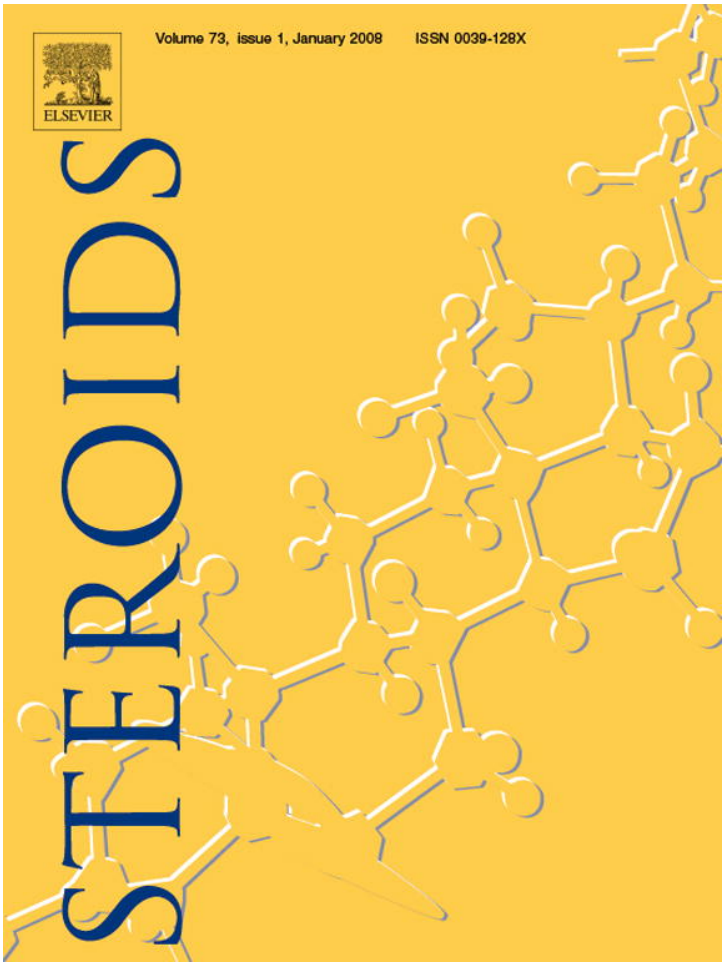

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Diagnostic value of salivary cortisol in end stage renal disease

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ABSTRACT

Objectives: Salivary cortisol has been proposed a surrogate marker for free serum cortisol measurements. The aim of this study was to ascertain the diagnostic value of basal and stimulated salivary cortisol for the detection of adrenal insufficiency (AI) in hypotensive end stage renal disease (ESRD) patients. Basal salivary cortisol and basal total serum cortisol were studied in order to determine the accuracy of both biomarkers in predicting AI.
Patients and methods: Twenty-nine ESRD patients with sustained hypotension were investigated for possible AI. Salivary cortisol was assessed at baseline and 30 min after 25 μg ACTH i.m. (LDTs). The dosage of salivary aldosterone was performed in salivary cortisol hyporesponders. Basal blood samples were drawn for steroids, renin and ACTH measurements.
Results: A clear separation between patients with normal and impaired adrenal function was obtained through salivary cortisol levels at 30 min after ACTH. AI was detected in six cases (21%) through impaired salivary cortisol responses; stimulated salivary aldosterone helped to differentiate primary ($n=3$) from secondary AI ($n=3$). ROC curves showed that cutoff values for basal SAF $\leq$4.4 nM and serum cortisol $\leq$232.0 nM suggest AI (sensitivities: 93% and 69%; specificities: 86.4% and 91%, respectively).
Conclusions: We conclude that ACTH stimulated SAF is an accurate biomarker for the diagnosis of AI in hypotensive ESRD patients. Neither basal salivary cortisol nor serum cortisol showed 100% sensitivities for the detection of AI.

