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Investigation of the Hypothalamo-pituitary-adrenal (HPA) axis: a contemporary synthesis

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Abstract
The hypothalamo-pituitary-adrenal (HPA) axis is one of the main components of the stress system. Maintenance of normal physiological events, which include stress responses to internal or external stimuli in the body, depends on appropriate HPA axis function. In the case of severe cortisol deficiency, especially when there is a triggering factor, the patient may develop a life-threatening adrenal crisis which may result in death unless early diagnosis and adequate treatment are carried out. The maintenance of normal physiology and survival depend upon a sufficient level of cortisol in the circulation. Life-long glucocorticoid replacement therapy, in most cases meeting but not exceeding the need of the patient, is essential for normal life expectancy and maintenance of the quality of life. To enable this, the initial step should be the correct diagnosis of adrenal insufficiency (AI) which requires careful evaluation of the HPA axis, a highly dynamic endocrine system. The diagnosis of AI in patients with frank manifestations is not challenging. These patients do not need dynamic tests, and basal cortisol is usually enough to give a correct diagnosis. However, most cases of secondary adrenal insufficiency (SAI) take place in a gray zone when clinical manifestations are mild. In this situation, more complicated methods that can simulate the response of the HPA axis to a major stress are required. Numerous studies in the assessment of HPA axis have been published in the world literature. In this review, the tests used in the diagnosis of secondary AI or in the investigation of suspected HPA axis insufficiency are discussed in detail, and in the light of this, various recommendations are made.

Keywords Hypothalamo-pituitary-adrenal (HPA) axis · Cortisol · Insulin tolerance test · ACTH stimulation test · Glucagon stimulation test · Metyrapone test

1 Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is one of the main components of the stress system. HPA axis dysfunction may be due to disorders involving the hypothalamus, pituitary or adrenals. As a result of cortisol deficiency, the body becomes unable to cope with stressful events which include infections, trauma, surgery or severe emotional stress. The diagnosis of adrenal insufficiency (AI) depends on a combination of a medical history, physical examination, and hormonal investigations including basal and dynamic tests. The diagnosis of secondary adrenal insufficiency (SAI) in patients with frank manifestations due to SAI is not challenging; these patients do not need dynamic tests and morning basal cortisol is usually enough to provide a correct diagnosis of SAI. Numerous studies in the assessment of HPA axis have been published in the literature since Thomas Addison's classical report on AI in 1855 [1, 2].

2 Physiology of the HPA axis

The neurons in the paraventricular nucleus (PVN) express corticotrophin-releasing hormone (CRH) and its co-secretagogue arginine vasopressin (AVP), and other neuropeptides that modulate the HPA axis [3]. The pituitary gland - which is designated as the 'master gland of the endocrine system' - is located under the hypothalamus and is connected to the hypothalamus via the pituitary stalk (infundibulum). Corticotroph cells in the anterior pituitary gland synthesise and secrete

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ACTH which stimulates the secretion of cortisol from the adrenal glands in response to stress, as well as increasing adrenal androgens [4]. In addition, plasma binding proteins, sex steroids, and the autonomic nervous system may all play some role in the regulation of the HPA axis.

Cortisol negatively regulates CRH, AVP and ACTH synthesis and secretion [5]. The negative feedback effect of cortisol occurs via mineralocorticoid receptors (MRs) and/or glucocorticoid receptors (GRs) located at multiple sites in the brain and in the pituitary, with rapid and slow both genomic and non-genomic effects [6].

3 Investigation of the HPA axis

3.1 Medical history and physical examination

Clinical judgment is still the most valuable tool in the initial suspicion of AI, but not enough on its own as a tool in the diagnosis of AI. Nevertheless, it is still important in spite of remarkable technical progress in the measurement of basal cortisol, ACTH and the other relevant hormones, together with a variety of hormonal stimulation tests. In mild to moderate cases, postural hypotension and tachycardia, fatigue, anorexia, weight loss, decreased libido, hypoglycaemia, and eosinophilia may be seen. Vascular collapse, which may be fatal, can be seen in patients in adrenal crisis since cortisol is essential for the maintenance of peripheral vascular tone. Unlike primary adrenal insufficiency (PAI), SAI demonstrates a less severe clinical presentation due to the sparing of aldosterone secretion, and additionally lacks the findings of hyperpigmentation and marked hyperkalaemia. AI is not an 'all-or-none' phenomenon [7], and thus the presence of pituitary or hypothalamic pathology requires investigation of the HPA axis, even if there are no obvious symptoms of AI. On the other hand, the diagnosis of SAI based on clinical findings alone is not sufficient in itself to start glucocorticoid replacement therapy (GRT) without hormonal confirmation, unless acute adrenal failure is apparently present when more detailed investigation may need to be delayed. SAI may be associated with the deficiency(s) of other anterior pituitary hormone(s) or may be isolated, the latter being less frequent except in hypophysitis.

3.2 Hormonal investigation

The final result of HPA axis insufficiency is the decreased secretion of cortisol from the adrenal glands, whatever the cause. All the tests used in the investigation of HPA axis depend upon the estimation of cortisol since measurement of ACTH levels after stimulation of the HPA axis have not been shown to have any additional benefit [8]. More or less, all the tests assess the integrity of the HPA axis as a whole, but none of them is able to identify the precise location of the abnormality responsible for reduced cortisol production.

When evaluating the HPA axis, the 'sufficient' cortisol response to dynamic tests is based on the cortisol responses given by healthy people when stressed. The study by Plumpton and Besser was probably the first one assessing the HPA axis as a response to major surgery [9]. Approximately 50 years later, a similar study using more modern highly specific cortisol assays revealed a positive correlation between the cortisol responses measured and severity of the operation [10]. The peak cortisol responses was 30%–38% lower than the previous study, presumably due to current highly-developed surgical and anaesthetic practices, as well changes in cortisol assays. A recent study also confirmed that serum cortisol levels during surgery are affected by the type of surgery, age, sex, surgical and anaesthetic technique [11]. It should therefore be emphasised that a normal response to a stimulation test may simply be a response within the normal range of a control population, but this does not necessarily equate with levels adequate for severe stressors such as surgery or trauma.

3.2.1 Methodological problems

The definition of the normal cortisol cut-off response to ACTH stimulation test may depend upon the method used for the measurement of cortisol [12]. In a recent study, the *Elecsys Cortisol* assay and *Elecsys Cortisol II* assay were compared in samples from dynamic tests. Cortisol values measured by new assay were found to be about 30% lower, and they suggested 374 nmol/L (13.5 μg/dl) as the revised cut-off in place of 500 nmol/L (18 μg/dl) for the insulin tolerance test [13]. Mean cortisol concentration measured by GC-MS was found to be significantly lower than cortisol measured by immunoassay in males for all five assays, and in females who were not on the oral contraceptive pill (OCP), for all but the *Architect* and *Access* assays. Post-ACTH cortisol levels measured by immunoassays were significantly higher in males and non-OCP females when compared to those in whom cortisol was measured by GC-MS [14]. Immunological tests including *Immulite* and *Roche* platforms were found to have similar results to LC-MS/MS despite higher median cortisol levels [15]. The assays used in the measurement of cortisol are of thus of major importance.

4 Basal hormone levels

4.1 Basal cortisol

Measurement of basal cortisol is generally the first and the easiest step to investigate the HPA axis. The best time for the measurement was suggested as before 09.00 h with a cut-off level of 375 nmol/L (13.5 μg/dl), as the median cortisol level decreases

by approximately 30 nmol/L (1.08 μg/dl) per hour between 07.00 h and 12.00 h [16]. Various cut-off levels for basal serum cortisol have been suggested depending on the study population and reference test used. An upper basal cortisol cut-off level of 285 nmol/l (9.8 μg/dl) and a lower cut-off level of 98 nmol/l (3 μg/dl) were shown to reduce the number of subjects requiring stimulation tests [17]. Preoperative basal cortisol levels of <6 μg/dl and > 18 μg/dl could predict insufficient and sufficient cortisol responses respectively to an insulin tolerance test (ITT) in patients undergoing pituitary surgery. In the postoperative first month, a basal cortisol level < 7 μg/dl was able to predict an insufficient cortisol response to the ITT with great accuracy [18]. Basal cortisol was also tested for its ability to obviate the need for high-dose ACTH stimulation test (HDST) (with 250 μg *Synacthen/Cosyntropin = ACTH* (1, 24)) i.m. in patients with pituitary disorders. The authors accepted >550 nmol/l (20 μg/dl) as an adequate cortisol response to Synacthen and concluded that dynamic testing is not necessary if the basal cortisol is <100 nmol/l (3 μg/dl) or >330 nmol/l (12 μg/dl), while the HDST is *required* in patients with basal cortisol levels between 100 and 214 nmol/l (3–7.7 μg/dl) and *suggested* in patients with a basal cortisol between 214 and 330 nmol/l (7.7–12 μg/dl) when clinical risk factors including previous cranial radiotherapy or deficiencies of other anterior pituitary hormones are present [19]. Strong linear correlations were detected between basal serum cortisol levels and cortisol responses to the HDST at 30 min and 60 min during the test [20].

In a retrospective observational study including 346 patients, it was suggested that a basal morning serum cortisol value ≥400 nmol/l (14.4 μg/dl) could predict a normal cortisol response to the i.m. HDST [21]. Recently, in 416 patients, a basal cortisol level < 85 nmol/L (3 μg/dl) (specificity 99.7%) and > 350 nmol/L (12.6 μg/dl) (sensitivity 98.9%) were able to eliminate the need for 30% of low-dose Synacthen tests (LDST) with 1 μg i.v. synthetic ACTH [22]. The HDST could also have been avoided in a significant number of patients by utilising basal cortisol levels [23].

Assessment of the HPA axis is crucially important in the determination of recovery of adrenal function in patients with pituitary disease associated with SAI. Patients who had an initial basal cortisol level >175 nmol/L (6.3 μg/dl) were shown to have an almost 50% chance of recovery of HPA axis function [24]. A basal cortisol level of ≥300 nmol/l (10.8 μg/dl) measured on the post-operative second day after pituitary surgery was suggested to be a predictive marker of normal HPA axis according to the LDST 3 months after surgery in a study carried out in 83 patients. Peak cortisol level, either at 20 or 30 min, of ≥500 nmol/l (18 μg/dl) after the LDST, was accepted as an adequate cortisol response [25]. Glucocorticoid use is the most common cause of SAI due to a supressed HPA axis, and a timely decision on recovery of the HPA axis is essential to avoid unnecessary GC administration: in such patients; an early morning cortisol of ≥8.8 μg/dl was reported to be an independent predictor of adrenocortical recovery [26].

