 with ACTH, 3 with cortisol) but also led to 5 false-positive ECS patients (3 with ACTH, 2 with cortisol).

Finally, the post-CRH peaks of ACTH and cortisol were analyzed. In terms of sensitivity, specificity, and AUC, however, post-CRH peaks of both parameters demonstrated a rather poor diagnostic outcome (Tables 2, 3).

Overnight 8 mg dexamethasone suppression test

The overnight 8 mg DST was conducted in 237 patients (228 CD (96%), 9 ECS (4%)). The median %-decrease of cortisol after 8 mg dexamethasone was 80% (IQR 26%) in patients with CD, and 40% (IQR 71%) in patients with ECS, respectively. As illustrated in Table 4 and Figure 4, ROC analysis revealed an optimal cut-off of ≥55% (sensitivity 80%, specificity 78%, AUC 0.75).

The outcome of a published cut-off of ≥50% for the %-suppression of cortisol during the overnight 8 mg DST was also evaluated. In our cohort, a comparable sensitivity (83% vs. 80%) and an identical AUC (0.75) but a lower specificity (67% vs. 78%) compared to our newly calculated cut-off of ≥55% were observed.

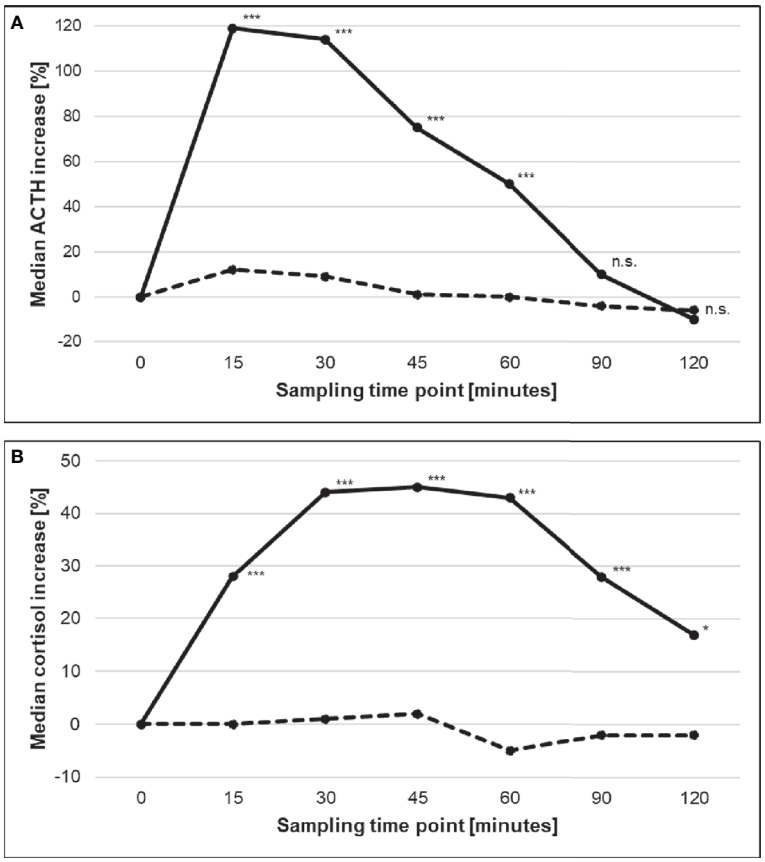

FIGURE 1
Median %-increases of **(A)** ACTH and **(B)** cortisol during the CRH stimulation test in patients with CD (solid line) and ECS (dashed line). Stars indicate statistical significant differences between both sub-entities (* <0.05; *** <0.001). ACTH, adrenocorticotropin; CD, Cushing's disease; CRH, corticotropin-releasing hormone; ECS, ectopic Cushing's syndrome. n.s., not significant

Combination of ACTH and cortisol during the CRH stimulation test

As outlined in Table 5, the combined analysis of ACTH and cortisol during the CRH stimulation test did not reveal any diagnostic benefit (as illustrated by comparable results for sensitivity, specificity, AUC, positive predictive value, and negative predictive value) compared to the analysis of any of these two parameters alone.

