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The Low-Dose ACTH test: Usefulness of Combined Analysis of Serum and Salivary Maximum Cortisol Response in Paediatrics.

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Context: The low-dose1-µgACTH test (LDT) is widely used to assess central adrenal insufficiency (CAI); however, the serum cortisol cut-off value is controversial. Salivary cortisol (SC) could be a more accurate measurement for CAI.
Objective: to assess a new maximum cut off value of serum cortisol after LDT in paediatric patients, taking into account the measurement of both serum and salivary cortisol
Design and Setting: prospective study in a paediatric tertiary referral center.
Working Hypothesis: the combine analysis of serum and salivary cortisol response to LDT might improve LDT for CAI diagnosis.
Participant and outcome measurement: 145 pediatric patients underwent LDT. Serum and salivary cortisol levels were measured. Sufficient response (CAS) was established accordingly to the reference serum cortisol cut-off value ≥497 nmol/L
Results: LDT study showed CAS in 72 and CAI in 73 patients**.** Considering the lower quartile of maximum salivary cortisol value (21 nmol/L) in CAS group (Gr), an intermediate (InCAI) and a real (RCAI)Gr were defined within the CAIGr. Regarding the median maximum value of serum cortisol levels in the InCAIGr, a new serum cortisol cut-off value of 450 nmol/L was established. Furthermore, 91 % of the patients in the RCAIG were below this cut-off value.
Conclusion: The combined evaluation of maximum serum and salivary cortisol levels to LDT might be useful to define an InCAIGr and avoid unnecessary hormone replacement therapy. However, rigorous patient follow-up is required

We propose the combined evaluation of maximum serum total and salivary cortisol responses after low doses ACTH test for Central Adrenal Insufficiency diagnosis in paediatrics.

Introduction

Adrenal insufficiency (AI) is a life –threatening disorder that can result from primary or central (C) adrenal disease due to impairment of hypothalamic-pituitary axis.

The diagnosis of AI is challenging because of its unspecific clinical signs, and the limitations of diagnostic tests of adrenal reserve. Diagnosis relies, mainly for the CAI, on laboratory testing of serum cortisol levels. In the latest years the low dose 1- µgr ACTH test (LDT) for the diagnosis of CAI has gained wide acceptance. (1-7). However, the lack of

cortisol assays standardization, the different test protocols applied as the timing of blood sample after ACTH stimulation (30 or 60 minutes), the preparation of 1ug of ACTH and the high variability in the serum cortisol levels diagnostic thresholds, challenge the reliability of the dynamics test of the hypothalamic-pituitary adrenal axis, (2,8-9), particularly in paediatric patients in whom the gold standard tests, insulin tolerance test (ITT) and metyrapone test, are considered of risk. In addition, a correct diagnosis of CAI is required, since over diagnosis may lead to unnecessary and detrimental hormone replacement therapy.

The current accepted maximum serum total cortisol (TC) cut-off of 497 nmol/L after ACTH test (10-11) for a CAI diagnosis has been revised in the latest years, principally for the LDT. Lower serum TC cut-off levels are proposed to improve the specificity. (2,11). In a paediatric meta-analysis Kazlauskaite et al, (1) showed that after a LDT, a serum TC levels less than 441.4 nmol/L was highly considered to predict CAI. However, maximum serum TC levels response to LDT between 441.4 -606.9 nmol/L was considered as an indeterminate area and CAI could not be defined.

Serum free cortisol (FC) level has been shown to provide a more accurate measurement of the circulating glucocorticoid status than serum TC (12), but measuring it, is not generally available.

Salivary cortisol (SC) is mostly in the free form and is in equilibrium with plasma FC, therefore, it is a surrogate for the concentration of serum FC. Its measurement correlates well with both, total and biological active serum FC(13). Several studies (14-17) have proposed that salivary sampling might be useful to define adrenal function after Synthetic ACTH test in both, healthy volunteers as well as in patients suspected of having adrenal insufficiency.

Therefore, we have hypothesized that the combine analysis of the maximum serum TC and SC response to LDT might improve the diagnosis of CAI in paediatric population.

In this study, a new LDT cut-off value of serum TC levels taking into account the measurement of both serum TC and SC was established in a pediatric population. In addition, an intermediate group was defined to allow the avoidance of hormone replacement therapy.