1. Introduction

In the early 1980s Umeda et al. [1] described that salivary cortisol reflected serum unbound cortisol fraction and later Vining et al. [2] found that its concentration was unaffected by saliva flow rate. Thereafter the assessment of salivary cortisol has become a valuable alternative to blood-borne analysis. Due to the non-invasiveness and laboratory independence of sampling, salivary cortisol can be measured at almost unlimited frequency, under a wide variety of clinical settings [3]. Studies have demonstrated predictable responses in salivary cortisol concentrations following known stimuli as synthetic ACTH, proposing salivary cortisol as a surrogate marker for serum free cortisol levels in the dynamic evaluation of the adrenal

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axis mainly when altered binding globulins are present [4–10].

Based on these data we focused our research on the application of this salivary biomarker to the evaluation of ambulatory patients with chronic illnesses in whom adrenal impairment is suspected. In patients with end stage renal disease (ESRD) undergoing dialysis treatment, hypotension is a major cardiovascular complication [11] related to an unfavorable clinical outcome [12]. In an analysis of 4500 patients in hemodialysis [13] it was found that low-systolic blood pressure was associated with high mortality risk. Currently data on adrenal function in hypotensive ESRD patients remain limited [14,15]. Knowing that hypotension is considered a key marker of adrenal insufficiency, a recent study conducted on patients with chronic renal failure and sustained hypotension revealed a high frequency of adrenal impairment while assessing adrenal steroids in response to ACTH [16].

Recently the usefulness of a rapid and less invasive intramuscular low-dose ACTH salivary test (LDTs) for the detection of adrenal insufficient states in non-critically ill patients was reported by us [17]. As described, deficient adrenal secretion was diagnosed when subnormal responses of salivary neutral steroids (cortisol and aldosterone) were obtained after the stimulation with synthetic ACTH (25 μg i.m.) [7].

In view of previous data a further investigation was undertaken to detect adrenal hypo-function by using the LDTs test in ESRD patients in whom sustained hypotension affected dialysis adequacy and life quality. In order to define if basal salivary cortisol and basal total serum cortisol offer information for the detection of adrenal insufficiency, the cutoff values and sensitivities of both biomarkers were studied.

2. Experimental

2.1. Patients

This study included 29 patients with chronic renal failure (CRF) secondary to: hypertension ($n=6$), renal stones ($n=2$), diabetes mellitus ($n=2$), systemic lupus erythematosus ($n=1$), chronic pyelonephritis ($n=1$), interstitial nephritis ($n=4$), renal polycystosis ($n=3$), uremic hemolytic syndrome ($n=1$) and unknown etiologies ($n=9$). All patients were on dialysis replacement therapy (28 patients were on hemodialysis three times a week, and 1 on continuous ambulatory peritoneal dialysis), had sustained hypotension (systolic blood pressure levels <100 mm/Hg, median 90.0) and normal serum albumin levels (4.04 ± 0.3 g/dl). Three patients had a past history of steroid therapy not very well documented due to multiple admissions to intensive care units where variable intravenous doses of glucocorticoids were administered. Patients were not taking β-blocking agents, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, diuretics, steroids or drugs that block adrenal steroidogenesis.

The study was approved by the Research Ethics Committee, School of Medicine, University of Buenos Aires and all participants in the study gave written informed consent.

2.2. Study protocol

After confirming the integrity of salivary gland function (salivary flow rates not differing from those found in controls) [16] all patients were studied the day before the third dialytic procedure within a week.

2.2.1. ACTH stimulation test

From 8.00 to 9.00 a.m., after being upright for at least 2 h, the patients were instructed to collect 3.5 ml of whole saliva by directly spitting into polypropylene tubes and a simultaneous blood sample was drawn in each case. A dose of 25 μg of synthetic human β1-24ACTH (Synacthen; Novartis Pharma AG, Basel, Switzerland; provided through Novartis SA, Argentina), prepared as previously described [7], was directly injected into the deltoid muscle. Salivary samples were obtained 30 min after intramuscular ACTH stimulation. The supernatant obtained after centrifugation of saliva ($1000 \times g$, 10 min) was kept at −20 °C for later salivary steroid measurements. Basal plasma (for ACTH) and serum samples (for renin, cortisol and aldosterone) were frozen at −20 °C until assayed. When salivary cortisol did not increase normally after ACTH, saliva samples obtained after corticotrophin stimulus were assayed for salivary aldosterone to get more information about the integrity of adrenal cortex.