Combination of the CRH stimulation test and the overnight 8 mg dexamethasone suppression test

Both dynamic testing procedures were carried out in 205 patients (197 CD (96%), 8 ECS (4%)). Overall, various combinations of the CRH stimulation test (i.e., with ACTH only, with cortisol only, or with both ACTH and cortisol) and the overnight 8 mg DST had comparable discriminatory power to the single tests (Table 5). However, if at least one of the two tests (i.e., either the CRH stimulation test or the overnight 8 mg DST) indicated CD, the correct diagnosis was established in 93.0-96.0% of cases (as shown in Supplementary Figure 3).

Discussion

CRH stimulation test and overnight 8 mg DST are dynamic testing procedures widely applied for the differentiation of ACTH-dependent CS (1, 9, 23, 28). In our study, we investigated the diagnostic outcome of both tests in a large number of well-characterized patients with confirmed diagnoses. We observed that ACTH and cortisol responses during the CRH stimulation test had comparable diagnostic value, and that sampling beyond 60 minutes after CRH stimulation did not provide diagnostic benefits. The overnight 8 mg DST demonstrated equivalent sensitivity but lower specificity. If both dynamic testing procedures (i.e., the CRH

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TABLE 2 Diagnostic outcome of ACTH during the CRH stimulation test.

ACTH	Time (min)	Cut-off	J	Sens. (%)	Spec. (%)	AUC	PPV (%)	NPV (%)	p-value
Baseline level (pg/ml)	0	110	0.47	89	58	0.70	97	25	–
Post-CRH %-increase	15	≥55%	0.58	73	85	0.82	99	19	<0.001
	30	≥31%	0.68	83	85	0.81	99	25	<0.001
	60	≥14%	0.56	71	85	0.76	99	18	<0.001
	90	≥14%	0.35	48	87	0.62	97	15	n.s.
	120	≥17%	0.20	29	91	0.58	97	12	n.s.
Post-CRH peak level (pg/ml)	–	160	0.15	42	73	0.56	96	8	–

ACTH, adrenocorticotropin; AUC, area under the curve; CRH, corticotropin-releasing hormone; J, Youden's index; NPV, negative predictive value; n.s., not significant; PPV, positive predictive value; Sens., sensitivity; Spec., specificity.

test with any parameter and the overnight 8 mg DST) were carried out simultaneously and any test outcome indicated CD, this was true in ≥ 93.0% of cases.

The CRH stimulation test is considered the most reliable non-invasive dynamic test in differentiating CD and ECS (9, 23, 25). Recently, high sensitivities for the non-stimulated baseline parameters were reported in a series of 101 patients with ACTH-dependent Cushing's syndrome (87% for ACTH vs. 93% for cortisol) (29). Although we observed comparable sensitivities (89% for ACTH vs. 87% for cortisol), specificity was remarkably lower (each 58% in our study vs. reported data of 69% for ACTH and 93% for cortisol). Accordingly, we have the impression that additional CRH stimulation appears justified.

In our series, the optimal cut-offs for the post-CRH %-increase at 30 minutes (≥31% for ACTH and ≥12% for cortisol) demonstrated comparable sensitivity (83% vs. 82%) and only moderate differences in specificity (85% vs. 89%).

Compared to the literature, however, the post-CRH %-increases of ACTH that were observed in our study had remarkably lower specificities despite similar sensitivities (9, 22, 23, 28). A possible explanation is certainly the limited number of ECS patients in other studies, making false-positive results per se less likely. In fact, it is well known that some neuroendocrine tumors and bronchial carcinoids (despite excessively high ACTH levels) still respond to a CRH stimulus (30), and this was also true for 8 (31%) of our ECS patients. In particular, four ECS cases with low baseline levels of ACTH and cortisol showed a remarkable post-CRH increase of both parameters, what is possibly related to a diminished negative feedback inhibition of the hypothalamus-pituitary-adrenal axis (which is considered to be a typical feature in ECS) (23, 31). On the contrary, four other ECS cases had incongruent results (post-CRH increase only of cortisol, n=3; post-CRH increase only of ACTH, n=1), most likely reflecting false-positive results (e.g. due to multiple sampling time points, as a tendency towards higher ACTH and cortisol levels was observed over time).