In general, then, basal cortisol is a good indicator of HPA axis in most of the patients with suspected AI, limiting the number of patients requiring dynamic tests, and is an appropriate first test in such patients.

4.2 Free cortisol

Serum cortisol levels may be affected by the levels of cortisol binding globulin (CBG). Free cortisol measurement may be of value in case of conditions affecting CBG levels. Peechakara et al. compared the serum total and free cortisol responses to different doses of Synacthen (LDST i.v., medium-dose ACTH stimulation test (MDST i.m. 25 μg) and the HDST i.m. in 10 patients with hypothalamo-pituitary disease and in 12 healthy control subjects. It was found that a serum free cortisol cut-off of 0.9 μg/dl at 30 min could be used as pass criterion during the LDST i.v., MDST and HDST i.m. and 1.3 μg/dl at 60 min during HDST [27]. However, it would be more useful if all the tests would have been performed in a more standardised pattern in that study. In another study, a cut-off level of 0.9 μg/dl for a peak serum free cortisol response to the HDST was suggested to be used to make a differential diagnosis between patients with AI and healthy subjects [28]. The free cortisol levels during the ACTH stimulation test can be especially helpful in females on oestrogen therapy such as the OCP, in patients who present with apparent clinical manifestations of AI but have normal total cortisol levels, or in patients who are suspected to have CBG abnormalities such as serious illness [29]. However, currently the routine measurement of serum free cortisol level is not practical and very limited due to its complicated analysis [30].

4.3 Basal ACTH

Determination of plasma ACTH is important for the differential diagnosis of AI. A plasma ACTH level > 300 ng/L (66 pmol/L) stimulates cortisol synthesis to its maximum [31]. A cortisol level < 140 nmol/L (5 μg/dL) with an elevated ACTH in a patient is highly predictive of PAI [31–34]. The elevation in ACTH generally precedes hypocortisolaemia in PAI. However, it is not easy to set a specific cut-off level for ACTH because of the analytical bias in ACTH assays [35, 36]. The *Endocrine Society* recommends two-times the upper limit of the plasma ACTH reference interval as a cut-off for the diagnosis of PAI [37]. Clinical findings, and the use of steroid and non-steroid medications, should be taken into account when interpreting the levels of ACTH [38]. Another problem with the measurement of ACTH is related to its sampling procedure, as the sample needs to be taken into a chilled tube and placed on ice immediately; if not, degradation of this peptide hormone can occur and lead to inappropriately low ACTH levels [39, 40]. Thus, plasma ACTH levels are

important for the differential diagnosis of AI, but the accurate estimation of ACTH can be difficult.

4.4 Basal DHEAS

The secretion of DHEAS from the adrenal gland, which is the most abundant steroid hormone in the circulation, is controlled by ACTH. Almost all DHEAS in the circulation is secreted by the adrenal glands, with only a slight contribution from the testes in men. DHEAS has a long half-life and lacks diurnal variation, making the measurement possible at any time of the day with widely-available assays [41]. However, age and gender specific ranges for DHEAS are essential for a proper evaluation, since levels decrease with age and are lower in women [42].

Some authors have suggested the use of DHEAS as a marker for the assesment of HPA axis integrity in patients with a pituitary tumour [43, 44]. Patients with SAI were found to have lower basal and ACTH-stimulated cortisol, DHEA, and DHEAS levels, and a higher baseline cortisol to DHEA molar ratio which increased further after the LDST [44]. Adrenal androgen secretion is impaired before loss of cortisol secretion in patients with impaired HPA axis function [45, 46]. This may be due to stimulation of DHEA secretion with intra-adrenal cortisol in a dose-dependent fashion, presumably via inhibition of 3β-hydroxysteroid dehydrogenase type II activity [47]. As a result, a small reduction of intra-adrenal cortisol concentration in early AI may lead to decreased DHEA secretion from the adrenal glands. Teleologically, this may be regarded as a means whereby DHEAS is sacrificed to the more significant cortisol.

In patients younger than 30 years of age, a Z-score of (calculated using age- and gender-specific references) less than −2.0 for DHEAS showed 100% sensitivity and specificity in terms of estimating HPA axis dysfunction, but was less useful in older patients [48]. Normal age- and gender-specific DHEAS levels predicted a sufficient cortisol response to the LDST with a sensitivity of 87.1% and a specificity of 86.7%. The authors suggested a DHEAS ratio (DHEAS divided by the lower limit of respective reference range of the substrates) of more than 1.78 as a minimum in order to identify intact HPA integrity [49]. Thus, DHEAS levels can be used only as an additional or adjunctive tool in the assessment of HPA axis function.

5 Dynamic tests

5.1 Insulin tolerance test (Table 1)

The use of the ITT as a test in the investigation of pituitary disorders goes back 80 years ago. The studies using the ITT are summarized in Table 1 [8, 9, 17, 18, 50–76]. The ITT had been found valuable in the differential diagnosis of panhypopopituitarism and anorexia nervosa or primary hypothyroidism by demonstrating unresponsiveness to hypoglycaemia [77]. Although 0.15 U/kg of insulin was used in earlier studies [50], an insulin dose of 0.1 U/kg is usually sufficient to achieve adequate hypoglycaemia (glucose level <40 mg/dl, 2.2 mmol/L, in venous blood) during the ITT. If the fasting blood glucose is ≥100 mg/dl, then a second dose of insulin is usually needed [78]. An optimised calculation method for insulin dosage in place of conventional method in the ITT has also been proposed [79]. Recently, we have shown that symptomatic hypoglycaemia, even without a decrease of glucose to <40 mg/dl during the ITT, is also sufficient to stimulate the HPA and GH axes in suspected patients [76]. The other causes of inadequate hypoglycaemia during the ITT can be active or persistent acromegaly or Cushing's disease as both are associated with insulin resistance, and with such diagnoses higher doses of insulin have been recommended (0.2–0.3 U/kg).

The reproducibility of the ITT has not been extensively investigated in healthy subjects despite its general acceptance as a gold-standard test in the investigation of the HPA axis as well as the GH axis for many years. Although less variable than GH responses, peak cortisol responses to the ITT do vary even within the same subject, and the reproducibility of the ITT in the assessment of the HPA axis is not perfect [55].

Normally, serum cortisol responses to the ITT are measured basally and at 30, 60, 90 and 120 min after achievement of hypoglycaemia. A significant positive correlation has also been detected between the peak ACTH and the peak cortisol concentrations during the ITT. However, as noted above, it is not possible to define an acceptable cut-off value for the peak plasma ACTH responses to ITT because of wide variations in levels [8].

On the other hand, the ITT is not free of side-effects, some of which may be serious. The test itself can be unpleasant for some patients, is time-consuming, and needs to be performed in a specialised centre by experienced medical staff [80]. The contra-indications to the ITT consist of coronary artery disease, ischaemic cerebral disease, and seizure disorders. In one large study including 220 patients who had an ITT, 2% had adverse events; one of the patients developed chest pain and finally had coronary artery by-pass surgery, while four patients developed blackouts. The depth of hypoglycaemia was lower than the targeted level for a prolonged period in a significant number of the patients. Nevertheless, overall the adverse events were few and they were not related to the depth of hypoglycaemia [81]. Glucose infusion may shorten the duration of hypoglycaemia without changing the stimulated cortisol levels. In one study a glucose infusion was shown to reduce plasma adrenaline/epinephrine levels and ameliorate patient discomfort after hypoglycaemia, but this procedure of 'reversal' is not in uniform use [82]. In general, the ITT remains the gold-standard test which is usually well tolerated and provides accurate information on both cortisol and GH levels, but should always be carried out by experienced personnel.

The cut-offs for a sufficient peak cortisol responses to ITT are summarised in Table 1. The rise in plasma cortisol

Table 1 Studies using the insulin tolerance test

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
Plumpton and Besser 1969 [9]	20 healthy 20 patients receiving GCC who underwent major surgical procedure ITT 0, 30, 45, 90. min Ins dose: 0.15 U/kg	Fluorometric assay	Max increase in cortisol: 500 ± 38 (166–830) Cut-off suggested for max increase in cortisol: 138 Peak cortisol: 886 ± 41 (580–1330) Cut-off suggested for peak cortisol: 550	NA	
Greenwood et al. 1966 [50]	38 healthy, 54 ITT Age: 18–34 yrs. ITT 0, 30, 60, 90, 120 min Ins dose: 0.15 U/kg	Fluorometric assay	Peak cortisol reached in 60 min 28 healthy: Max increase in cortisol: 500 ± 38 (166–830) Peak cortisol: 780 ± 139 (600–1040)	Glu is lower, 90th min cortisol is higher in females	
Landon et al. 1966 [51]	34 patients, 19 men age: 16–73 yrs. ITT 0, 30, 60, 90, 120 min Ins dose: 0.15 U/kg metyrapone, ACTH infusion	Fluorometric assay	Max increase in cortisol: 350 ± 94 Peak cortisol: 717 ± 127	NA	25 patients had compatible results in all 3 tests. 3 patients had sufficient cortisol response to ACTH, insufficient response to metyrapone and ITT
Jones et al. 1994 [52]	129 patients, 49 men Age: 39 (16–65) yrs. ITT 0, 30, 45, 60, 90. 120 min Ins dose: 0.15 U/kg	RIA	Cut-off accepted: 580	NA	Basal cortisol <100 was suggested as insufficient Screening cortisol >500 was suggested as sufficient
Hurel et al. 1996 [53]	27 healthy, 16 men Age: 30 ± 8 yrs. 166 patients, 55 men Age: 35 ± 12 yrs. ITT ins dose: 0.1–0.2 u/kg 0, 30,60, 90,120 min HDST		Cut-off accepted: 520 ITT is suggested to be carried out in patients with a 30th min cortisol response between 350 and 600	NA	Basal and peak cortisol responses to ITT and HDST are correlated.
Ammari et al. 1996 [54]	30 patients, 15 men Age: 19–66 yrs. ITT 0, 30, 60, 90, 120 Insulin dose: 0.1 U/kg HDST	RIA	Cut-off accepted: 550	NA	Discrepancy of ITT and HDST 50–100%
Vestergaard et al. 1997 [55]	16 healthy, 8 men Age: 31(26–50) yrs. 2 ITTs, 2 HDSTs were carried out. ITT −30, 0, hypoglycemia, 30, 60 min Ins dose 0.15 U/kg	RIA	ITT peak cortisol reached after 42–86 min ITT peak cortisol: 585 ± 77 (448–775) 2 volunteers had peak cortisol response <500 in the 1st ITT 3 volunteers had peak cortisol response <500 in the 2nd ITT	None	Reproducibility for cortisol is good Peak cortisol in HDST is higher than ITT Peak cortisol levels of ITT and HDST are correlated
Erturk et al. 1998 [56]	193 patients, 73 men Age: 43 ± 14 yrs. ITT 0, 30,60, 90 min Ins dose: 0.15 u/kg	Chemiluminescence ACTH (RIA)	Cut-off accepted: 500 Peak ACTH: 30th min Peak cortisol: 60th min	NA	Basal and peak cortisol levels are correlated Too much variation in ACTH responses
Ambrosi et al. 1998 [57]	57 patients, 31 men ITT 0, 30, 45, 60, 90 min Ins dose: 0.1–0.15 u/kg LDST: 0, 30, 40, 60 min	RIA	Cut-off accepted: 500		LDST (cut-off: 500) Positive predictive value: 77%, Negative predictive value: 91%