Salivary steroid measurements: salivary cortisol and salivary aldosterone were measured by RIA (Diagnostic Products Corporation, Los Angels, CA, USA) in saliva samples as previously described [6]. Salivary cortisol was expressed as nM and the minimal detectable salivary cortisol concentration was 0.5 nM. Salivary cortisol intra- and interassay coefficients of variation (CVs) were less than 6% and 13%, respectively. Salivary aldosterone was expressed as pM and the minimal detectable dose was 13.0 pM. The intra- and interassay CVs were less than 8% and 12%, respectively.

2.3. Blood assessments

Serum cortisol levels (nM) were assessed by RIA (Coat a Count; Diagnostic Products Corporation, Los Angeles, CA). The minimal detectable dose was 6.0 nM. The intra- and interassay CVs were less than 5.0% and 6.0%, respectively. Serum aldosterone levels (pM) were assessed by RIA (Diagnostic Products Corporation, Los Angeles, CA). The detection limit for serum aldosterone assay was 33.0 pM. The intra- and interassay CVs were less than 6.0% and 12.0%, respectively.

Plasma ACTH (pg/ml) was measured by IRMA (Diagnostic Systems Laboratories, Webster, TX, USA). The detection limit was 1.3 pg/ml. The intra- and interassay CVs were less than 9.4% and 8%, respectively.

Serum renin (pM) was assayed by IRMA (Diagnostic Systems Laboratories, Webster, TX, USA). The minimum detectable concentration was 0.06 pM. The intra- and interassay CVs were less than 3% and 4%, respectively.

2.4. Reference values (from 0800 to 0900 h)

Basal values in 21 healthy subjects—serum cortisol: 139–500 nM, serum aldosterone: 138–500 pM, salivary cortisol: 2.5–18.0 nM; salivary aldosterone: 20–70 pM; plasma ACTH:

5–50 pg/ml; serum renin: 0.55–3.10 pM. In 27 already known hypo-adrenal patients basal salivary cortisol ranged from 0.5 to 5.5 nM and basal total serum cortisol: 38.0–303.0 nM.

Criteria used to define a normal salivary steroid response 30 min after ACTH i.m. [7] and locally established reference values for salivary cortisol and salivary aldosterone (5th and 95th percentiles) were 20–70 nmol/l and 100–340 pmol/l, respectively, for healthy subjects ($n = 50$) after 25 μg of ACTH.

2.5. Statistical analysis

Results are expressed as mean ± S.D. unless otherwise specified. Data were analyzed by analysis of variance (ANOVA). Correlations between serum and salivary steroids levels were evaluated by Spearman analysis. The ROC (receiver operating characteristic) curve was employed to graphically demonstrate the sensitivities and specificities of the different diagnostic tests (baseline salivary and serum cortisol assessments). In order to estimate the cutoff values, we used ROC analysis for the two diagnostic settings, which were optimized for sensitivity. The areas below the ROC curves (global accuracy) of baseline salivary and serum cortisol were calculated and compared as described by Hanley and Mc Neil [18]. Statistical analysis was performed with program Medcalc for Windows version 7.4.3.1. p values less than 0.05 were considered statistically significant.

3. Results

3.1. Salivary cortisol response to ACTH

Individual basal and stimulated salivary cortisol levels are shown in Fig. 1. Subjects # 1–23 achieved normal salivary cortisol concentrations after ACTH stimulus, confirming adequate cortisol secretion.