A pertinent finding of our study is the diagnostic value of cortisol analysis during the CRH stimulation test, a result that is different to a former manuscript on a subgroup of our current study cohort (23). Nevertheless, our current findings have also been reported by others (9, 18, 22, 24, 32). In two studies involving stimulation with human CRH (as in our study), post-CRH cortisol cut-offs of ≥14% (9) and ≥17% (24) resulted in sensitivities of 85% and 90%, and specificities of 100% and 85%, respectively. Although both reported cut-offs are well comparable to our current cut-off of ≥12%, discrepancies regarding sensitivity and specificity may possibly be explained by a) the remarkably lower number of CD patients in former publications (i.e., 101 and 167 in former vs. 420 in this series) and b) the different study outlines (e.g. overall instead of time-point specific analysis of the %-increase) (24). Regarding the latter point, for instance, the trend in ECS patients towards higher ACTH and cortisol levels over time (that was already mentioned above) may result in more false-positive results if overall instead of time-point specific cut-offs are applied.

With respect to other studies (20, 24, 31), contradictory results regarding the analytical merits of cortisol during the CRH-stimulation test may also be explained by the use of ovine CRH instead of human CRH. According to some authors, ovine CRH results in a prolonged and more pronounced response of both ACTH and cortisol due to a longer plasma half-life and a lower metabolic clearance rate (22, 33). On the other hand, other studies reported comparable effects of human and ovine CRH (20, 21). A direct comparison between the two compounds would certainly be of interest, however, their commercial availability is limited (oCRH is not available in Europe and United States, and hCRH is not available in the United States).

Considering that the maximal discriminatory power of post-CRH %-increase of ACTH and cortisol was achieved at 30 minutes, it is our impression that a duration of the test beyond 60 minutes does not appear to be useful. This confirms what was already reported elsewhere (9, 23, 34).

The high-dose DST represents an alternative to the CRH stimulation test in the in the differentiation of CD and ECS (28). Several protocols are known, but the overnight 8 mg DST represents one of the most widely applied variants. Confirming what has been already reported by others (19, 23), we observed that this test demonstrated lower diagnostic accuracy than the CRH stimulation test (hence, this procedure is also not recommended in a current consensus paper (16)). With respect to our study, application of the newly generated optimal cut-offs led to similar

