

Can the Gold Standard Be Beaten? How Reliable Are Various Modifications of the Synacthen Test Compared to the Insulin Tolerance Test

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Summary

Criteria for the evaluation of the insulin tolerance test (ITT) and Synacthen test are still a matter of debate. The objective of the study was to make a comparison of serum and salivary cortisol during four stimulation tests. Sixty four healthy volunteers underwent the ITT, the Synacthen test with 1 (LDST), 10 (MDST) and 250 (HDST) µg dose of ACTH. Maximum serum cortisol response was observed at the 90 min of the ITT (49 %), HDST (89 %) and MDST (56 %) and at the 40 min of the LDST (44 %). Results expressed as 95 % confidence intervals: 408.0-843.6 and 289.5-868.1 nmol/l in the IIT at 60 and 90 min. In the HDST and the MDST serum cortisol reached the maximum at 90 min 542.6-1245.5 and 444.2-871.3 nmol/l. Levels of salivary cortisol followed the same pattern as serum cortisol. Salivary cortisol reached the maximum response in the HDST and the MDST at 90 min and at 40 min in the LDST. We confirmed good reliability of all tests with respect to timing of response and maximum response compared to the ITT. We proved that the MDST test can provide the similar response in serum cortisol to the HDST. Measuring either salivary cortisol or ACTH levels did not provide any additional benefit then measuring serum cortisol by itself.

Key words

Insulin tolerance test • Synacthen tests • Serum cortisol • Salivary cortisol

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Introduction

Assessments of hypothalamic-pituitary-adrenal (HPA) axis insufficiency are an ongoing challenge. Misdiagnoses of adrenal insufficiency (AI) because of false negative stimulation test results should be minimized by using an optimal test with the highest sensitivity and specificity.

The gold standard is still regarded to be the insulin tolerance test (ITT), but the cut-off level for the ITT is still unclear. In many centres the ITT is avoided due to several limitations. An easily performed alternative, the Synacthen test, was introduced 45 years ago, but there are still a lot of controversies and issues about the choice of test.

Moreover, it is still unknown whether morning cortisol can sufficiently predict adrenal gland capacity. A morning serum cortisol (SeC) below cut-off of 103 nmol/l (Deutschbein *et al.* 2009) and over 381 nmol/l were suggested (Maguire *et al.* 2008, Hana *et al.* 2015). In such cases, if AI is still suspected, then a stimulation test is recommended.

The standard Synacthen test (HDST) is usually

performed with a 250 µg dose of ACTH (Ospina *et al.* 2016). Nevertheless, this dose can overstimulate the adrenal gland, particularly in cases of a mild form of AI, and can cause difficulties with diagnosis. To avoid misinterpretation, it has been suggested to use a more physiological dose of Synacthen. The dose 1 µg of Synacthen (the low dose Synacthen test, LDST) has also been widely used, but this test has got many pitfalls.

The selection of either the HDST or LDST is still a controversial topic. There is a lack of standard protocols for the performance of the LDST, the methods of Synacthen administration, timing of the blood test or the cut-off (Wallace *et al.* 2009, Chatha *et al.* 2010). The 1 µg ACTH dose can only be applied intravenously. Only 24 % of the 1 µg dose may reach circulation administered intramuscularly (Dickstein 1998). The loss of ACTH during reconstitution of commercial ampule and during application is a crucial argument against the LDST (Dickstein *et al.* 2008, Murphy *et al.* 1998). Dickstein also examined the question of ACTH stability in low concentration solutions and confirmed its stability and biological efficacy (Dickstein *et al.* 2008).

Our interventional/observational study aimed to compare the response of cortisol in serum and saliva to the ITT and to three doses of Synacthen. We also tested medium dose 10 µg of Synacthen whether it may provide a sufficient response and decrease misdiagnosis of AI. We attempt to answer the still-unresolved question of the best test of HPA insufficiency.