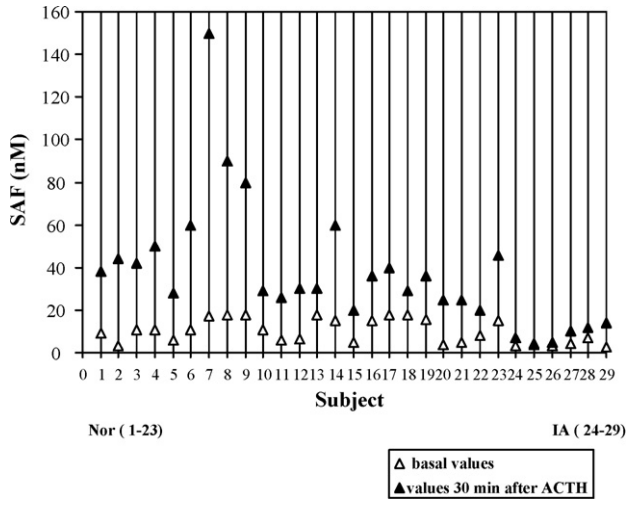

Fig. 1 – Salivary cortisol levels (SAF) at baseline (Δ) and 30 min after (▲) 25 μg ACTH i.m. (LDTs) in 29 hypotensive end stage renal disease patients. Normal range for SAF responses to LDTs (5th–95th percentiles): 20.0–70.0 nM. Nor (1–23): normal responders in SAF; IA (24–29): hypo-responders in SAF.

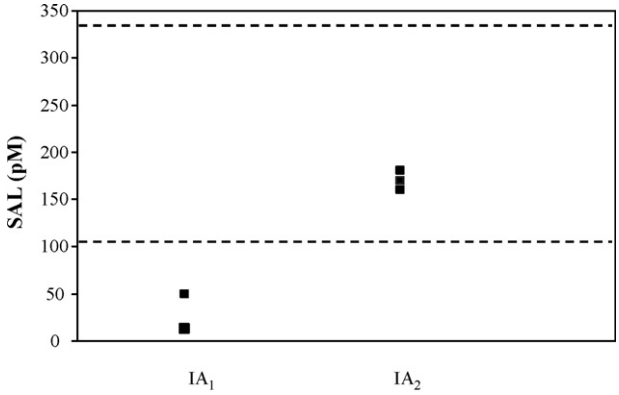

Fig. 2 – Salivary aldosterone (SAL) 30 min after 25 μg ACTH i.m. (LDTs) in six hypotensive end stage renal disease patients (ESRD) with blunted salivary cortisol response. Dotted lines indicate the normal range (5th–95th percentiles) for SAL responses to LDTs. IA_1: primary adrenal insufficiency, IA_2: secondary adrenal insufficiency.

The remaining six patients (# 24–29) failed to normally increase salivary cortisol levels after ACTH (7.7 ± 5.13 nM), demonstrating impairment of cortisol secretion after ACTH stimulation (Fig. 1). In these patients the response of aldosterone to ACTH was investigated in order to obtain further information about the etiology of the adrenal dysfunction.

Patients # 1–23 showed normal ACTH (28.5 ± 15.0 pg/ml) and renin levels (1.93 ± 1.0 pM) according to normal salivary cortisol increases after ACTH.

3.2. Contribution of salivary aldosterone assessment in response to ACTH to differentiate the etiology of adrenal insufficiency

After ACTH stimulation # 24–26 showed blunted salivary aldosterone responses (Fig. 2, group AI1) while patients # 27–29 demonstrated normal salivary aldosterone responses (Fig. 2, group AI2). These two different responses suggested a complete involvement of adrenal cortex (# 24–26) while a secondary origin of adrenal dysfunction was suggested in patients # 27–29. Table 1 shows the circulating hormonal levels in these patients. Higher ACTH and renin levels (except in # 24 who had total nephrectomy) reconfirmed the primary etiology of the adrenal insufficiency in patients # 24–26. Meanwhile patients # 27–29 showed normal ACTH and renin concentrations, supporting the extra-adrenal etiology of the dysfunction.