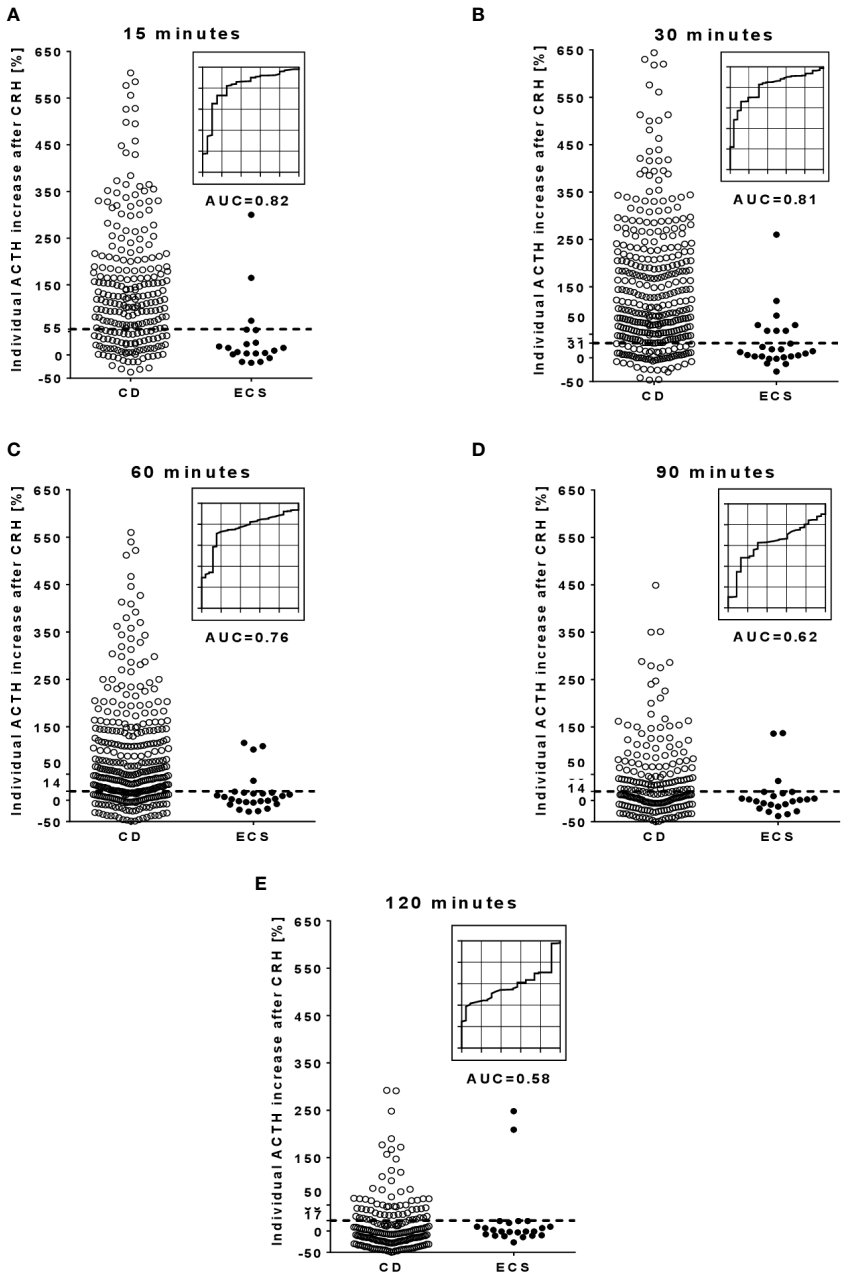

FIGURE 2
Individual %-increase of ACTH after CRH and corresponding ROC curves at different time points during the CRH stimulation test. (**A**, at 15 minutes; **B**, at 30 minutes; **C**, at 60 minutes; **D**, at 90 minutes; **E**, at 120 minutes). The dotted lines in the scatter plots illustrate the optimal cut-off for the post-CRH %-increase of ACTH. Few outlier results are not reported in the scatter plots: 13 CD patients at 15 minutes, 20 CD patients at 30 minutes, 11 CD patients at 60 minutes, and 3 CD patients at 90 minutes. ACTH, adrenocorticotropin; AUC, area under the curve; CD, Cushing's disease; CRH, corticotropin-releasing hormone; ECS, ectopic Cushing's syndrome; ROC, receiver operator characteristic.

sensitivities (overnight 8 mg DST: 80%; CRH stimulation test: 83% for ACTH, 82% for cortisol), however, specificity was lower (overnight 8 mg DST: 78%; CRH stimulation test: 85% for ACTH, 89% for cortisol). A possible explanation might be the persistent dexamethasone responsiveness of some neuroendocrine tumors (30, 35). Of note, the low number of our ECS patients undergoing an overnight 8 mg DST (n=9) certainly represents a relevant limitation of our current analysis.