Subjects and Methods

Eligible participants were recruited by a poster in the Institute of Endocrinology, Prague, Czech Republic. 64 healthy volunteers (33 men, 31 women) with mean age 41.4 and mean BMI 25.5 kg/m², with no history of endocrine disorder, serious disease, treatment with corticosteroids and estrogens were included. All subjects signed informed consent forms prior to inclusion. The study was approved by the Regional Ethical committee, and performed according to the principles of Good Clinical Practice (CPMP/ICH/135/95).

The following tests were performed consecutively, with a minimum interval of one week between each test: ITT (n=57), HDST (n=62), MDST (n=61) and LDST (n=64) and in the morning hours (08:00-09:00) after overnight fasting.

An intravenous cannula was inserted in the

forearm vein. After 30 min of rest a baseline blood and saliva sample were taken (time 0) and then serum and saliva samples were taken simultaneously at defined intervals during each test. We evaluated serum cortisol (SeC) and salivary cortisol (SaC) and, in addition, ACTH and CRH during the ITT.

For the ITT, insulin (Humulin R, Eli Lilly, Indianapolis, IN, USA) was administered intravenously at a dose of 0.15 IU per kg of body weight. Blood samples were taken at 20, 30, 40, 60, 90 and 120 min after the administration of insulin.

ACTH (tetracosactid 250 µg/1 ml, Synacthen, Novartis) was used for each Synacthen test. A standard commercial ampule of Synacthen was reconstituted to doses of 10 µg and 1 µg ACTH right before each test by adding 250 ml of isotonic saline, respectively, to the 250 µg ACTH/1 ml ampule.

For the HDST, a standard dose of 250 µg of ACTH was administered intravenously and then blood was drawn at 30, 60 and 90 min.

For the medium dose Synacthen test (MDST), 10 ml of diluted Synacthen solution (diluted as described above) containing 10 µg of ACTH were administered intravenously and then blood samples were drawn at 30, 60 and 90 min.

The LDST was performed by intravenous administration of 1 ml of Synacthen solution (diluted as described above) containing 1 µg of ACTH. Blood samples were drawn at 20, 30, 40 and 60 min.

Blood samples were taken into Vacuette tubes (plastic tubes for sampling coagulating blood with a coagulation activator and separation gel). Serum was obtained by centrifugation for 5 min at 2000g at 4 °C, and then stored at -20 °C. Saliva was sampled at regular intervals during each test. Saliva samples were collected in Sarstedt Salivette saliva examination tubes type 51.1534, centrifuged for 5 min at 2000g at 4 °C, and frozen at -20 °C.

Laboratory method

Serum and salivary cortisol were analyzed by the chemiluminiscent immunoassay (CLIA) Centaur XP Siemens (CLIA) method. Sensitivity was 5.5 nmol/l.

ACTH was analyzed by IRMA kits (Brahms Diagnostica, Hennigsdorf, Germany). Sensitivity was 1.2 pg/ml. The intra- and interassay variability was less than 5.0 % and 10.0 %, respectively.

Corticotropin Releasing Factor (CRH) was analyzed by RIA kits (Phoenix Pharmaceuticals, Inc,

Karlsruhe, Germany). The sensitivity of the CRF RIA kit was 23.9 ± 2.5 pg/tube. The interassay and within-assay variation coefficients at 16 pg/tube were 8.4 % and 3.8 %, respectively.

Statistical analysis

Statistical analysis was performed using the STATISTICA Version 12 (StatSoft Inc, Prague, Czech Republic) program. Confidence interval was obtained using MedCalc software, version 12.7.7.0 (MedCalc software, Mariakerke, Belgium).

Results

Serum cortisol (SeC)

ITT

95 % of subjects achieved SeC levels of over 500 nmol/l at 60 min. The remaining three (5 %) subjects had SeC levels of 427, 494 and 484 nmol/l at 60 min; this was the maximum response for two of them (3.4 %), the third subject reached a level of over 500 nmol/l at 90 min. Two false positive results were detected in the ITT. Surprisingly, these subjects achieved a normal response in all three Synacthen tests. 84 % of subjects reached SeC levels over 550 nmol/l, and 72 % were over 600 nmol/l at 60 min, 61 % at 40 min, and 90 % were over 500 nmol/l at 90 min. All subjects reached sufficient hypoglycemia during the ITT.