Subjects and Methods

Subjects

The study group consisted of 145 pediatric ambulatory patients suspected of having CAI that underwent LDT at our Institution. Clinical characteristic and diagnosis are showed in **table 1**. Diagnoses leading to the indication of the LDT were: chronic corticosteroid treatment: n=18, 12.4%, multiple pituitary insufficiencies, n=79, 54.4% (idiopathic and secondary to central nervous system (CNS) tumors or their treatments,CNS malformations such as Septo-optic-dysplasia), and miscellaneous (Prader Willi syndrome, Autoimmune disease, genetic syndromes), n=48,33.1%.

Suspected CAI was defined by the presence of history and/or clinical compatible with CAI (orthostatic hypotension, abdominal pain, nausea and vomiting), fasting morning, serum TC levels less than 276 nmol/L and ACTH inappropriate low for serum TC levels. Patients with other hormonal deficiency were on adequate replacement therapy and those on chronic physiological hydrocortisone therapy (doses: 9 mg/m2/day) were evaluated with at least 48 hs of therapy suspension. (18)

Puberty was defined as testis volume ≥ 4 ml in males and breast Tanner stage 2 in females.

Exclusion criteria were: girls on estrogen therapy, hypothalamus-pituitary tumor surgery less than 1 year previously, primary adrenal insufficiency evidenced by high levels of ACTH.

Low dose ACTH test (LDT)

LDT was performed in an out patient setting, in the morning accordingly to previous report (18). Briefly: one ampoule of 250 µg /ml of tetracosactide acetate was diluted with Cl Na

0.9% to reach the appropriated concentration by personnel with long-term experience in the dilution process. Each dose was stored at 4˚C for up to 12hs before administration. A dose of 1µg per m2, up to 1.5µg total dose, was administered as intravenous bolus. Saliva samples were obtained using the Salivette tubes containing synthetic swabs.or hyssop. Patients were fasting and those older than 5 years were requested to rinse their mouth with water just before obtaining the first sample.

Serum and salivary cortisol were measure simultaneously, at baseline, 30 and 60 minutes, after synthetic ACTH injection.

The highest value of the tested parameters, at either 30 or 60 minutes, was regarded as the maximum response value. Reference cut-off value ≥ 18 µg /dl (497 nmol/l) of serum TC levels was considered as a sufficient response (10-11,19)

Patients were classified in two groups (Gr) according to the reference serum TC levels cut- off (10) : a CA sufficient response (CAS) Gr and a CA Insufficient (I) response (CAI) Gr. In addition, taking into account the lower quartile of SC in CAS Gr, CAI patients were sub-classified into an intermediate (In) and Real (R) CAI Gr.

This study was approved by the Ethics Committee of the Garrahan Paediatric Hospital. Informed consent was obtained from parents and patients.

Assays

Serum Cortisol was measured by Immulite 2000 Chemioluminescent . Intra-assay CV were for level 1: 103 nmol/L = 6% and level 2: 540.7 nmol/L, 3.9% Interassay CV for level 1= 6.9% level 2 = 4.2%

Salivary cortisol was measure using enzyme immunoassay (ELISA) Euroimmun. Intra-assay CV were for level 1: 5.5 nmol/Ll, 4.2% and level 2: 35.8 nmol/L , 3.2% Interassay CV for level1: 7.9% and for level2: 4.7%

Statistical analysis

The statistical analysis was performed using Statistix 2000 Analytical Software

Data not distributed homogenously, were analyzed by nonparametric tests.

Data are reported as mean (SD) or median (interquartile ranges) when the Shapiro Wilk test showed normal or not normal distribution respectively.

Correlation between maximum TC and SC was evaluated by Spearman s rho correlation analysis.

Receiver operating characteristic (ROC) curve was drawn to determine the performance of serum cortisol for predicting adrenal insufficiency, using new salivary cortisol cut-off

The sensitivity, specificity and likelihood ratios were calculated.

Comparisons between two groups were made by Wilcoxon Rank Sum Test.

Comparison among groups of maximum serum TC response was made with one way AOV and Bonferroni comparison of means, as maximum cortisol response had a normal distribution.

Statistical significant was considered with p value less than 0.05.