Our suggested optimal cut-off of ≥55% for dexamethasone suppressed serum cortisol was well comparable to a published threshold of ≥53% (19). Both cut-offs, however, demonstrated a remarkably discrepant diagnostic outcome, as illustrated by

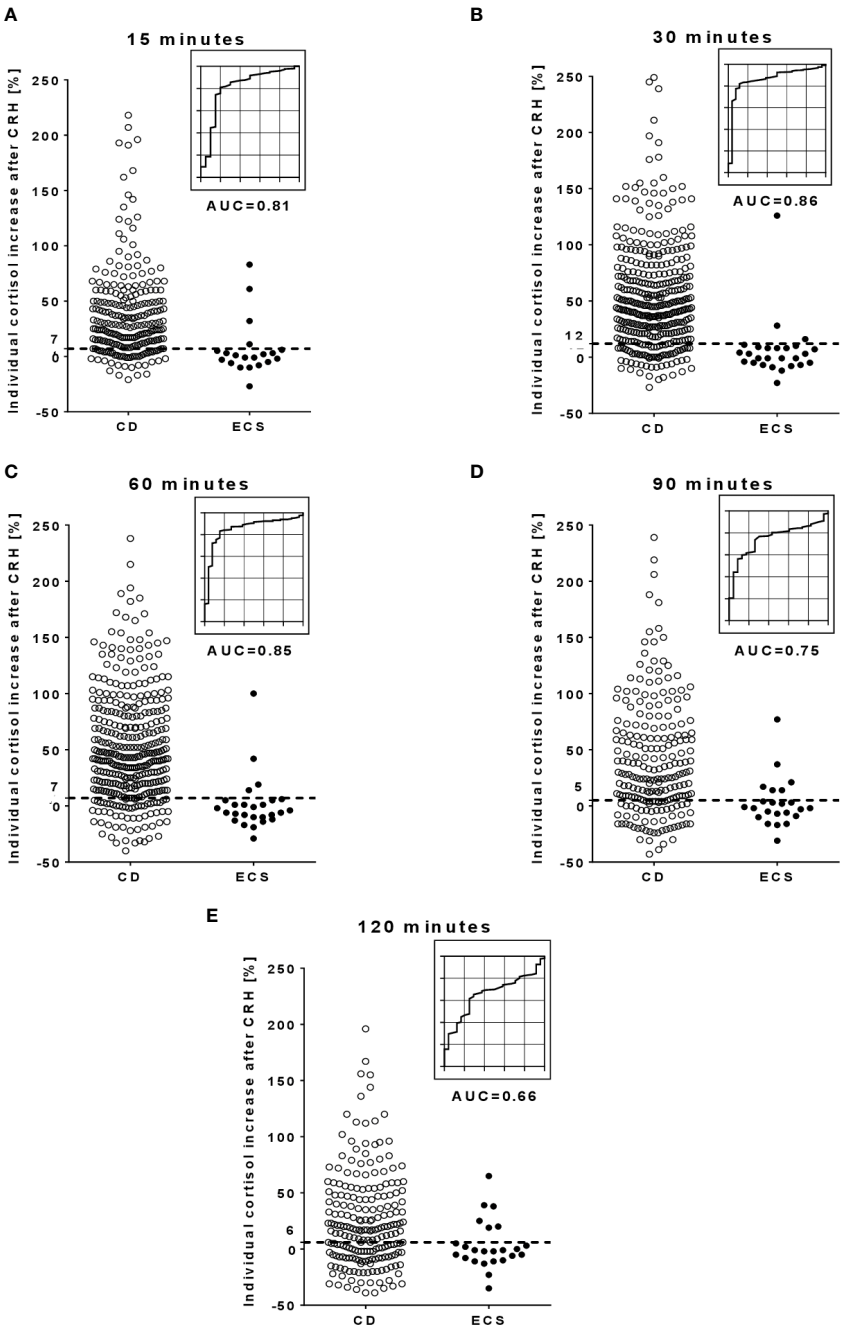

FIGURE 3
Individual %-increase of cortisol after CRH and corresponding ROC curves at different time points during the CRH stimulation test. (**A**, at 15 minutes; **B**, at 30 minutes; **C**, at 60 minutes; **D**, at 90 minutes; **E**, at 120 minutes). The dotted lines in the scatter plots illustrate the optimal cut-off for the post-CRH %-increase of cortisol. Few outlier results are not reported in the scatter plots: 1 CD patients at 15 minutes, 7 CD patients at 30 minutes, 9 CD patients at 60 minutes, 4 CD patients at 90 minutes, and 2 CD patients at 120 minutes. AUC, area under the curve; CD, Cushing's disease; CRH, corticotropin-releasing hormone; ECS, ectopic Cushing's syndrome; ROC, receiver operator characteristic.