Table 1 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
Dullaart et al. 1999 [58]	80 patients, (132 ITT) ITT 0, 30, 45, 60, 90 min Ins dose: 0.15–0.2 u/kg CRH stim test UFC	RIA	Cut-off accepted: 500	NA	If ITT is sufficient peak cortisol response to ITT is higher than the response to CRH If ITT is insufficient peak cortisol response to CRH is higher than the response to ITT
Abdu et al. 1999 [59]	64 patients 42 ITT, LDST, HDST Age: 47.5 ± 11.5 (28–70) yrs. 22 LDST, HDST ITT −15, 0, 30, 45, 60, 90,120 min Ins dose: 0.1 u/kg LDST: 0, 20, 30, 40, 60 HDST: 0, 30, 60	Immunoassay	Cut-off accepted: 500	NA	Correlation is good between tests
Gonzalbez et al. 2000 [60]	30 healthy, 14 men LDST, HDST Age: 34 yrs. 20 healthy ITT ITT −15, 0, 30, 45, 60, 90 min Ins dose: 0.1–0.15 u/kg	Chemiluminescence immunoassay	Peak cortisol reached in 30 min Peak cortisol for ITT: 5th percentile: 539 10th percentile 562 50th percentile 654	None	Cut-off suggested for LDST: 500 HDST:600
Courtney et al. 2000 [61]	33 patients 18 men Age: 49 (19–99) yrs. Postoperative 3rd day ITT ITT −15, 0, 30, 45, 60, 90 min 0.1–0.2 u/kg Postperative 7th day: metyrapone	RIA	Cut-off accepted: 550	NA	6 of 7 patients with subnormal ITT response had insufficient response to metyrapone
Lange et al. 2002 [62]	255 patients 126 men Age: 35 (14–80) yrs. ITT −15, 0, 15, 30, 45, 60, 75, 90 min Ins dose: 0.1 u/kg	HPLC (yrs: 1991–1995) Fluorescence immunometric assay (yrs: 1995–1997) Fluorometric assay (yrs:1997–2002)	Cut-off accepted: 500	NA	
Schmidt et al. 2003 [17]	54 patients, 27 men Age: 46.6 ± 2.5 yrs. 20 healthy, 12 men Age: 35.8 ± 3.9 yrs. ITT 0, 15, 30, 45, 60, 90, 120 min ins dose: 0.15 u/kg CRH	Competetive immunoassay	Cut-off accepted: 500 Peak cortisol values not given for healthy	NA	Peak cortisol levels are correlated in 2 tests
Born et al. 2003 [8]	125 patients, 65 men 15 healthy, 12 men ITT −15, 0, 30, 45, 60, 90 min Ins dose: 0.1–0.15 U/kg	Chemiluminescence using an autoanalyser (ACTH and cortisol)	Peak cortisol response to ITT in healthy: 690 ± 72 2.5th percentile: 545 97.5th percentile: 715 Cut-off accepted for patients: 500 (Treatment given <470)	NA	ACTH measurement is not sensitive
Finucane et al. 2008 [63]	197 patients, 80 men Age: 41 ± 16 (13–76) yrs. ITT dk −15, 0, 15, 30, 45, 60, 90, 120 min Ins dose: 0.1–0.2 U/kg	Fluoroimmunoassay	Cut-off accepted: 500 Peak cortisol: 60th min	NA	Basal cortisol ≥393 eliminates the need of dynamic tests
	31 patients, 14 men	RIA	Cut-off accepted: 500	NA	

Table 1 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
Giordano et al. 2008 [64]	Age: 45.8 ± 2.4 yrs. ITT 0, 15, 30, 45, 60 min Ins dose: 0.1 U/kg Metyrapone HDST LDST VLDST (0.06 μg)				Neither metyrapone nor ACTH stimulation tests are completely reliable when compared with ITT
Deutschbein et al. 2009 [65]	77 patients, 41 men Age: 44.2 ± 1.8 yrs. ITT 0, 15, 30, 45, 60, 90, 120 min	Competetive immunoassay	Cut-off accepted: 500	NA	Perform dynamic test if basal cortisol is between 100 and 470
Karaca et al. 2010 (18)	64 patients, 32 men Age: 43 ± 13 yrs. Preoperative and postoperative 1st month ITT −15, 0, 30, 60, 90, 120 Ins dose: 0.1 U/kg Postoperative basal cortisol on 2-6th days	RIA	Cut-off accepted: 500	NA	Perform ITT if preoperatve basal cortisol is between 165 and 496 Replacement suggested for patients with a postoperative cortisol response lower than 194 on 2nd day, 222 on 3rd day, 194 on 4th day, 165 on 5th day, 83 on 6th day
Berg et al. 2010 [66]	36 patients, 23 men Age: 18–78 yrs. Postoperative 3rd-12th month ITT −10, 0, 15, 30, 45, 60, 90, 120 min Ins dose: 0.15 u/kg	Competetive immunoassay	Cut-off accepted: 500	NA	
Ferrante et al. 2012 [67]	55 patients, 18 men ITT, ITT 0, 30, 60, 90, 120 min Ins dose: 0.15 u/kg HDST	ECLIA electrochemiluminescence immunoassay	Cut-off accepted: 500	NA	Suggested cut-off for HDST 30th min: 500 60th min: 600
Cho et al. 2014 [68]	180 healthy, 49 men Age: 15–70 yrs. ITT, LDST, HDST ITT 0, 30, 60, 90, 120 min Ins dose: 0.15 u/kg LDST: 0, 20, 30, 40 HDST: 0, 30, 60	RIA	Peak cortisol response to ITT in healthy 95th percentile: 410 Peak cortisol in ITT: 600 ± 155 (387–980)	NA	Suggested cut-off for LDST: 500 HDST: 600
Simsek et al. 2015 [69]	129 patients, 53 men Age: 43 ± 10 yrs. ITT −15, 0, 30, 60, 90, 120 Ins dose: 0.1 U/kg LDST, GST	RIA	Cut-off accepted: 500 Suggested cut-off for ITT when LDST and GST have concordant results: 300 (97% sens, 75% spec)	NA	
Simunkova et al. 2015 [70]	60 healthy, 30 men Age: 38 ± 10 yrs. ITT 0, 30, 60 min Ins dose: 0.1–0.2 U/kg LDST, MDST, HDST	LCMS/MS	Peak cortisol: 60th min Basal cortisol: 350 ± 144 30th min: 379 ± 111 60th min: 578 ± 142	No sex difference in cortisol responses	Cortisol/cortisone ratio is higher in females during ITT
Kacheva et al. 2015 [71]	118 patients, 66 men Age: 47.7 ± 13.6 yrs. ITT 0, 30, 60, 90,120 min	Commercial assay Copeptin sandwich immunoluminometric assay	Cut-off accepted: 500 Peak cortisol: 60th min Peak copeptin: 45th min	Copeptin response to ITT is higher in females	Copeptin response is higher in patients with intact pituitary function

Table 1 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
	Ins dose: 0.1–0.25 U/kg				
Cerina et al. 2016 [72]	70 patients, 28 men Age: 47 (33–60) yrs. Postoperative basal cortisol on day 3 and 6 ITT on postoperative 6th month ITT 0, 10, 20, 30, 60, 120 min Ins dose: 0.1–0.2 U/kg	Electrochemiluminescence immunoassay	Cut-off accepted: 500		Cut-off suggested for basal cortisol on day 3: 343 day 6: 320
Kosak et al. 2017 [73]	64 healthy, 33 men Age: 41.4 yrs. ITT (in 57) 0, 20, 30, 40, 60, 90, 120 min Ins dose 0.15 U /kg HDST (in 62) MDST (in 61) LDST (in 64)	CLIA chemiluminescence immunoassay	95% achieved peak cortisol >500 at 30 min 2 (2%) had peak cortisol<500 but they passed the other tests	NA	
Cadegiani et al. 2017 [74]	12 non-active healthy 25 healthy athlete 14 overtraining syndrome ITT basal, during hypoglycemia, 30 min after hypoglycemia Ins dose 0.1 U/kg	electrochemiluminescence	Peak cortisol<470 in 66.7% of non-active healthy, but none in healthy athletes	NA	
Taieb et al. 2018 [75]	81 patients, 44 men Age: 35.8 ± 19.6 ITT 0, 10, 20, 30, 45, 60, 90, 120 min Ins dose: 0.1 U/kg GST	RIA	Cut-off accepted: 500	NA	Cut-off suggested for GST: 462 has a concordance of 86.4% with ITT
Simsek et al. 2020 [76]	135 patients, 52 men 118 achieved glu < 40 mg/dl 17 couldn't achieve glu < 40 mg/dl ITT: −15, 0, 30, 60, 90, 120 Ins dose: 0.1–0.2 U/kg	RIA	Cut-off accepted: 500	NA	Confirmation of hypoglycemia (serum glu < 40 mg/dl) was not associated with a higher response of cortisol to ITT

LDST: 1 μg ACTH, MDST: 10 μg ACTH, HDST: 250 μg ACTH, ins: insulin

including either 7 μg/dl above the baseline or doubling the basal cortisol concentration was associated with very high false positive and negative rates, and *changes* in cortisol are not recommended as the criterion to predict HPA axis integrity [56]. The evolution of other dynamic tests after the ITT has led to many attempts to ascertain normative values for the ITT in order to compare to newer dynamic test procedures.