Conversion factor for nmol/L to µg/dl: divide by 27.59

Results:

The clinical characteristics of the 145 patients included in the study are shown in **table 1**. There was a predominance of boys vs. females, p<0.01(88 males and 57 females) and pubertal patients, p<0.01(86 pubertal and 59 pre-pubertal).

In 86.1% of the patients, reference serum TC cut- off level response after LDT was achieved at 30min while in 13.9% of them occurred at 60 min.

In accordance with maximum serum TC cut-off levels (serum TC ≥ 497 nmol/L) previously reported (10), 72 patients were considered to have a sufficient adrenal response

(CAS Gr) while 73 patients had insufficient response (CAI Gr) . The median (interquartile range) of maximum serum TC and SC levels in the CAS Gr were 587.6 nmol/L (546.2-971), and 31.4 nmol/L (21-43.5), and in the CAI Gr were 400 nmol/L (336.5-452.4), and 16 nmol/L (10-28.6), respectively. As it is shown in Table 2 median SC values were significantly higher in the CAS Gr in comparison to CAI Gr, $p<0.01$.

A significant positive correlation between maximum serum TC and SC response to LDT was found, p Spearman; 0.6; $p<0.001$.

In order to define a new serum TC cut off level, the lower quartile of maximum SC (21 nmol/L) in the CAS Gr was applied in CAI Gr. In this way, two sub groups of patients were defined: an Intermediate (In) CAI Gr and a Real (R) CAI Gr wherein SC was higher than/ equal to or less than 21 nmol/L respectively**. Fig 1 panel A.**

Median (interquartile range) maximum serum TC and SC (nmol/L) response values were as follows : In-CAI Gr (n= 28 patients) 450 (392- 463.5) and 32 (24.2-35.5), respectively and R-CAI Gr, (n= 45 patients) 364 (311.7-422) and 11.3 (8.8-15), respectively. **Fig1 panel B**

Significant differences in maximum serum TC level responses were found among CAS Gr, In-CAI Gr, and R-CAI Gr ($p< 0.001$). Considering the median maximum TC (450 nmol/L) in the In-CAI Gr as a new cut off, 91 % of the patients in the RCAI Gr had TC bellow the cut-off value. As a result, In the In-CAI Gr 14/28 (50%) had TC between 450 and 494 nmol/L and SC ≥ 21 nmol/L. **Fig 1 panel B.**

Roc curve analysis revealed that ≥450nmo/L has a sensitivity (95% C.I) of 63.5(50.4-75.3) and specificity(95% C.I) 82.9(73.0-90.3), +LR:3.72, -LR: 0.44

In **Fig 2** individuals, values are depicted accordingly to the reference maximum serum TC cut off value and the new maximum serum and salivary cortisol cut off values.

However, accordingly to the SC value defined within the CAS Gr, a number of patients had discrepant TC and SC responses in the three groups. In the case of CAS Gr, 17 out of 72 patients (23.6%) had SC less than 21 nmol/L. In the In- CAI Gr 14/28 patients (50%) had serum TC levels below 450 nmol/L but SC ≥ 21 nmol/L. For medical reasons, in four patients hydrocortisone replacement therapy was started. In the remaining patients, no clinical features of adrenal insufficiency were detected and several normal serum basal cortisol levels were found during at least 0.5-3.64.years of follow up.

In the R-CAI Gr, 4 out of 45 patients (8.8%) had serum TC levels greater than 450 nmol/L but SC less than 21 nmol/L. At least during 1-1.88 years of follow -up none of these patients received hydrocortisone replacement therapy since no clinical sings of adrenal insufficiency were detected and several normal serum basal TC levels were found. **Fig 2**

Discussion

In this study the combined analysis of maximum serum total and salivary cortisol levels response to LDT in order to define a new maximum serum cortisol cut off level, resulted in the establishment of an intermediate group and the avoidance of unnecessary hydrocortisone replacement therapy in a considerable percentage of patients.

The assessment of hypothalamic-pituitary-adrenal (HAP) axis is still an ongoing challenge. On this line, in the meta-analysis by Ospina et al.(20) the authors determined the diagnostic accuracy of ACTH tests (low and high dose), in both adults and pediatrics patients. The results showed low to moderate accuracy because of low sensitivity. For this reason, when positive ACTH test is detected, it can be considered as an acceptable reliable test. However they are not as reliable in ruling out the condition when negative. (20)

In this study, we propose the combined evaluation of maximum serum total and salivary cortisol responses after LDT, in order to obtain a more accurate serum TC cut off for CAI diagnosis in a paediatric population suspected of having CAI.