sensitivities of 80% and 88%, and specificities of 78% and 90%, respectively (with the lower values observed in our own study). However, if we applied the conventional cut-off of ≥50%, we identified a comparable sensitivity (of 83%) and specificity (of 67%) to what has been previously reported elsewhere (i.e., a sensitivity of 81%, and a specificity of 67%) (19).

Interestingly, if the CRH stimulation test and the overnight 8 mg DST were analyzed in combination, sensitivity and

TABLE 3 Diagnostic outcome of cortisol during the CRH stimulation test.

Cortisol	Time (min)	Cut-off	J	Sens. (%)	Spec. (%)	AUC	PPV (%)	NPV (%)	p-value
Baseline level (nmol/l)	0	883	0.45	87	58	0.72	97	21	–
Post-CRH %-increase	15	≥7%	0.61	81	80	0.81	98	26	<0.001
	30	≥12%	0.71	82	89	0.86	99	25	<0.001
	60	≥11%	0.69	78	91	0.83	99	21	<0.001
	90	≥7%	0.68	83	85	0.85	99	26	<0.001
	120	≥5%	0.48	74	74	0.75	97	22	<0.001
Post-CRH peak level (nmol/l)	–	1048	0.22	68	54	0.57	96	10	–

AUC, area under the curve; CRH, corticotropin-releasing hormone; J, Youden's index; NPV, negative predictive value; PPV, positive predictive value; Sens., sensitivity; Spec., specificity.

TABLE 4 Diagnostic outcome of the overnight 8 mg dexamethasone suppression test.

Cortisol	Cut-off	J	Sens. (%)	Spec. (%)	AUC	PPV (%)	NPV (%)	p-value
%-suppression	≥55%	0.58	80	78	0.75	99	14	<0.05

ACTH, adrenocorticotropin; AUC, area under the curve; overnight 8 mg DST, overnight 8 mg dexamethasone suppression test; J, Youden's index; NPV, negative predictive value; PPV, positive predictive value; Sens., sensitivity; Spec., specificity.

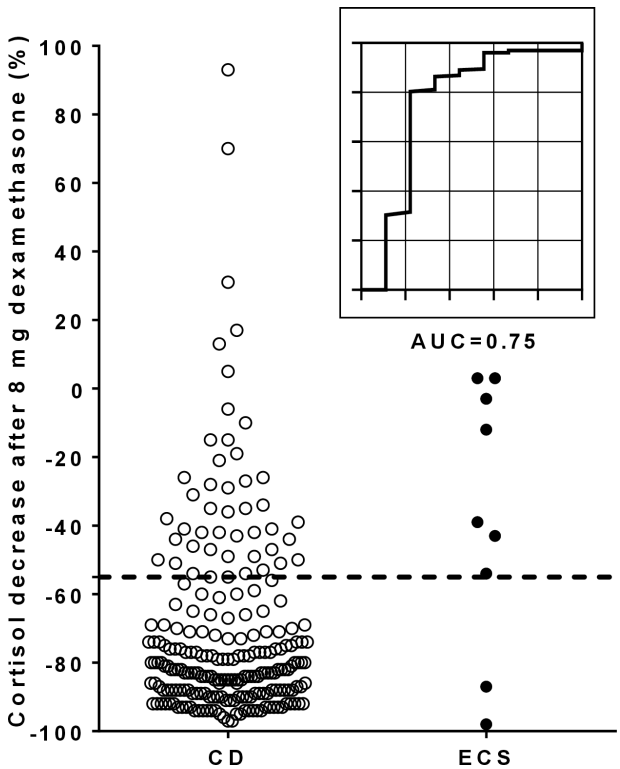

FIGURE 4
Individual %-suppression of cortisol after dexamethasone and corresponding ROC curve for the overnight 8 mg dexamethasone suppression test. The dotted line in the scatter plot illustrates the optimal cut-off of 55% for the %-suppression of cortisol after dexamethasone. One CD patient with an outlier result of 628% is not reported in the scatter plot. AUC, area under the curve; CD, Cushing's disease; ECS, ectopic Cushing's syndrome; ROC, receiver operating characteristic.