HDST

All subjects reached SeC levels of over 500 nmol/l after stimulation with 250 µg of Synacthen at 60 min and at 90 min. 97 % of subjects at 30 min. 98 % and 97 % subjects at 60 min and 90 min had SeC over 550 nmol/l, respectively.

MDST

98 % subjects exceeded the cut-off 500 nmol/l, 97 % over 550 nmol/l at 60 min.

95 % were over 500 nmol/l and 79 % over 550 nmol/l at 30 min. At 90 min, 97 % were over 500 nmol/l, 92 % over 550 nmol/l, and 89 % over 600 nmol/l.

LDST

At 60 min, only 67 % of subjects had reached a SeC level of over 500 nmol/l, though 48 % over 550 nmol/l.

A vast majority of subjects, 98 % and 86 %, were over 500 nmol/l and 550 nmol/l at 40 min, respectively.

95 % and 86 % of the subjects were over 500 nmol/l and 550 nmol/l at 30 min, respectively.

At 20 min, 92 % and 83 % were over 500 nmol/l and 550 nmol/l.

One subject reached a SeC over 500 nmol/l by 20 min and then SeC gradually declined.

More details are shown in Table 1 and Figures 1, 2, 3 and 4.

Distribution of the maximal SeC response in all tests

The distribution of maximum SeC responses according to the time of occurrence differed in all of the tests. The prevalence of the maximum response was 49 % at 90 min during the ITT, 89 % at 90 min during the HDST, 56 % at 90 min during the MDST, and 44 % at 40 min during the LDST. The LDST had the widest maximal response distribution of all tests. For more details see Table 2.

Table 1. Serum cortisol levels (nmol/l) at basal condition and after the stimulation expressed as 95 % confidence intervals.

	ITT	HDST	MDST	LDST
<i>Confidence interval %</i>	2.5-97.5	2.5-97.5	2.5-97.5	2.5-97.5
<i>0 min</i>	184.8-610.7	170.8-653.6	159.1-584.0	132.3-660.1
<i>20 min</i>	161.6-557.3	-	-	444.5-808.6
<i>30 min</i>	137.7-596.6	484.5-991.4	476.2-719.1	490.1-786.8
<i>40 min</i>	242.0-698.7	-	-	501.1-771.7
<i>60 min</i>	408.0-843.6	554.6-1013.8	508.6-839.3	341.9-775.5
<i>90 min</i>	289.5-868.1	542.6-1245.5	444.2-871.3	-
<i>120 min</i>	307.4-827.2	-	-	-