Using the recommended threshold for stimulated serum TC of 497 nmol/L (10), 72 patients of 145 (49.6%) were considered as having CA sufficient response, CAS Gr, whereas 73 (50.3%) had a subnormal response, belonging to the CAI Gr. Subsequently, SC, a surrogate of the biological active FC was measured simultaneously with serum TC through the LDT.

In agreement with previously reported studies a positive and significative correlation between maximum serum TC and SC in the LDT was obtained, (16,21) suggesting that SC might be useful to be considered to evaluate maximum serumTC cut- off level at LDT. We did not find any correlation between basal serum TC and SC levels (data not shown), probably due that SC falls bellow detectable levels during periods when serum TC concentration are too low (22).

According to the reference cut-off (10), most serum- TC- response was obtained at 30min (86%), in agreement with other studies (7, 15-16). However, in 14% of the patients it was observed at 60 min after LDT. Therefore, a longer LDT should be evaluated to avoid misdiagnosis.

Although median SC between sufficient and insufficient groups was significantly different, there was considerable overlap.

In Cetinkaya et al study (16) maximum SC cut off levels to define CAI by the ROC curve analysis, using reference serum TC cut off levels, (10) showed good specificity, but very low sensitivity. Moreover, in agreement with other study (19) we might propose that SC as the unique measurement at LDT is not useful for the assessment of CAI diagnosis.

Furthermore, in the absence of differences in the protocol as well as the methodology applied, different serum SC cut -off value was reported (23).

Considering the first quartile of SC cut-off value in the CASGr distribution, two subgroups of patients were defined in the CAI Gr: a R- CAI and an In CAI Gr establishing 450 nmol/L as a new maximum serum TC cut- off value. The reasonability of this new serum TC cut-off value is supported by the fact that in the RCAIGr, 91% of patients had serum TC levels bellow this new cut-off value

In agreement with Ospina et al meta analysis study, similar positive and negative likelihood ratio was obtained(20)

Moreover, in the paediatric metanalysis study by Kazlauskaite et al (1) and in adult populations studies (8, 24) a similar serum TC cut off value was recommended.

In this cohort 24% of patients had a discrepancy between maximum serum TC and SC levels to LDT response. Several reasons might explain these different responses to LDT. It could be speculated that higher or lower serum CBG levels modify serum CBG bound cortisol fraction concentration. Accordingly to serum CBG levels, serum non-CBG bound cortisol fraction and therefore SC concentration change (25-26). However, in this cohort serum CBG levels and serum Cortisol distribution between serums specific binding proteins were not measured.

Another explanation for this discrepancy might be related to SC metabolism. 11betahydroxi steroid dehydrogenase type2 which convert cortisol to cortisone is highly express in the salivary glands (22). Thus, normal variation in SC levels could be the consequence of interindividual differences in cortisol metabolism. On this line, there are evidences that stimulated salivary cortisone provide a better reflection of serum cortisol (22, 27-28).

Gender and age specific difference in hypothalamus-pituitary-adrenal axis activity during childhood has been described (29-30), Attempts to establish reference sex and age SC range are being analysed in the CIRCOT data set (31). Concerning to this point although there was a significative predominance of male and pubertal patients, the proportion of them in the subgroups analysed were similar.

A limitation of the present study is related to the fact that the new serum TC value cut-off to the LDT proposed was confirmed in a short but not in long-term follow up of patients. For safety reasons in paediatric population, we could neither validate the cut off with the gold standard tests (ITT and/or metyrapone).

Another constraint is the uncertainty that currently surrounds patients in whom discrepancies between the results of the serum TC and SC emerged in the course of the LDT. Nevertheless, the present study does suggest that measurements of SC may improve the interpretation of the LDT and allows further insight not given by testing based on serum TC or SC separately.

In addition, the same study using LC MS/MS methodology might be done. However, currently normal serum and salivary cortisol/ cortisone after LDT stimulation measured by LC-MS/MS values are poorly known in pediatric population (32-34).