TABLE 5 Diagnostic outcome of a) the combined analysis of ACTH and cortisol at each time point during the CRH stimulation test and b) various combinations of the CRH stimulation test and the overnight 8 mg suppression test.

	Time	J	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)
Combined analysis of ACTH and cortisol during the CRH stimulation test						
Optimal cut-offs for ACTH & cortisol during the CRH stimulation test*	15 min	0.47	67	80	98	17
	30 min	0.61	76	85	99	20
	60 min	0.44	67	77	98	15
	90 min	0.25	38	87	97	13
	120 min	0.04	26	78	92	10
Combinations of CRH stimulation test and overnight 8 mg dexamethasone suppression test						
ACTH at 30 min during the CRH stimulation test (≥31%) & overnight 8 mg DST (≥55%)		0.42	67	75	98	9
Cortisol at 30 min during the CRH stimulation test (≥12%) & overnight 8 mg DST (≥55%)		0.46	71	75	99	10
ACTH & Cortisol at 30 min during the CRH stimulation test & overnight 8 mg DST		0.39	64	75	98	9

ACTH, adrenocorticotropin; AUC, area under the curve; CRH, corticotropin-releasing hormone; overnight 8 mg DST, overnight 8 mg dexamethasone suppression test; J, Youden's index; NPV, negative predictive value; PPV, positive predictive value; Sens., sensitivity; Spec., specificity; * the newly generated optimal cut-offs for ACTH and cortisol are provided in Tables 2, 3.

specificity decreased substantially (to 64-71% and to 75%, depending on the particular combination), while the positive predictive value remained remarkably high (always ≥98%). In two other studies, higher sensitivities (of 76% and 81%) and specificities (of 89% and 100%) were reported (20, 36). This discrepancy could probably (at least in part) be explained by the highly variable numbers of patients with CD (ranging from 148 to 420) and ECS (ranging from 8 to 26) in the threes studies. Nevertheless, each single test obviously allowed for a better diagnostic outcome. Accordingly, one could argue that a diagnostic routine approach (with both testing procedures being carried out in each individual) appears questionable. Recently, however, it was shown that a concordant positive result to both dynamic tests may be sufficient to reliably diagnose CD in patients with negative MRI but subsequently confirmed small pituitary microadenomas (36). Furthermore, if both dynamic testing procedures were applied simultaneously and at least one test indicated CD, we observed that this finding was true in ≥93.0% of our cases. In other words, the vast majority of patients with CD who undergo pituitary surgery on the basis of such test combinations will be adequately treated.

Due to its retrospective and multicentric nature, our current study has certainly some important limitations (e.g. center-specific laboratory testing procedures, few individuals with both tests, some individuals with relatively low basal ACTH levels despite confirmed ACTH-dependent Cushing's syndrome). However, we have the impression that these aspects reliably reflect real-world settings. Furthermore, although some authors reported assay-specific spurious ACTH levels leading to diagnostic and therapeutic obstacles (37), the center-specific analytical methodology virtually remained the same over time (in particular, only one center changed its cortisol assay). One of the most relevant boundaries is probably the low number of ECS cases that were also not comparably distributed among the six study centers. Possible gender-specific differences in test outcomes could therefore not be evaluated (as only 17 females and 12 males with ECS were enrolled). The following facts are also relevant limitations: a) data on tumor grade was not systematically assessed (as a substantially variable secretion pattern of ACTH and probably also of CRH in high- and low-differentiated tumors has to be assumed); b) radiological procedures relevantly improved over time (possibly, some of our older ECS cases had a false-negative imaging); c) only a single baseline value before administration of hCRH was analyzed (and not a mean from the two time points -15 minutes and 0 minutes). Finally, it has to be pointed out that percent increases and their respective cut-offs always have to be interpreted with caution (and should be reserved for patients with baseline levels of ACTH and cortisol in a suspiciously elevated range).