Although the ITT has been accepted as the gold-standard test to investigate the HPA axis for decades, and has the advantage of also evaluating the GH axis, the unpleasant effects of hypoglycaemia, some contraindications, uncertain reproducibility and the requirement of experienced medical staff, limit the use of ITT in daily clinical practice. On the other hand, new cut-off levels for the peak cortisol responses to ITT are also needed with newer assays (see above).

5.2 ACTH stimulation test (Table 2)

Synthetic ACTH stimulates the adrenal gland directly and give rise to the synthesis of both glucocorticoids and sex steroids. The theory on which the test is based is that in PAI there will be little or no response in a damaged adrenal, while a lack of hypothalamo-pituitary function will lead over time to adrenal atrophy. A decreased cortisol response to ACTH cannot discriminate primary or secondary AI due to resultant atrophy of the adrenal glands in SAI. The ACTH stimulation test (also called the short *Synacthen* test (SST), *Cosyntropin* stimulation test, rapid ACTH stimulation test) has been commonly used as an alternative test to the ITT [83]. In the early days purified i.m. ACTH was used and uric acid/creatinine ratio in urine and eosinophil count as a response to ACTH were measured instead of cortisol, but cortisol is now measured directly. The ACTH stimulation test may be performed as an HDST or LDST. Long-acting i.m. depot Synacthen, which is commonly available worldwide, has been suggested as a reliable and safe test in the investigation HPA axis insufficiency where short-acting Synacthen is unavailable as the responses over the first 60 min are identical [84]. The studies using the ACTH stimulation test are summarized in Table 2 [12, 15, 20, 31, 85–102].

5.2.1 High dose ACTH stimulation test (HDST)

The high dose ACTH stimulation test has been used in the diagnosis of AI for more than 50 years around the world. In one of the first studies, the HDST i.v. was investigated in healthy subjects, patients with Addison's disease and SAI. In all control subjects, peak cortisol values were found to be equal or higher than 18 μg/dl [103]. The peak cortisol response to the HDST was found to be significantly correlated with the peak cortisol response to the ITT in the studies that followed [88], although another study revealed a clear discrepancy between ITT and HDST in terms of cortisol responses [54].

The optimal time for measurement of cortisol response to an HDST is generally at 30 min. However, a recent study has shown that both 30 and 60 min cortisol responses have an adequate index of consistency, but the same is not true in terms of absolute agreement, particularly when a SAI is suspected: 10% of patients with a subnormal response at 30 min had a normal response in 60 min in cases of SAI [20]. Except in a few early studies which used the i.m. route, HDST is usually performed by i.v injection [103] (Table 2).

When the HDST was compared with the ITT, the necessity for the presence of both criteria for the hypoglycaemia: i) a glucose level of <2.2 mmol/L and ii) neuroglycopenic symptoms such as sweating were thought to be the reason for similar results obtained from both tests [88]. The ITT and i.m. HDST were compared in 166 consecutive patients with suspected SAI compared to the results obtained from healthy volunteers. The HDST 30 min cortisol response of 600 nmol/l (21.6 μg/dl) could be used in place of the ITT to rule out the possibility of HPA axis insufficiency in 94.4% of the patients [53].

In a retrospective study including 399 patients, the cortisol level was measured at baseline, 30 and 60 min in the HDST. A peak cortisol level ≥ 20 μg/dl was suggested as a sufficient single criterion for normal adrenocortical function [104]. The peak cortisol response in the ITT has also been accepted the criterion for a normal ACTH stimulation test in most of the studies published so far in the literature. However, recent data suggest that each test should have its own cut-off value [68, 69, 105]. Cho et al. proposed cut-off values of 15, 18, 20 μg/dl for the ITT, LDST and HDST respectively in normal subjects and 16 μg/dl for the LDST, 17 or 18 μg/dl for HDST in patients with pituitary disorders, in order to determine HPA axis sufficiency [68]. The HDST can also be used to determine adrenocortical recovery with increased diagnostic accuracy when combined with a subsequent random morning cortisol [101].

The cost of Synacthen shows great variation across countries, and is not universally available. It has also varied over time as some commercial operations have taken up the licences for its manufacture and massively increased these costs without any clear clinical reason. Ben-Shlomo et al. implemented an electronic medical record (EMR) system protocol, and by using this system they reduced the number of wasted tests and maximised staff time and resources [106]. Long-acting porcine sequence ACTH known as *Acton Prolongatum*® was suggested to be used instead of Cosyntropin in the diagnosis of adrenal insufficiency when Cosyntropin is unavailable [107]. Very recently, the nasal administration of tetracosactide was shown to generate similar serum cortisol response, while measurement of salivary cortisol or cortisone provided a non-invasive test [108]. However, where the drug is available, the HDST is a simple and relatively accurate way to assess HPA function, and as long as it is not used in acute situations (as immediately post-pituitary surgery as the adrenal requires time to atrophy), it will remain in extensive use.

Table 2 Studies using the ACTH stimulation test

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
Landon J et al. 1965 [85]	50 pts. who received GCC >1 yr 58 healthy subjects ACTH 10 IU/h for 5 h: 0, 30, 60, 90, 120, 180, 240, 300 min	Fluorometric assay	Healthy 0 min: 350 ± 110 30 min: 775 ± 135 60 min: 913 ± 145 90 min: 990 ± 165 120 min: 1050 ± 220 180 min: 1153 ± 170 240 min: 1224 ± 170 300 min: 1326 ± 190 lower cortisol response in GCC treated pts	No sex difference	
Wood et al. 1965 The Lancet [86]	66 healthy subjects 40 asthma pts. on GCC 22 pts. who stopped GCC tx one wk. to 12 mts previously 9 pts. with suspicious Addison's disease ACTH 250 μg IM 0, 30 min	Fluorometric assay	Healthy: 0 min: 405 ± 104 30 min: 866 ± 142 Increase: 460 ± 106	No age or sex difference	The test was shown to be reproducible in 10 pts
Kehlet et al. 1973 [87]	28 patients who never received GCC before major surgery 28 normal subjects 48 patients who were on GCC tx before major surgery ACTH 250 μg IV: 0, 30 min Surgery: at the time of skin incision, after 1 h and at intervals throughout 24 h postoperatively	Fluorometric assay	Normal: ACTH test: 30 min >666 Increase >333 Surgery: after 1 h: >830 after 3–4 h > 1275 31 of 48 pts. on GCC tx had impaired response to ACTH, 25 of these 31 pts. had impaired response 1 h after skin incision, 16 of those 25 had normal response after 3–4 h None of them required GCC tx	NA	Correlation of ACTH test and response to major surgery is good
Kehlet et al. 1976 [88]	25 pts. w suspected SAI ACTH 250 μg IV: 0, 30 min ITT	Fluorometric assay	Increase in cortisol after ACTH or ITT is poor in predicting the outcome of the tests in individual pts	NA	ITT and ACTH tests are correlated
Cunningham et al. 1983 [89]	35 pts. ACTH 250 μg IM: 0,60 min Metyrapone	RIA	Cut-off accepted: 500 11 pts. had discordant results, all had normal response to ACTH and subnormal response to metyrapone	NA	
Dickstein et al. 1990 [90]	ACTH 0, 5, 15, 30 min Doses: 10 healthy 250, 10, 5 μg IV 10 healthy 250, 1 μg IV 6 healthy different times of the day w/wo pretx of dxm 10 pts. on long-term GCC tx	RIA	Pretx with dxm resulted in lower baseline, 5 and 15th min cortisol responses, but 30th min cortisol responses were similar 30th min cortisol responses were similar in the morning and in the afternoon, but 5 and 15th min cortisol responses were higher in the afternoon Peak cortisol response to ACTH is more reliable than increment	NA	30 and 60th min cortisol responses were similar in 250 and 5 μg ACTH test 30th min cortisol responses were similar in 250 and 1 μg ACTH test, but 60th min cortisol response in 1 μg ACTH test was lower than in 250 μg ACTH Long-term GCC tx resulted in lower cortisol reponses
Clark et al. 1998 [12]	100 healthy, 33 men 44 pts., 21 men w pituitary disease	Immunoassay 4 different types	Pts median (5th–95th percentile) 0: 290 (113–650)	No age difference	

Table 2 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
	ACTH 250 μg IV: 0, 30, 60 min		30: 748 (302–1000) increment: 373 (112–667) Healthy 0: 349 (164–870) 30: 811 (626–1431) increment: 488 (289–776)	Higher stimulated cortisol levels in females in all assays	
Soule et al. 2000 [91]	21 healthy, 6 men 65 pts., 26 men w pituitary disease ACTH 1 μg IV: −15, 0, 20, 30, 40, 60 min in all Metyrapone test in pts	Chemiluminescense immunoassay	Cut-off suggested for 30th min cortisol: 414 Peak cortisol response achieved at 20th min in 21%, 30th min in 44.7%, 40th min in 28.2%, 60th min in 5.9%	NA	Sens: 50% Spec:100% compared to metyrapone test
Agha et al. 2006 [92]	148 pts. ACTH 250 μg IV: 0, 30 group 1: 30 min cortisol >550 group 2: 30 min cortisol: 510–550	Chemiluminescense immunoassay	Cut-off suggested: 550 for pass 510–550: borderline no routine GCC <510 fail 137 of 148 pts. did not show AI during follow-up In the remaining 11 pts., −7 developed SAI after a surgery or RT −2 clinical uncertain cases, −1 pt. in group 1, 1 pt. in group 2 developed AI	NA	
Dekkers et al. 2011 [93]	207 pts., 80 men ACTH 1 μg IV: 0, 30 ACTH 250 μg IV: 0, 30	Fluosence immunoassay	Cut-off accepted: 550	NA	30 min cortisol responses were correlated in low and high dose ACTH test discordance: 16% for cut-off 550 11% for cut-off 500
Karaca et al. 2011 [94]	55 healthy, 28 men ACTH 1 μg IV: 0, 30, 60, 90, 120 ACTH 250 μg IV: 0, 30, 60, 90, 120 Glucagon	RIA	Cut-off suggested for peak cortisol response: ACTH 250 μg: 550 ACTH 1 μg: 344 GST: 250 250 μg test should be continued up to at least 90th in order to avoid false positive results	No age and sex difference	Lower cortisol response in low dose ACTH than in high dose Peak cortisol responses are correlated in low and high dose ACTH, but not with glucagon test
Lekkakou et al. 2013 [95]	21 young, 8 men (age: 43 ± 10) 40 elderly, 20 men (age: 79 ± 6) healthy ACTH 1 μg IV: 0, 30, 60	Chemiluminescence immunoassay	Cut-off accepted: 497	Elderly men had lower cortisol responses in both 30th and 60th min	
Chitale et al. 2013 [96]	250 pts. from one centre, 134 pts. from another centre in UK ACTH 250 μg IV: 0, 30, 60	Chemiluminescense immunoassay	Cut-off suggested: 550 31% of pts. had suboptimal response at 30 min, but passed at 60 min	NA	
Bancos et al. 2015 [31]	295 pts., 77 men ACTH 1 μg IV: 0, 30, 60 Cortisol and free cortisol	Immunoenzymatic assay	Cut-off accepted: 500 60 min cortisol is higher than 30 min cortisol	Discordance was seen in 7% of females on estrogen tx	Discordance of total and free cortisol is 1%