Conclusion

We suggest the following algorithm for the diagnosis of CAI**,** depicted in Fig 3. As it is shown**,** after LDT, maximum serum TC response could be sufficient or insufficient. In the CAI Gr, SC could be of aid to differentiate an intermediate from a real insufficient group.

The recognition of a new lower cut-off of serum TC together with SC cut-off proposed, allows avoidance of unnecessary hormone replacement therapy in an intermediate group in which hormonal treatment relies on medical judgment and/ or the presence of symptoms of AI. However, if medical treatment is withheld; a rigorous patient follow-up is required, the latter apply specially to discordant results in the In-CAIGr and R-CAI Gr in whom outcome are of difficult interpretation.

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Disclosure summary:
The authors declare that they have nothing to disclose

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Fig 1. Panel A. Maximum salivary cortisol post LDT in CAS Gr , CAI Gr , and in the two subgroups. In-CAI, R-CAI obtained according to first quartile of CAS Gr. CAS: central adrenal sufficient, Gr: group, CAI central adrenal insufficient, In: intermediate, R: real. Panel B. Maximum serum TC response post LDT by groups CAS, In-CAI, R-CAI.

LDT: low dose1- µgr ACTH test; CAS: central adrenal sufficient; TC: total cortisol, In: intermediate, R: real. Conversion factor for nmol/L to µg/dl: divide by 27.59

Fig 2. Individuals values of maximum serum and salivary cortisol to LDT distributed according to the reference serum cortisol cut off value and the new serum and salivary cortisol cut off values. TC: total cortisol, SC: salivary cortisol. panel A: Intermediate central adrenal insufficient (In CAI) group (Gr) , panel B : Real central adrenal insufficient (R-CAI) Gr, panel C: central adrenal sufficient (CAS) Gr. Black circles: patients without hormone replacement treatment. White circles: patients in whom hormone replacement treatment was initiated or continued after LDT results. Dotted line: reference cut –off. Solid line: new serum and salivary cortisol cut-off

FIG 3. Algorithm. After LDT, maximum serum TC ≥497 nmol/Ll could be considered as sufficient response. In the intermediate group with serum TC between 450-497 nmol/L and SC ≥21 nmol/L, the response could also be considered as sufficient with clinical regards. In the intermediate group, patients with serum TC <450 nmol/L and SC ≥21 nmol/L the response is of difficult interpretation, as it is in patients in R-CAI Gr with serum TC between 450-497 nmol/L and SC <21 nmol/L . The initiation or not of substitutive treatment in the latest groups is under individual medial criteria , in case of deciding not to initiate hormone replacement treatment an strict clinical and biochemical follow-up should be carried out. When serum TC is <450 nmol/L and SC is < 21 nmol/L, the response could be considered real insufficient. Gray color represents the intermediate group. Discontinue arrows correspond to discordant results in each intermediate and real insufficient group. LDT: low dose1- µgr ACTH test; TC: total cortisol; SC salivary cortisol. Conversion factor for nmol/L to µg/dl: divide by 27.59

Table1. Clinical Features and Diagnosis of 145 patients included in the study.

Age mean ±SD, year	**11.3±4.8**
Sex Male n (%)	**88 (60.6)***
Female n (%)	**57 (39.3)**
Tanner pubertal status n (%)	
Pre pubertal	**59 (40.7)**
Pubertal	**86 (59.3)****
Patients etiologies	
Chronic corticoid treatment n (%)	**18 (12.4)**
Multiple pituitary insufficiencies #. n(%) (## diagnosis and [abcd] treatment)	**79 (54.5)**
Miscellaneous: PWS, autoimmune disease, Genetic syndromes n (%)	**48 (33.1)**

* male vs female p<0.01

** pubertal vs prepubertal p<0.01

Puberty was defined as testis volume ≥ 4 ml in males and breast Tanner stage 2 in females.

Multiple pituitary insufficiency (idiopathic and secondary to central nervous tumor (CNS),

PWS: Prader Willi Syndrome.

Hormonal deficiency were as follow (%): GH and TSH: (43.6); isolated TSH: (21); Isolated GH: (11.2); GH and LH-FSH: (8.4); isolated Diabetes Insipidus (DI): (5.6); TSH and DI: (4.2); GH and DI: (2.8) GH,TSH and DI:(1.4); TSH and LH-FSH: (1.4).