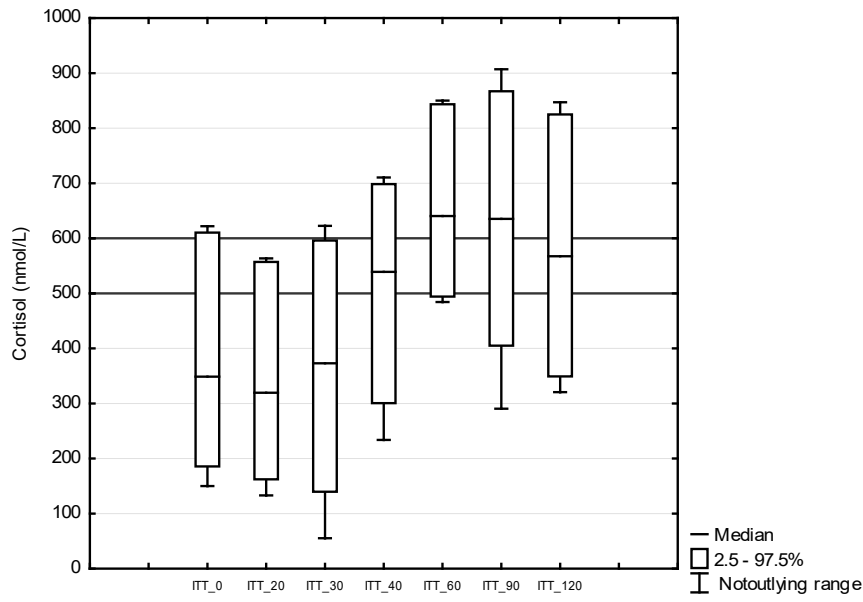


Fig. 1. Serum cortisol levels during the ITT. Cut off limits of 500 and 600 nmol/l are shown.

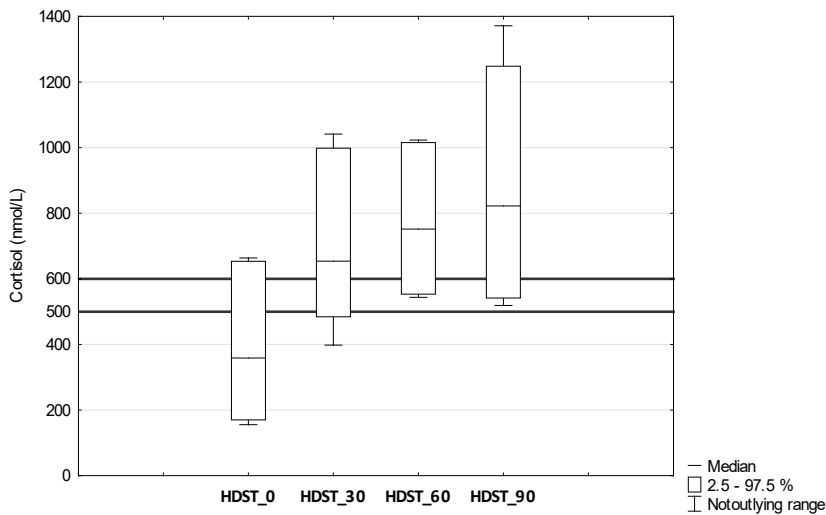


Fig. 2. Serum cortisol levels during the HDST. Cut off limits of 500 and 600 nmol/l are shown.

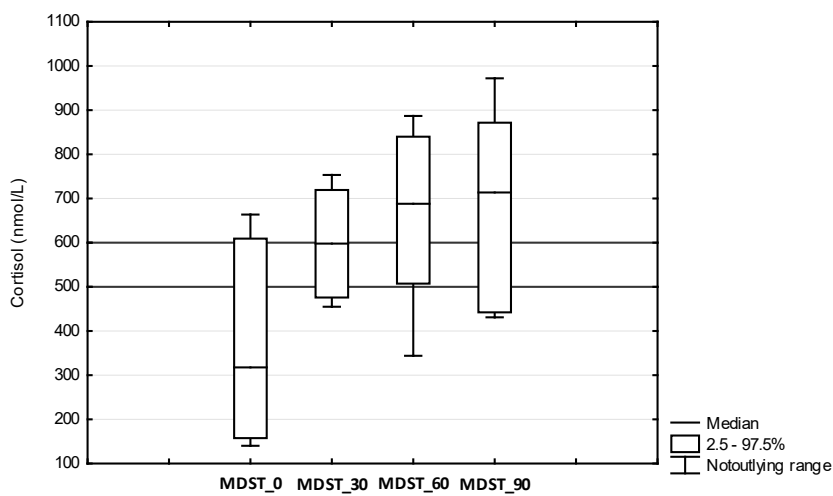


Fig. 3. Serum cortisol levels during the MDST. Cut off limits of 500 and 600 nmol/l are shown.