In conclusion, ACTH and cortisol measurement 30 minutes after CRH stimulation showed a comparable diagnostic outcome. The overnight 8 mg DST has significantly lower specificity than the CRH stimulation test. Finally, a duration of more than 60 minutes for the CRH stimulation test does not provide substantial diagnostic benefits. Further diagnostic procedures (e.g. BIPSS) may be omitted in cases where both dynamic tests indicate CD, however, the final decision on the required means has to be made on an individual basis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by local ethics committee approval numbers 85/12 in

Wurzburg and Berlin, NCH-01-21 in Milan, 152-10 in Munich, 353/2013BO2 in Tubingen, and 1457/2016 in Vienna). All patients provided written informed consent.

Author contributions

TD designed the research. MD, VT, and TD performed the statistical analyses and drafted the manuscript. All authors collected samples and clinical data from patients, contributed to writing the manuscript, and approved the final version to be published.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.955945/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Center-specific outcome of ACTH analysis during the CRH stimulation test.

SUPPLEMENTARY FIGURE 2
Center-specific outcome of cortisol analysis during the CRH stimulation test.

SUPPLEMENTARY FIGURE 3
Diagnostic outcome of the combined analysis of the CRH stimulation test (with analysis of ACTH only, with analysis of cortisol only, and with analysis of both ACTH and cortisol) and the overnight 8 mg dexamethasone suppression test. The tables at the bottom of the graph provide details on incongruent test results. A '+' indicates a true positive test result (i.e., the ACTH source was correctly identified according to the pre-defined 'gold standard' criteria), whereas a '-' indicates a false negative test result (i.e., the ACTH source was falsely calssified according to the pre-defined 'gold standard' criteria).

SUPPLEMENTARY TABLE 1
Center-specific number of patients undergoing a CRH stimulation test.

SUPPLEMENTARY TABLE 2
Individual outcome of the CRH stimulation test in patients with ectopic Cushing´s syndrome.

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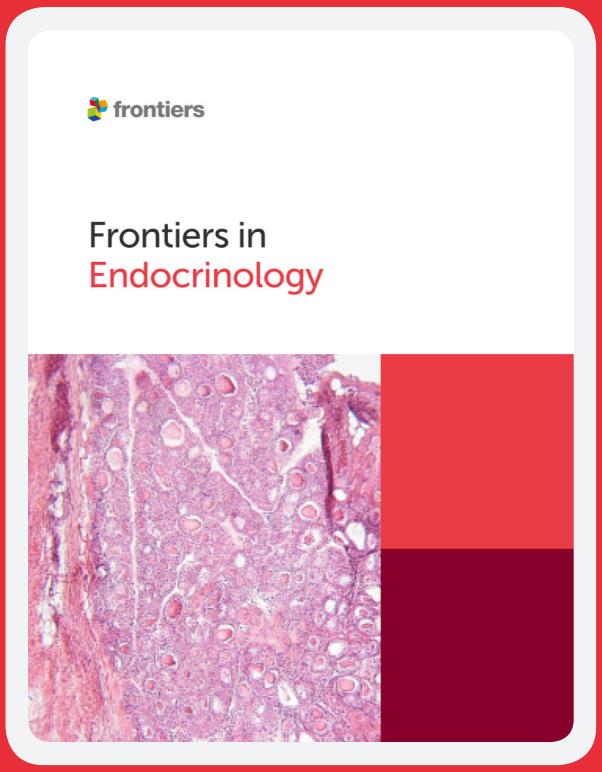

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