The pituitary insufficiency group consisted of patients with the following diagnosis (%) : CNS tumors and their treatments(a: surgery, chemotherapy and radiotherapy), b: medical treatment, c: chemotherapy, d: chemotherapy and rediotherapy (31.6) : (Chiasmatic-hypothalamic glioma[a], posterior fossa medulloblastoma[a] , pineoblastoma[a], giant prolactinoma[b]) , Langerhans cell histiocytosis[c] (7.5), leukemia and retinoblastoma[d] (6.3), idiopathic hypopituitarism (32.9), septo-optic dysplasia and other CNS malformation. (21.5)

Table2: Clinical features and hormonal laboratory findings of 145 patients included in the study. LDT: Maximum serum and salivary cortisol response.

Group	CAS	CAI	CAI	
			Intermediate	Real
Patients: n	72	73	28	45
Age mean±SD (year)	10.4±5.5	12.2±4.0	11.9±3.1	12.4±4.4
Sex n (%)				
Male	40	48	18 (37.5)	30 (62.5)
Female	32	25	10 (40.0)	15 (60.0)
Tanner pubertal				
status n (%)	37	22	9 (40.9)	13(59.0)
Prepubetal	35	51	19 (37.2)	32 (62.0)
Pubertal				
LDT maximum TC (median; interquartile range) nmol/L				
	587.6	400	450*	364.1
	546.2-971	336.5-452.4	391.7-463.5	311.7-422.1
LDT maximum SC (median; interquartile range) nmol/L				
	31.4**	16	32***	11.3
	21- 43.5	10-28.6	24.2-35.5	8.8-15.1

CAS : Central Adrenal Sufficient response. CAI: central adrenal insufficient response. LDT: low dose1- µgr ACTH test. TC: total cortisol, SC: salivary cortisol. Pubertal was defined as testis volume ≥ 4 ml in males and breast Tanner stage 2 in females.
Total cortisol Intermediate vs Real CAI * p<0.01
LDT saivary cortisol CAS vs CAI **p<0.01
LDT saivary cortisol Intermediate vs Real ***p<0.01
Conversion factor for µg/dl to nmol/l: multiply by 27.59