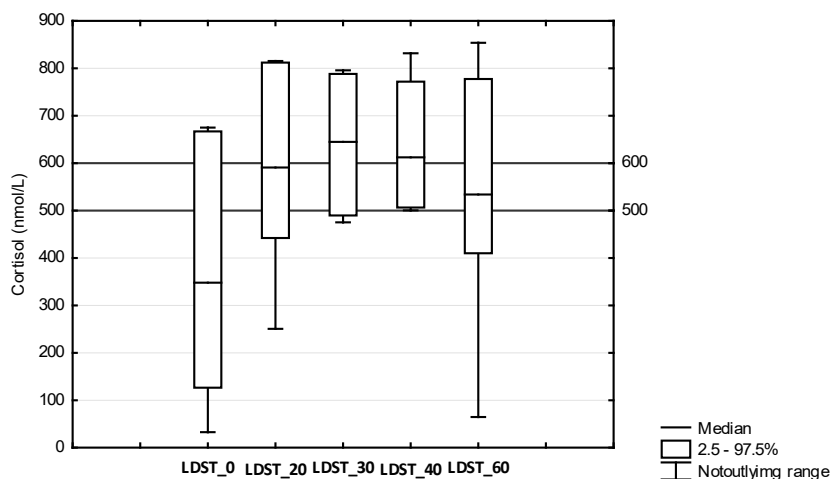


Fig. 4. Serum cortisol levels during the LDST. Cut off limits of 500 and 600 nmol/l are shown.

Table 2. Number of subjects reaching used cut off for serum cortisol for evaluation of stimulation test.

	>500 nmol/l	>550 nmol/l	>600 nmol/l
HDST - 60 min	62/62 (100 %)	61/62 (98.4 %)	58/62 (93.5 %)
HDST - 90 min	62/62 (100 %)	60/62 (96.8 %)	58/62 (93.5 %)
MDST - 60 min	60/61 (98.4 %)	59/61 (96.7 %)	53/61 (86.9 %)
MDST - 90 min	59/61 (96.7 %)	56/61 (91.8 %)	54/61 (88.5 %)
LDST - 30 min	61/64 (95.3 %)	55/64 (85.9 %)	43/64 (67.2 %)
LDST - 40 min	63/64 (98.4 %)	55/64 (85.9 %)	40/64 (62.5 %)
ITT - 60 min	54/57 (94.7 %)	48/57 (84.2 %)	41/57 (71.9 %)
ITT - 90 min	51/57 (89.5 %)	44/57 (77.2 %)	36/57 (63.2 %)

Table 3. Salivary cortisol levels (nmol/l) at basal condition and after the stimulation expressed as 95 % confidence intervals.

	ITT	HDST	MDST	LDST
Confidence interval %	2.5-97.5	2.5-97.5	2.5-97.5	2.5-97.5
0 min	3.7-51.6	2.5-42.0	3.7-33.9	2.0-45.4
20 min	3.2-51.6	-	-	5.2-72.2
30 min	3.3-50.3	7.9-97.3	9.0-82.1	8.1-76.6
40 min	4.7-61.1	-	-	10.4-76.9
60 min	9.6-73.6	12.1-126.0	15.7-105.0	7.0-59.9
90 min	8.9-78.0	12.1-158.4	12.5-114.7	-
120 min	5.5-96.3	-	-	-

Salivary cortisol (SaC)

ITT

Cortisol increments during the ITT expressed as 95 % confidence intervals are shown in Table 3. The range between the lowest and highest SaC levels was quite wide (3.38-55.35 nmol/l). 7 out of 35 subjects

(20 %) provided adequate amount of saliva during the ITT and had low levels of salivary cortisol during the entire ITT, failing to reach the suggested cut-off 20 nmol/l (Contreras *et al.* 2004). For the remainder of the subjects, the maximal response of SaC was observed at 90 min (median 45.79 nmol/l).

ACTH tests

The SaC concentration correlated with SeC, and the maximal responses of SaC after Synacthen stimulations were similar to SeC. The maximal responses of SaC were observed at 90 min during the HDST, at 60 min during the MDST and at 30 min during the LDST.