Table 2 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
			cut-off suggested for free cortisol 0 min: 271 ng/dl 30th min: 873 ng/dl 60th min: 1190 ng/dl		
Mak et al. 2017 [97]	56 healthy, 28 men 171 pts., 82 men ACTH 1 μg IV: 0, 30, 60 Cortisol, salivary cortisol and cortisone	Chemiluminescence immunoassay Salivary cortisol and cortisone: LC-MS/MS	Cut-off suggested for Peak cortisol: 376 Peak salivary cortisol: 8.6 nmol/L Peak salivary cortisone: 33.5 nmol/L	NA	
Struja et al. 2017 (98)	804 pts., 370 men w suspicious AI 400 pts. ACTH 250 μg IV: 0, 30, 60 404 pts. ACTH 1 μg IV: 0, 30	Immunoassay	Cut-off accepted for 250 μg peak: 550 Cut-off accepted for 30 min cortisol in 1 μg: 500	NA	Cut-off suggested for basal cortisol: ≥450: - PV: 98.7% ≤100: +PV: 93.2%
Ortiz-Flores et al. 2018 [20]	370 pts., 133 men 150 women who were tested to rule out NCAH ACTH 250 μg IV: 0, 30, 60	2 different immunoassays	Cut-off accepted: 500 Cut-off suggested for assay 1: 470 for men and women 690 for women taking OCP Cut-off suggested for assay 2: 441 for men 414 for women 579 for women taking OCP		Overdiagnosis with classic cut-off values 60th min sampling is more specific in suspected secondary AI
Ueland et al. 2018 [15]	138 healthy, 54 men, 17 on OCP 94 pts. w suspicious AI or NCAH	LC-MS/MS Also with 2 immunoassays in 54 healthy	Cut-off suggested for LC-MS/MS 412 for 30th min 485 for 60th min tendency for a lower cortisol response at a later start time of the test	No effects of age and sex	Cortisol levels measured by immunoassay are higher than LC-MS/MS
Mongioi et al. 2019 [99]	103 patients, 20 men ACTH 1 μg IV: 0, 20, 30	ELISA	Cut-off suggested 401.5 nmol/L sens: 100% spec 93.9% Cut-off accepted 500 nmol/L: sens 100% spec 67.3%		Cortisol measurement at 20 min increases the accuracy of LDST
Garg et al. 2020 [100]	63 pts., 44 men ACTH 1 μg IV: 0, 30 preoperatively and 12 weeks after surgery	Chemiluminescense immunoassay	Cut-off accepted: 441 nmol/L for 30th min cortisol		Postoperative day 3 cortisol ≥8 μg/dL: 73% sens, 79% spec cortisol ≥5.8 μg/dL 93% sens and 67% spec in predicting eucortisolism at 12 weeks.
Pofi [101]	776 pts., 335 men with reversible causes of AI at least 2 tests were performed ACTH 250 μg IV: 0, 30	Immunoassay	Cut-off accepted: 450 nmol/L for 30th min cortisol 30 min cortisol >350 nmol/L best predicted the HPA axis recovery		Subgroup analysis (GCC exposure) A delta cortisol best predicted the recovery None of the pts. with a delta cortisol <100 nmol/L and a subsequent 1-year random cortisol<200 nmol/L recovered
Pofi [102]	109 pts., 71 men who underwent TSS for a pituitary adenoma All patients underwent a preoperative ACTH 250 μg IV: 0, 30 Postoperative 8th day basal cortisol Postoperative 6th week ACTH 250 μg IV: 0, 30	Immunoassay	Cut-off accepted: Basal cortisol: 350 nmol/L 30 min cortisol: 430 nmol/L Cut-off suggested for basal cortisol: 370 nmol/L		A tumour volume >9 ml predict hypopituitarism at 6th week

SAI: secondary adrenal insufficiency

5.2.2 Low dose ACTH stimulation test (*LDST*)

The lower sensitivity of the HDST in detecting especially mild SAI resulted in seeking for different doses of ACTH stimulation test. Dickstein et al. reported similar cortisol responses to HDST in both healthy volunteers and in patients on long-term steroid treatment for 2–4 yr. In contrast, the cortisol response to the LDST in patients was significantly lower than in normal subjects at 30 min [90]. The unique advantage of the LDST is its capability to reveal a mild SAI overlooked by more powerful tests such as the ITT and HDST. The sensitivity and specifity of the LDST is 71% and 93% respectively when compared to the ITT [57]. It is also safe and inexpensive [57, 58]. Abdu et al. suggested that the LDST could not only replace the HDST, but also the ITT, for the initial investigation of the HPA axis in patients with pituitary disease [59]. The LDST was found to be more concordant with the ITT than the HDST in the investigation of the HPA axis immediately after pituitary surgery [110]. However, performing any test straight after surgery in clinical practice is debatable [111].

The LDST with a 30 min sampling time point may be used instead of the HDST in the diagnosis of especially mild SAI since cortisol measured at 30 min during the LDST is similar to that obtained in the HDST in healthy subjects [112].

The optimal time points of sampling for cortisol were suggested to be at baseline and 30 and 40 min after the i.v. ACTH administration [113]. Recently, 103 patients with suspected PAI and SAI were evaluated with the LDST and a cut-off value of 402 nmol/L (14.5 μg/dl) was suggested with 100% sensitivity and 93.9% specifity in predicting normal HPA axis function, according to clinical follow-up and HDST in suspicious cases [99]. However, they did not perform a confirmatory test in all patients.

In healthy adults with an age range of 25–69 years, and a normal cortisol response to the HDST, the lowest peak cortisol response obtained after the LDST was found to be 12.5 μg/dl [94]. Gonzalbez et al. reported similar serum cortisol responses to the HDST, LDST and the ITT in healthy volunteers [60]. Although the LDST and the HDST resulted in statistically similar cortisol responses in suspected AI, the levels were slightly higher in the HDST [93]. Nevertheless, this study did not show any clear advantage of the LDST over the HDST.

The widespread use of salivary cortisol (SC) in recent years has led to studies on their use during the LDST. Peak SC and cortisone to LDST which were measured by LC-MS/MS have been suggested to perform well in the diagnosis of AI with similar accuracy [97]. However, since the data are scanty, it is too early to recommend SC measurement after ACTH stimulation test instead of serum cortisol, but the concept is promising [114].

One of the controversial issues regarding the LDST is related to the plastic intravenous line which may be responsible for subnormal cortisol response because of Cosyntropin adherence to the tube and insufficient delivery of the dosage [115, 116]. In healthy adults, a 2.5 cm plastic tube does not alter delivered Cosyntropin dosage or cortisol stimulation compared to direct i.v. Cosyntropin administration [117]. Another concern with the LDST is the stability of Cosyntropin after dilution. However, it has been reported that 1 μg ACTH (1–24 ACTH) was stable when refrigerated at 4C in saline in plastic tubes for 4 months [90].

In conclusion, the LDST with appropriate cut-off levels may also be used in the diagnosis of SAI particularly when a mild or recent-onset SAI is suspected in place of the HDST.

5.3 Glucagon stimulation test (GST) (Table 3)

Studies using the GST are reviewed in Table 3 [66, 69, 94, 105, 118–129]. Glucagon is able to stimulate both GH and HPA axes when administered either subcutaneously or intramuscularly [130]. Although the cortisol response to glucagon s.c. was assumed to be ACTH-dependent, it is not known for certain whether glucagon stimulates the synthesis and/or secretion of ACTH directly from the pituitary. The GST is characterised by significantly increased copeptin, which correlates well with ACTH levels [127]. Glucagon was not able to stimulate ACTH and cortisol secretion when it was given i.v. On the other hand, i.m. glucagon was shown to be as effective as i.v hCRH and more effective than vasopressin on ACTH and cortisol secretion in the same study. It seems that the stimulatory effect of i.m. glucagon on ACTH secretion from corticotroph cells is not selectively mediated by endogenous hCRH or AVP, which have only additive effects on ACTH secretion [131, 132]. Ghrelin has also been assessed as to whether it was associated with the stimulatory effect of glucagon or hypoglycaemia on HPA and GH axes. However, it was shown that ghrelin does *not* mediate the ACTH or GH responses to either the GST or the ITT [133]. The fall in blood glucose level was also found not to be responsible for cortisol release either [122]. While some have suggested that it is the nausea associated with glucagon injection that is a non-specific stressor, the possible underlying mechanisms regarding the stimulatory effects of glucagon on cortisol and GH curently remain unclear.