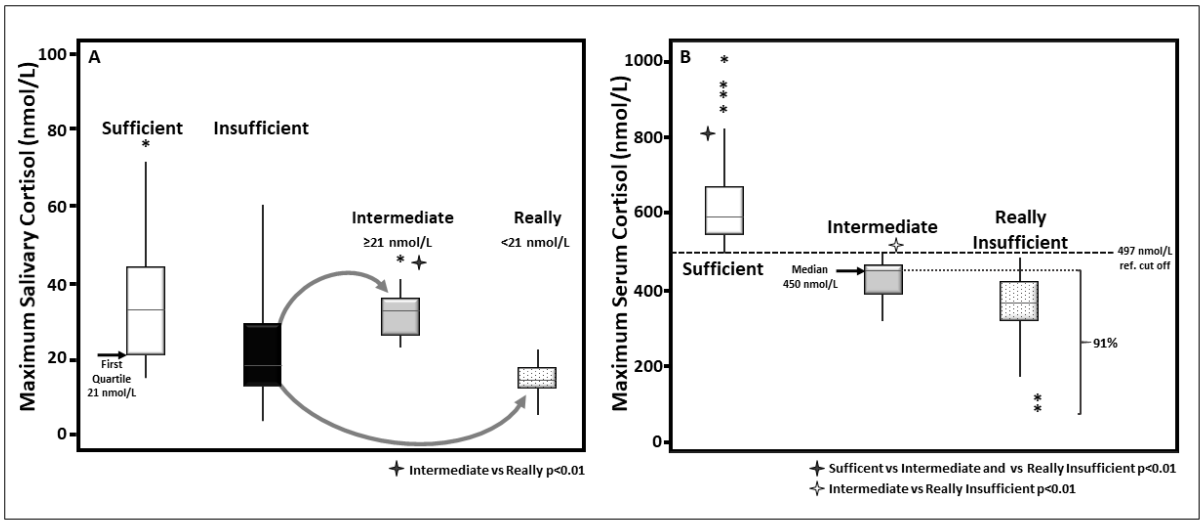

Figure 1

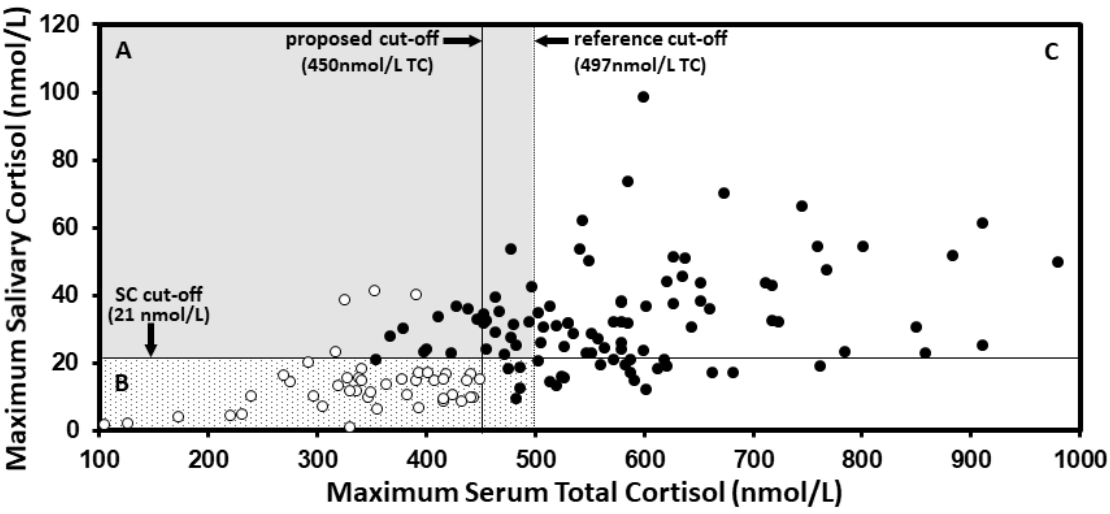

Figure 2

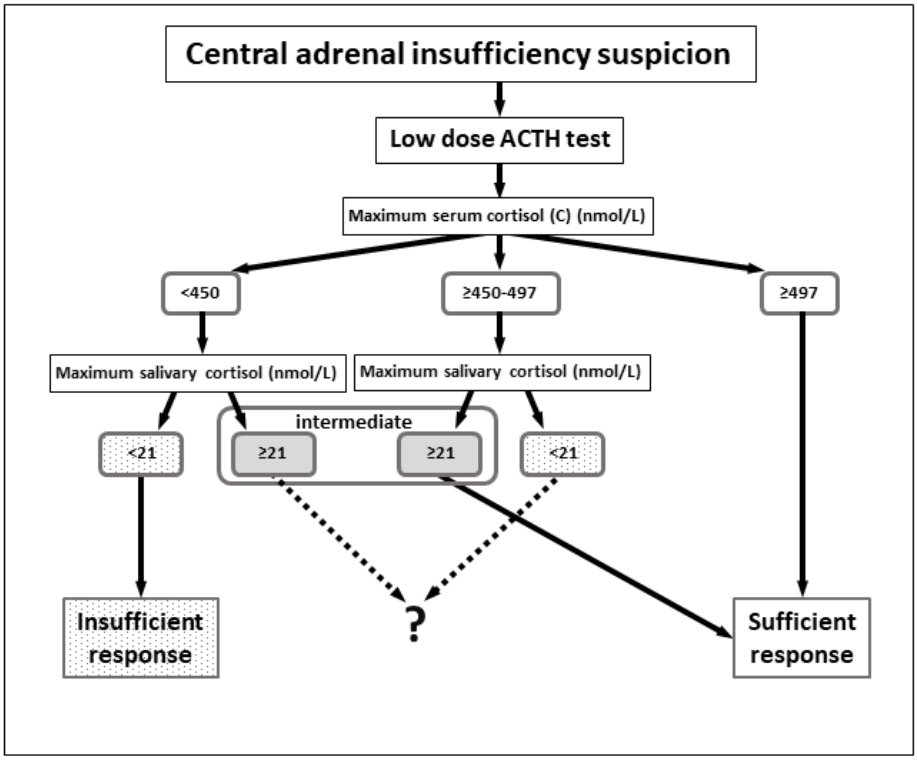

Figure 3