CRH and ACTH during the ITT

The results of CRH reaction to insulin-induced hypoglycemia were heterogeneous and inconclusive. The maximum response of ACTH in ITT was observed at 40 min (median 258.6 nmol/l) then ACTH rapidly declined. Results expressed as median ACTH concentrations during the ITT were 23.7 ng/l at baseline, and 25.5, 45.1, 258.6, 256.9, 56.9 and 29.6 ng/l at 20, 30, 40, 60, 90 and 120 min, respectively.

Discussion

The ITT showed similar results as the HDST and MDST tests in the percentage of subjects with sufficient responses with respect to the time of occurrence and cut-off. The same cut-offs for cortisol during the LDST were reached earlier, however, mostly at 30 and 40 min. This confirmed that the cortisol response is dose dependent (Dickstein *et al.* 1991) and that the MDST can provide comparable responses as the HDST. The percentage of false positives in all tests increased with increased cut-off levels of serum cortisol. We used three different cut-offs of cortisol for all 4 tests to evaluate the sensitivity of these cut-off levels, and logged the time that a response occurred. During the LSDT the maximal stimulated cortisol response is found between 20 and 30 min and during the HDST between 60 and 90 min (Nye *et al.* 1999, Rasmuson *et al.* 1996, Patel *et al.* 1999). Nevertheless, current recommendation for evaluation of maximum response is still 20 and 30 min for the LSDT and at 30 min for the HDST. Based on other dose response studies (Crowley *et al.* 1991, Dickstein *et al.* 1997) we decided to follow the cortisol response to 3 different ACTH stimuli for 90 min during the HDST and MDST tests, and more frequently for 60 min during the LDST. During the LDST the highest prevalence of maximal SeC response was at 40 min (44 %), compared to at 90 min during the MDST and HDST (maximal responses of 56 % and 89 %, respectively). Our results support frequent sampling during the first hour of the LDST. An additional blood test at 90 min during the MDST and HDST should also be considered. During the

ITT sampling at the 120 min it may be necessary to obtain a maximal SeC response. These delayed maximal SeC responses may be used to exclude AI or they may indicate mild AI and may be the first sign of AI (Dickstein *et al.* 1991, Park *et al.* 1999).

Interpretation of results and their specificity particularly depends on determining the maximal stimulated cortisol concentration or the lowest limit for a normal adrenal response (maximal response, cut-off). Generally, in healthy individuals the SeC response after administering 1 µg and 250 µg ACTH ranges from 390-770 nmol/l (Patel *et al.* 1999). However, no agreement yet exists on the cut-off limit of maximal serum cortisol response after stimulation. Some authors suggested 500 nmol/l, others 550 nmol/l or even 600 nmol/l (Hurel *et al.* 1996, Metha *et al.* 2005, Cho *et al.* 2014). Partial AI, with maximal cortisol levels from 510 to 550 nmol/l, is a term used by some authors (Agha *et al.* 2006). We evaluated the applicability of the three most frequently-used SeC cut-offs of 500, 550 and 600 nmol/l. Our results showed that the cut-off 500 nmol/l was most frequently reached in the ITT and LDST, the cut-off 550 nmol/l in the MDST, and 600 nmol/l in the HDST, regardless of the time of occurrence. Cortisol levels of 500-550 nmol/l were considered to be a maximal increase upon stimulation by 1 µg ACTH that corresponds to adrenal stimulation after the ITT with sensitivity of 94 % and specificity of 90 % (Tordjman *et al.* 2000, Rasmuson *et al.* 1996, Dickstein *et al.* 2001). We consider SeC level cut-off of 500 nmol/l for the LDST to be the optimal cut-off for distinguishing patients with an intact HPA. Based on our results, higher levels of 550 or even 600 nmol/l, as suggested by Abdu *et al.* (1999) would be inappropriate in terms of higher number of false negative results.