Forty five years ago, a prospective study including normal subjects and patients with pituitary tumours, showed a good agreement between the ITT and the subcutaneous glucagon (1 mg) test in terms of GH and cortisol responses [118]. The GST was shown to be reproducible and suggested as the test preferable to ITT as a screening procedure because of its safety, reliability and ease of use [120]. Because of the great variability in cortisol responses and increased unpleasant side

Table 3 Studies using the glucagon stimulation test

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
Spathis et al. 1974 [118]	15 healthy, 9 men 9 pituitary tumors 17 acromegaly 1 mg glucagon SC 0,30,60,90,120,150,180,210,240 min	Competetive protein binding	Peak cortisol: 200–510 Peak usually achieved in 150–180 min	NA	Excellent agreement with ITT in 17 patients
Waldhaus at al. 1976 [119]	15 healthy, 12 men 2 (pure), 4 (pure), 10 mg (Zn-protamine) glucagon IM in 4, 7 and 4 patients respectively −15 min, 0,1,2,3,4,5,6,7,8 h	Competetive protein binding	Significant increase in cortisol only with 4 mg glucagon after 3 h Mean peak cortisol: 650±137	NA	
Rao and Spathis 1987 [120]	97 healthy, 23 men 42 pts. 1 mg glucagon IM 0,90,120,150,180 min	RIA	Peak cortisol: 300–1225 In men: 689 ± 35 In women: 799 ± 23 Peak levels are achieved in vast majority in 150–180 min.	Higher cortisol response in women No age difference	
Littley et al. 1989 [121]	6 healthy men glucagon 1 mg SC ITT 8 am-2 pm 10 min interval	IRMA	Peak cortisol: 289–717 in GST 493–736 in ITT between 170, 240 min after glucagon between 60 and 120 min after insulin	NA	Similar cortisol but higher ACTH response after ITT than glucagon
Leong et al. 2001 [122]	374 pts., 177 men, 500 GST 1 mg glucagon SC, 1.5 mg for pts. weighing >90 kg) 0,90,120,150,180,210,240 min	RIA chemiluminescence	Cut-off accepted: 550 Peak cortisol response at 150–180 min	No difference in ages in pts. w/wo sx	
Agha et al. 2004 [123]	31 healthy, 22 men 1 mg glucagon IM	fluoroimmunoassay	Cut-off suggested: 450	NA	
Tanriverdi et al. 2007 [124]	22 healthy, 1 mg glucagon IM	RIA	Cut-off suggested: 298	NA	
Berg et al. 2010 [66]	49 pts., 26 men 3 months after pituitary surgery 1 mg glucagon SC, 1.5 mg for pts. weighing >90 kg) 0,90,120,150,180,210,240 min ITT	Competetive immunoassay	Cut-off accepted for ITT: 500 Cut-off suggested for GST: 277	No difference in ages in pts. w/wo sx	Lower cortisol responses in GST than in ITT
Karaca et al. 2011 [94]	55 healthy, 28 men 1 mg glucagon IM 0,90,120,150,180,210,240 min LDSST HDSST	RIA	Cut-off suggested: 251	No sex difference No age difference	Similar cortisol response after GST and LDSST Lower cortisol response after glucagon than HDSST
Cegla et al. 2013 [125]	78 pts., 50 men 61effective test 1 mg glucagon SC, 1.5 mg for pts. weighing >90 kg) 0,90,120,150,180 min metyrapone test	Chemiluminescent assay	Cut-off suggested 350 71% sens, 57% spec	NA	Significant discrepancey between metyrapone and GST
Yuen et al. 2013 [126]	515 GST, 171 men		Cut-off accepted: 251		

Table 3 (continued)

Publication	Study group and method	Cortisol assay	Cortisol response (nmol/L)	Effects of age and sex	Comparison with other tests
	fixed dose: 1 mg glucagon SC, 1.5 mg for pts. weighing >90 kg) over 3 h in one center (48 pts), 4 h in 3 centers (119 pts) weight-based: 0.03 mg/kg over 4 h (90 pts)		Peak cortisol achieved in 150–180 min in fixed dose, 210–240 min in weight-based regimen	Negative correlation between peak cortisol and age in weight-based but not in fixed dose	Lower glucose, peak cortisol and cortisol increment after fixed-dose than weight-based
Simsek et al. 2015 [69]	129 pts. 53 men 1 mg glucagon IM 0,90,120,150,180,210,240 min LDSST ITT	RIA	Cut-off accepted: 295	No effects of age or sex	Lower peak cortisol levels in ITT
Lewandowski et al. 2016 [127]	79 subjects, 16 men (32 healthy, 29 pts) 1 mg glucagon SC, 1.5 mg for pts. weighing >90 kg) 0,90,120,150,180 min	Cortisol: immunoassay Copeptin: sandwich immunoassay	Cut-off accepted: 450	No effect of sex on copeptin response	Copeptin response is correlated with ACTH at 150. min, but not with cortisol
Hamrahian et al. 2016 [128]	28 pts. (14 men), 14 healthy (6 men) fixed dose: 1 mg glucagon IM, 1.5 mg for pts. weighing >90 kg) weight-based: 0.03 mg/kg 0,90,120,150,180,210, 240 min ITT	Chemiluminescent assay	Cut-off suggested: 242 for fixed dose 309 for weight-based regimen	NA	Good correlation of peak cortisol in GST and ITT
Tavares et al. 2017 [129]	41 geriatric volunteers (67–88 yrs., 6 men) 1 mg glucagon IM, 0,90,120,150,180 min	IRMA	Peak cortisol range: 340–1166	Higher cortisol levels in elderly <80 yrs	
Ach et al. 2018 [105]	81 pts., 44 men 1 mg glucagon IM, 0,90,120,150,180, 210, 240 min ITT	RIA	Cut-off suggested: 460	NA	Good concordance between GST and ITT

effects, i.m. GST was recommended instead of an s.c. test [130], but the s.c. test remains in most use.

The GST is a long test which lasts 4 h with 7 cortisol levels are obtained at baseline, 90th, 120th, 150th, 180th, 210th and 240th minutes. The peak cortisol response was reported to be obtained at 180th min by 1 mg sc glucagon [118]. The peak cortisol responses to GST are obtained at 150–180 min in majority of volunteers [94, 105, 122]. The duration of the GST was shown to be reliably shortened to 3 h by including fixed-dose and weight-based regimens.

The cortisol response to glucagon was demonstrated to be lower in men than in women, but not affected by age or BMI. A high basal cortisol level was associated with lower cortisol responses to glucagon [120, 128, 129]. Fixed-dose (1 or 1.5 mg in patients >90 kg) glucagon was shown to be associated with lower cortisol responses when compared to a weight-based dosing (WB:0.03 mg/kg) [128]. Thus, the potential influencers of cortisol response to GST are gender, baseline cortisol (in terms of rise) and dose of glucagon used.

An important point in evaluating the HPA axis during the GST is the diagnostic cut-off points used. The mean peak cortisol response to the GST was found to be significantly lower than that obtained during the HDST, but similar to that obtained during LDST in healthy adults. The lowest peak cortisol response achieved during the GST was found to be 9.1 μg/dl in healthy adults [94]. Hamrahian et al. suggested that the GST may be an acceptable alternative to the ITT in the investigation of the HPA axis with cortisol cut-off points of 9 μg/dl for fixed dose-GST and 11 μg/dl for weight-based GST as appropriate criteria to diagnose SAI [128]. Another study showed a good concordance between the ITT and GST, but ROC analysis revealed a cut-off for a peak cortisol response to the GST as 16.7 μg/dl for HPA axis sufficiency [105] which was higher than in the previously mentioned studies. In a study including 129 patients, a peak cortisol response of <298 mmol/l (10.7 μg/dl) to the ITT was found to be 97% sensitive and 75% specific for determining SAI according to the confirmed concordant results of LDST and GST with local cut-off levels. The reproducibilities of the tests were reported as 88%, 83%, and 79% for the GST, LDST and ITT, respectively [69]. Any of three tests can be used in the investigation of the HPA axis, but it would be appropriate to individualise cut-off levels used for the diagnosis of AI.

Recently, we compared the GST, LDST and a new combination test of the LDST and GST in 41 adult patients with pituitary disorders and 20 healthy subjects. The combination test was performed by injection of 1 μg ACTH i.v. at the 180th minute of a standard GST, with blood samples for cortisol measurement obtained at 210 and 240 min: 3 patients with discordant results during the LDST and GST had normal cortisol responses to the combination test and were clinically HPA axis sufficient. It is possible that this test may provide additional information in patients with unequivocal results in the GST and ACTH stimulation tests [134].

The GST can be associated with side effects including occasional nausea with vomiting, mild flushing, sweating, and headache in <10% [66]. However, more recent studies reported side effects in 21.4% of the patients including severe symptomatic hypotension, dizziness and sweating [129]. The most common side effects related to glucagon were reported as nausea (24%) and vomiting (22.16%); symptomatic hypoglycaemia was not reported [105].

In conclusion, the GST is a good alternative in patients with contra-indications to the ITT who require assessment of both HPA and GH axes, and is in more frequent use in the very young and the elderly. However, the test results should be carefully interpreted keeping in mind that glucagon is a weaker stimulant of the HPA axis than the ITT or the HDST, that the dose of glucagon used may influence the results when the tested patient is over-weight or obese, and that patients may suffer nausea and/or vomiting.

5.4 Metyrapone test (Table 4)

Metyrapone is an inhibitor of adrenal 11-β-hydroxylase enzyme which is responsible for conversion of 11-deoxycortisol to cortisol (Fig. 1). Metyrapone-induced reduction in cortisol levels in the circulation leads to stimulation of the HPA axis and increases 11-deoxycortisol proximal to the blocked enzyme: 11-deoxycortisol is not able to effectively supress ACTH secretion from the pituitary. When compared to the stimulation tests such as the ITT and GST, the mechanism by which metyrapone affects the HPA axis is totally different and it is characterised by a negative feedback stimulus instead of direct stimulation of the hypothalamus and/or pitutary.

The metyrapone test has been used in order to investigate the HPA axis since 1950s [135]. It can be used when the ACTH stimulation test results in normal adrenal function but fails to exclude SAI. The studies using metyrapone test are summarized in Table 4 [136–146]. The single-dose metyrapone test has been shown to be a simple and reliable test as the standard metyrapone test (750 mg every four hours, six times) [139]. Overnight single-dose metyrapone (at 11 p.m. metyrapone 2 g for subjects <60 kg, 3 g for >60 kg) was found to be more sensitive than the ITT and HDST in detecting subtle degrees of HPA axis insufficiency [147]. However, a similar study later showed that metyrapone test was *not* better than an early morning cortisol level in the prediction of glucocorticoid need 6 months after pituitary surgery [146]. The short overnight metyrapone test was able to reveal more patients with ACTH deficiency after pituitary surgery when compared to the ITT, but it was not clear whether the patients who failed the metyrapone test but not the ITT required GRT [61]. However, in another study, the overnight metyrapone test and the ITT were found to produce very similar results [148].