Generally, the maximal stimulated cortisol response occurs 30 min after the administration of 0.5-1 µg of ACTH (Dickstein *et al.* 1991) and 60 min after 250 µg of ACTH. In our study, the HDST as well as the MDST showed the highest frequency for a SeC response (over 500 nmol/l) at 60 and 90 min. For the LDST maximal SeC levels were reached earlier, at 30 and 40 min, in line with the results of previous studies. According to the cut-off values and timing used in our study, the MDST gave a similar SeC response as the HDST. Unfortunately we did not follow SeC up to 120 min during those tests.

The question about comparability of ACTH tests and the ITT is still being studied; numerous studies

showed good correlation between 1 µg ACTH and the ITT, while other studies found that the ITT causes a greater maximal cortisol increase (Dickstein *et al.* 1991, Rasmuson *et al.* 1996, Gonzálbez *et al.* 2000, Giordano *et al.* 2008). One explanation may be insufficient hypoglycemia reached during the ITT (Hurel *et al.* 1996). In our study, the LDST provided similar response of the SeC as the ITT and, interestingly, 2 subjects failed to reach SeC over 500 nmol/l despite achieving adequate hypoglycemia.

Administration of 1 µg ACTH 1-24 resulted in similar levels of ACTH 100 ng/l in circulation as during the ITT (Alia *et al.* 2006, Nye *et al.* 1999). In our study, ACTH exceeded 100 ng/l, and the maximum SeC response was reached between 60 and 120 min with the highest prevalence (49 %) at 60 min. Our median ACTH was 256.9 ng/l at 60 min during the ITT, confirming proper ITT performance with all subjects achieving sufficient low-blood glucose levels. The two subjects failed to reach SeC levels of over 500 nmol/l (maximal responses were 427 and 494 nmol/l) and did not differ in their severity of hypoglycemia (1.3 and 1.2 nmol/l) or in BMI (20.1 and 28.5). Corresponding maximal ACTH levels in these subjects were 108.4 and 69.6 ng/l.

Making the interpretation of results even more difficult is the fact that there are circumstances that can lead to altered levels of cortisol binding proteins and albumin. The SaC measurement was introduced to circumvent this issue. One study has suggested a SaC cut-off of about 20 nmol/l, but no clear cut-off limit for adrenal insufficiency has yet been published (Contreras *et al.* 2004). Saliva cortisol levels are currently only useful as an additional tool for assessing HPA axis insufficiency. Compared to a previous study (Kosak *et al.* 2014), with 250 µg of Synacthen we found lower SaC values of 2.9-42 nmol/l before the test, and a wider range of SaC levels after stimulation: 7.93-97.2 nmol/l at 30 min and 12.1-126.0 nmol/l at 60 min (data expressed as 95 % confidence intervals). Therefore, measurements of SaC levels gave no additional benefit over serum cortisol, regardless of the type of test.

Additionally, the influence of gender on cortisol response has yet to be quantified (Roca *et al.* 2005, Roelfsema *et al.* 1993). We did not find a difference in

SeC response between men and women, and age was also excluded as an additional confounding factor.

Our assessment of CRH during the ITT confirmed previous observations that CRH levels in the periphery do not correlate, because extra-hypothalamic sources of CRH contribute to serum levels (Jeske *et al.* 1989). Measuring ACTH along with SeC during the ITT did not give any advantage for assessing the HPA axis (Born *et al.* 2003), ACTH correlated with SeC during the ITT. However, we do not suggest replacing SeC with ACTH measurements. At most, an evaluation of ACTH might be used as an additional marker during the ITT in some borderline clinical cases.

Conclusions

All the tests provided reliable evaluations of the HPA axis but each test needs its own cut-off. However, our data lacks comparisons with an appropriate group of patients with AI.

Our results suggest that the MDST may replace the HDST, with additional blood samples at 90 min. Moreover, blood tests during the LDST must be performed more frequently.

Salivary cortisol assessments did not show any benefits and we found no superiority of SaC over SeC in these stimulation tests. Synacthen test, especially MDST, may be suggested as a first – choice test in suspicion not only for primary, but also for secondary adrenal insufficiency with serum cortisol level 500 nmol/l as a sufficient cut-off limit.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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