Table 4 Studies using the metyrapone stimulation test

Publication	Study group and method	11-S/cortisol assay	11-S response (nmol/L)	Effects of age and sex	Comparison with other tests
Buus et al. 1962 [136]	7 healthy, 4 men 3 g 500 mg every 4 h to 3 healthy 3 g as 250 mg every 2 h to 2 healthy 6 g as 500 mg every 2 h to 2 healthy	Spec isotope dilution principle Double tracer technique	Metyrapone every 4 h is unable to supress cortisol production 3 g every 2 h can completely inhibit 11-beta hydroxylation	NA	The study defines the required dose of metyrapone to stimulate 11-S response
Metcalf et al. 1968 [137]	80 pts., 101 tests 6 g every 6 h for 48 h		Urinary oxogenic steroids were measured 21 pts. abnormal <50 mg/day 23 pts. normal >100 mg/day 17 pts. doubtful 50–100 mg/day 17 pts. marginal 50–60 mg/day 40 pts. normal 60–100 mg/day	NA	Clinical and lab findings of hypopituitarism
Strott et al. 1969 [138]	Healthy 6 pts. hypopituitarism 5 pts. (hypogonadism and/or GH deficiency) 750 mg metyrapone every 4 h	Modified competitive protein binding assay	Healthy 11-S 375-923 nmol/l Abnormal 11-S 0–200 nmol/l	NA	
Jubiz et al. 1970 [139]	30 healthy 11 pts. hypopituitarism 750 mg metyrapone every 4 h 30 mg/kg body weight metyrapone given orally at midnight body weight < 70 kg: 2 g body weight 70–90 kg: 2.5 g body weight > 90 kg: 3 g cortisol <2 μg/dl: sufficient inhibition of 11β-hydroxylase	11-S competitive protein binding assay ACTH-Radioimmunoassay	Max ACTH and 11-S response obtained at 8 am cortisol <2 μg/dl: sufficient inhibition of 11β hydroxylase healthy: 11-S: 200–490 nmol/l Abnormal 11-S: 0–150 nmol/l	NA	Comparable response in both regular and single dose of metyrapone
Meikle et al. 1975 [140]	34 healthy 30 mg/kg body weight metyrapone given orally at midnight	11-S and cortisol: radioimmunoassay	Normal: 11 S > 200 nmol/l	NA	
Spiger et al. 1975 [141]	137 pts.	11-S competitive protein binding and radioimmunoassay	11-S nmol/l normal response 473 ± 17 AI 112 ± 17 hyporesponse 106 ± 14 hyperresponse 750 ± 17	NA	
Staub et al. 1979 [142]	21 pts. with normal HPA axis according to basal cortisol, ACTH stimulation test or ITT	ACTH-measured by an antiserum Cortisol: radioimmunoassay	Peak ACTH at 7 am Mean peak ACTH 468 ng/l Mean peak ACTH 369 ng/l	NA	Slightly higher ACTH response after metyrapone compared to ITT
Dolman et al. 1979 [143]	104 healthy 7 primary AI 20 secondary AI 2 g metyrapone given orally at midnight	11-S, ACTH, cortisol were measured by radioimmunoassay	Primary AI: normal ACTH, negligible 11-S after metyrapone Secondary AI ACTH, negligible 11-S after metyrapone	NA	
Feek et al. 1981 [144]	19 pts. 2 g metyrapone given orally at midnight IM ACTH ITT 13 healthy assessed by metyrapone	ACTH:radioimmunoassay Cortisol:fluorometric assay	Timing of ACTH sampling is critical for an optimal test before 8 am		Poor correlation between ACTH response to metyrapone and cortisol response to ITT
	185 pts., 327 test	ACTH:varied by time	For ACTH: 150 ng/l		

Table 4 (continued)

Publication	Study group and method	11-S/cortisol assay	11-S response (nmol/L)	Effects of age and sex	Comparison with other tests
Berneis et al. 2002 [145]	2 g metyrapone given orally at midnight	Cortisol: chemiluminescense immunoassay 11-S: radioimmunoassay	11-S > 200 nmol/l sens 47% spec 82% 11-S > 260 nmol/l sens 67% spec 68% 11-S + cortisol >450 sens 71% spec 69%		Better sensitivity of combined 11-S and cortisol response to metyrapone
English et al. 2017 [146]	40 pts., Postoperative day 3–4: max cortisol Postoperative week 1 and 6: 30 mg/kg overnight metyrapone test Postoperative week 6 HDST Postoperative week 7 ITT	Metyrapone test: LC MSMS ITT: immunoassay HDST: immunoassay	Cortisol <200 nmol/L 11-S > 200 nmol/L Concordance compared to ITT at week 7 for metyrapone test at week 1: 78% Metyrapone test at week 6: 66% Max cortisol on day 3–4: 71% HDST at week 6:71% Concordance compared to GCC need at 6 months for metyrapone test at week 1: 81% Metyrapone test at week 6: 85% Max cortisol on day 3–4: 80% HDST at week 6:83% ITT at week 7: 82%		Meytrapone test was not better than basal cortisol in predicting long-term GCC requirement

A peak serum 11-deoxycortisol >7 μg/dl and simultaneous serum cortisol <10 μg/dl were accepted as the cut-off values for the metyrapone test [147]. The plasma ACTH level may stil not be sufficient enough despite a normal 11-deoxycortisol response in the setting of mild SAI [149]. The sum of 11-deoxycortisol and cortisol responses with a cut-off level of 450 nmol/l after single dose of 2.0 g metyrapone given at midnight may lead a better diagnostic accuracy when compared to 11-deoxycortisol measurement alone. On the other hand, an ACTH level > 150 ng/l after single dose of metyrapone was found to be valuable in detecting a safe pituitary response [145] and may increase the sensitivity of the test. Half of the patients with a subnormal response to metyrapone had a normal cortisol (>414 nmol/l, 15 μg/dl) response to the LDST [91].

Metyrapone was demonstrated to cause some side effects such as unusual limb sensations, nausea and vomiting, dizziness without postural hypotension, and nightmares in the 576 tests evaluated. Worsening of adrenal function was not reported in that study [148].

So, the metyrapone test seems to be very sensitive in the detection of SAI but does not have clear superiority over other tests, and the significance of such minor chages is unclear. Theoretically, as it explores feedback at a pituitary level, it may not be sensitive to changes in hypothalamic function. The requirement of both cortisol and 11-deoxycortisol estimation and its lack of universal availability limits its use in routine clinical practice.

5.5 Corticotrophin-releasing hormone test

During CRH stimulation test 100 μg of corticoliberin (human CRH) is administered i.v., and cortisol and ACTH levels are measured at baseline and 30, 45, and 60 min. The results of the CRH test have been reported to correlate with those of the ITT in patients with HPA axis suppression with GCs [150]. The mean and minimum peak cortisol response after hCRH in healthy controls were found to be 594.8 ± 21.7 nmol/l (21.4 ± 0.8 μg/dl) and 400 nmol/l (14.4 μg/dl), respectively. A peak cortisol level < 377 nmol/l (13.6 μg/dl) after the hCRH test was suggested to be optimal for the diagnosis of AI but the sensitivity of the test was low [17].

The cortisol response to CRH was demonstrated to show a poor correlation with that of the ITT, except in patients with overt AI, in another study. Although the CRH test was shown to provide better results in accuracy than the LDST and HDST taking the ITT as the reference [151], the cortisol response to CRH was highly variable in normal subjects. In the studies carried out later, the early post-operative CRH-test was reported to be insufficient to reliably predict adrenal function after pituitary surgery in all patients, and retesting became essential [152]. Recently, the sensitivity and specificity of CRH test was found to be 78% and 90%, respectively and its diagnostic performance was shown to be worse than a single basal cortisol measurement [153]. CRH is not widely available and it is expensive. The current data suggest that CRH stimulation test is unhelpful and unlikely to replace the other traditional tests in

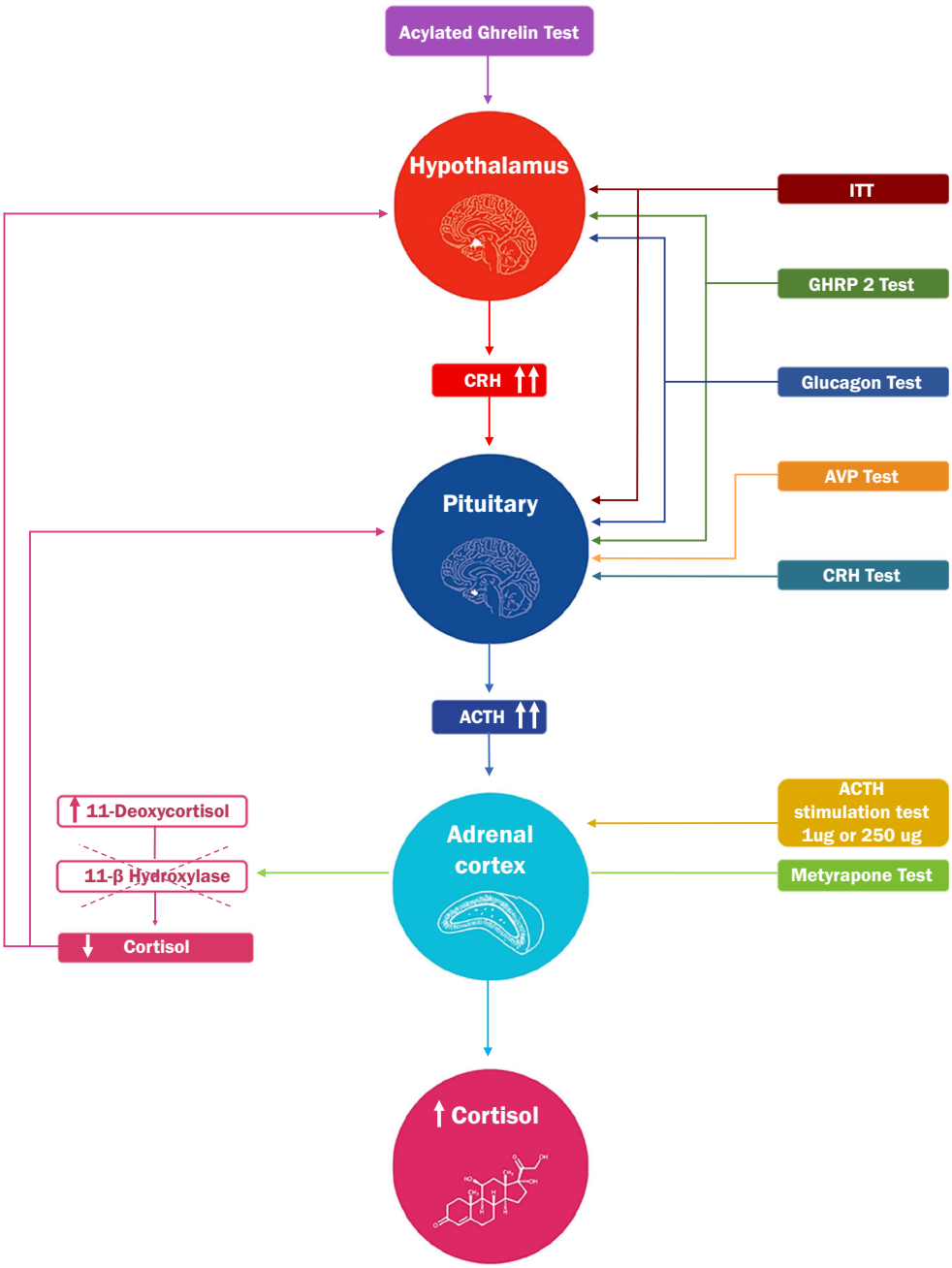

Fig. 1 Schematic representation of HPA axis stimulation via different tests and their assumed site of action

the investigation of the HPA axis. GH secretagogues stimulate GH secretion by binding to a GHS-R1 for which ghrelin is a natural ligand [154, 155]. Ghrelin stimulates the HPA axis through both CRH, and particularly AVP release from the hypothalamus [156]. GHRP2 stimulates HPA axis via the GH secretagogue in the hypothalamus [157] and ACTH directly from the pituitary [158]. However, neither GHRP nor ghrelin seem to have additional benefits to the traditional tests [154, 157–160].

6 Assessment of the HPA axis in patients with pituitary tumours undergoing pituitary surgery

Assessment of the HPA axis after pituitary surgery is crucially important not only to identify the patients who developed HPA axis insufficiency and require GRT, but also to avoid unnecessary glucocorticoid supplementation. Different doses of glucocorticoids are still given to patients undergoing pituitary surgery

on the day of surgery and post-operatively because of local traditions, fear of post-surgical hypopituitarism, and uncertainty about the definition of subclinical HPA axis insufficiency [161].

Non-functioning pituitary adenomas (NFPAs) may cause SAI due to mass effects or due to surgery performed to relieve compressive signs. Although decompression may improve hypocortisolism, it was shown to result in de novo hypocortisolism in 10.3% of patients with normal adrenal function before surgery. Only patients with a basal cortisol level of <8 μg/dl pre-operatively were suggested to be given GRT [162]. A cortisol level ≥ 15 μg/dl measured on the morning of the first post-operative day and a cortisol peak of ≥18 μg/dl to the MDST (25 μg) at post-operative 4–6 weeks were shown to be associated with normal HPA axis function [163]. Cortisol levels may be very low during the first part of surgery presumably due to anaesthetics, followed by a remarkable increase after intrasellar manipulation [164]. Recently, a retrospective study including 149 patients who underwent TSS for pituitary tumours assessed the place of recovery-room (RR) cortisol and found it to be the most accurate method when compared to day 1, 2 and 3 post-surgical basal cortisol. The RR cortisol threshold of 757.5 nmol/L (27.5 μg/dl) had 100% sensitivity and 70% specificity in the determination of necessity for long-term GRT [165]. The early post-operative basal cortisol was recommended to be a safe and simple measurement to guide (dis)continuation of GRT [153].

Pre-operative MDST and immediate post-operative MDST were found to have the highest sensitivity, accuracy, and positive predictive value (PPV) for a normal post-operative HPA axis, but pre-operative testing was found to be more cost effective (including costs of tests and hydrocortisone treatment) [166]. Neither the LDST nor the HDST were found to be reliable in determining the integrity of the HPA axis a week after pituitary surgery [167]. Although the LDST has been found to be more closely correlated with the ITT than the HDST immediately after pituitary surgery, none of them can completely correctly estimate the status of the HPA axis 3 months post-operatively [110]. Normalisation of HPA axis function can be seen in the first months after surgery. A recent study reported that a pre-operative SST 30-min cortisol cut-off level of 350 nmol/L (12.7 μg/dL) as the best predictor of HPA axis status [101]. However periodic testing is important post-operatively since recovery of the HPA axis can be seen in the postoperative 9–12 months [102].

The GST was proposed as a potential alternative to the ITT 3 months post-operatively for the assessment of GH reserve, but a poor test for ACTH reserve. A peak cortisol level < 500 nmol/l (18 μg/dl) was accepted as the criterion for the diagnosis of AI, but different criteria were not investigated for the ITT and GST, and the study did not contain a healthy population [66]. The metyrapone test was also not better than an early morning cortisol level in the prediction of glucocorticoid need 6 months after surgery [146].

In a study which aimed to limit GC exposure in patients undergoing TSS, patients with a normal HPA axis before TSS were not given peri-operative GC coverage and followed up by daily cortisol measurements. Of these patients, 45% received GC treatment for the following reasons: serum cortisol <5 μg/dl, cortisol between 5 and 12 μg/dl accompanied by manifestations of AI, moderate to severe post-operative hyponatraemia and severe headache, nausea and vomiting, fatigue or anorexia with cortisol >12 μg/dl. Only 14% of the patients were on GRT at 12 weeks. GRT was not found to be essential in most of the patients undergoing TSS [168]. The administration of corticosteroids peri-operatively in patients with an intact HPA axis is not only unnecessary, but also interferes with the assessment of the HPA axis after surgery. So, it was usggested that perioperative corticosteroid administration coud be safely witheld in these patients [169].

The definition of hypocortisolism as being a basal cortisol <8 μg/dL on 3rd post-operative day was suggested to be the single most significant predictor of hypocortisolism (a peak cortisol response to LDST <16 μg/dL) 12 weeks following surgery. The post-operative 3rd day basal cortisol was found to correctly predict eucortisolism 12 weeks after surgery with a sensitivity of 73% and specificity of 79% [100].

In a very recent prospective study including 92 patients without pre-operative AI and not receiving GRT, the 2nd postoperative day basal cortisol levels ≤3.2 μg/dL (89 nmol/L) and > 14 μg/dL (386 nmol/L) have been found to be diagnostic of SAI and normal function, respectively [170].

In conclusion, measurement of basal cortisol or an SST, if required, may be good alternatives for the preoperative evaluation of patients undergoing pituitary surgery with a cut-off value depending on the preferred dose of ACTH used. If the HPA axis is intact pre-operatively, then GRT can be avoided according to the clinical findings of the patient. Post-operatively, the best method for the evaluation of HPA axis is clinical follow-up with measurement of basal cortisol. Considering that the level of serum cortisol is highest immediately after surgery and decreases gradually, clinical findings of AI should be carefully evaluated dynamically. If clinical findings of SAI are present, GRT should be given until the next evaluation, probably at the first post-operative month. If clinical findings of AI are not present, then the basal serum cortisol level, depending on the post-operative day of measurement, will aid the decision of GRT. In the early post-operative period the HDST can lead to false negative results. So, when basal cortisol is inconclusive in the first month then an ITT, GST or LDST may be used. However, it should be kept in mind that the HPA axis may normalise during the post-operative 3 months. The assessment should be repeated 3 months after surgery.

7 The effects of age, sex and body mass indices on the HPA axis or on the cortisol response to dynamic stimulation tests

Current data in the literature suggest good maintenance of cortisol secretion with aging but a clear impairment of secretion of androgens in elderly subjects. A significant increase in the cortisol/DHEAS molar ratio occurs as a result of both physiological and pathological aging [171]. The cortisol responses to the HDST, LDST, ITT and GST in healthy volunteers did not show differences in terms of age and sex [15, 60, 94, 128, 172], but in a study evaluating the cortisol response to GST in elderly patients, the cortisol peak was found to be significantly different between subjects stratified by age < or >80 years (22.4 and 18.5 μg/dl, respectively) [129].

Studies regarding the effects of sex have also shown conflicting results. Mean daily cortisol levels were found to be lower in premenopausal women than men in subjects <50 years of age, but this effects of gender were not sustained after 50y [173]. Although basal and 1 μg ACTH-stimulated cortisol responses were not found to differ between older and younger individuals, older men had significantly lower cortisol responsiveness than older women [95]. There may be a complex effect of age, sex and menopausal status. In contrast, the HDST i.m.was found to be associated with higher peak cortisol levels and incremental responses in females than in males independent of age. The authors revealed the need of sex-specific cut-off levels for cortisol responses [12].

On the other hand, the peak calculated free cortisol and free cortisol index after LDST and HDST were found to be lower in women due to higher CBG levels despite similar serum cortisol responses [172]. Oral contraceptives (OCP) may also affect total cortisol levels, so premenopausal women on OCP (or pregnant women, where deemed appropriate) may need a separate reference limit for cortisol [14].

The BMI does not also seem to affect the cortisol response to dynamic stimulation tests including the LDST, HDST and GST [18] and daily cortisol secretion [173]. The effects of age, sex and BMI on the tests evaluating the HPA axis seem to be, at least clinically, negligible according to present data except in very elderly patients and OCP using or pregnant women. However, further detailed studies would help to better understand the effects of age, sex and BMI on HPA axis.

8 Conclusions

There is no gold standard test in the investigation of SAI meeting all the criteria of being safe, cheap, practical, easy, sensitive, specific and reproducible. Measurement of basal cortisol eliminates the need of dynamic test in majority of the patients when clinical findings are concordant. However, many patients with unequivocal basal cortisol levels may also require a dynamic test for a correct diagnosis. An ACTH-stimulation test or an ITT may be the first options for the evaluation of the HPA axis as dynamic tests, with 250 μg ACTH given i.m. or i.v., and serum cortisol measured at 30 mins, being most validated in clinical use, although the LDST may offer some advantages. The GST may be a good alternative to the ITT when this is contraindicated and GH axis evaluation is also required. Each test, for each time point and for each method used, requires its own minimum threshold of normality to assess the HPA axis and to determine the requirement of GRT. There may still be gray zones for cut-off levels used, then clinical judgement is essential.

Search Strategy: References in this review were identified through searches of PubMed articles by using the following terms: Insulin tolerance test, low dose test, short synacthen test, high dose synacthen test, insulin hypoglycemia test, insulin stress test, HPA axis, secondary AI, secondary adrenal failure, hypopituitarism.

*1 μg/dl was multiplied by 27.59 for conversion to nmol/L.

Compliance with ethical standards

Conflict of interest There is no conflict of interest of the authors.

Ethical approval There is no ethical approval or informed consent since the article is a review article, not a study.

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