3.3. Accuracy of baseline salivary cortisol and serum cortisol levels to predict adrenal insufficiency

Baseline salivary cortisol concentrations correlated significantly with serum cortisol levels in all patients ($r = 0.68$, $p = 0.001$).

Individual values of basal salivary cortisol and serum cortisol levels in ESRD patients with normal and abnormal responses to ACTH are shown in Fig. 3. Basal serum cortisol concentrations were similar ($p = 0.199$) in normal

Table 1 – Basal serum hormones levels in six ESRD patients with adrenal insufficiency

Patient	Cortisol (nM)	Aldosterone (pM)	ACTH (pg/ml)	Renin (pM)	Adrenal insufficiency
24[a]	200.0	60.0	92.0	0.11	Primary
25	452.0	122.0	59.0	14.30	Primary
26	396.0	290.0	79.0	12.00	Primary
27	276.0	400.0	10.0	1.32	Secondary
28	221.0	555.0	12.0	3.00	Secondary
29	232.0	350.0	14.0	2.8	Secondary

Reference values (5th and 95th percentiles)—cortisol: 139.0–500.0 nM, aldosterone: 138.0–500.0 pM, ACTH: 5.0–50.0 pg/ml, renin: 0.55–3.10 pM.

[a] This patient had total nephrectomy. It explains the low-renin value.

responders (352.0 ± 90.0 nM) and in patients with adrenal insufficiency (296.0 ± 104.0 nM). By contrast, baseline salivary cortisol levels were significantly lower ($p = 0.002$) in adrenal insufficient patients (4.1 ± 1.6 nM) than in normal responders (11.5 ± 5.3 nM), although a clear separation could not be distinguished.

To define the diagnostic accuracy of baseline salivary cortisol and serum cortisol levels as a screening test for adrenal insufficiency, data from 21 healthy subjects with normal salivary cortisol response and 27 patients with subnormal salivary cortisol response to LDTs (see Section 2) were added to ESRD data to perform ROC curves (Fig. 4, $n = 77$). In normal responders ($n = 44$) mean salivary cortisol (10.2 nM) and mean serum cortisol (338.0 nM) were significantly higher than in hypo-responders (2.8 and 174.0 nM, respectively; $p = 0.001$). For salivary cortisol, ROC graphic shows an area under the curve (AUC) of 0.945, with 95% trust interval (CI) = 0.867–0.984 and standard error (S.E.) of 2.6%. AUC for serum cortisol is 0.86, CI = 0.762–0.929 and S.E. = 4.2%. The areas under the ROC curves are not equal to 1, so there is not a perfect separation between values from the two groups. Salivary cortisol value ≤4.4 nM had a sensitivity of 93.9% and 86.4% specificity to detect adrenal insufficiency. A cutoff value ≤232.0 nM for serum cortisol showed the best sensitivity (69.7%) and specificity (90.9%) to detect adrenal hypo-function.

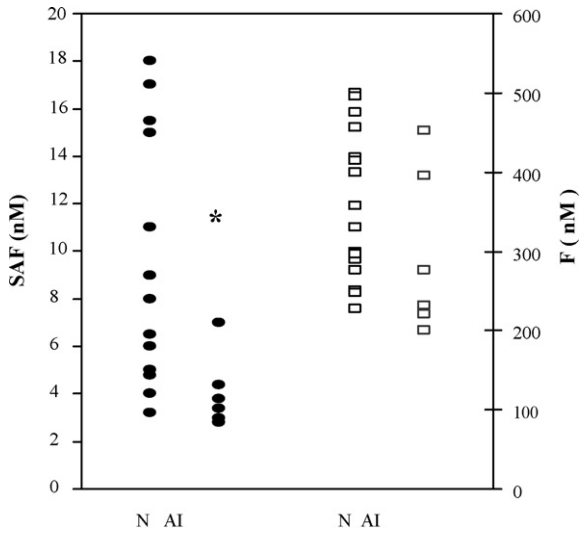

Fig. 3 – Morning baseline salivary cortisol (SAF) and total serum cortisol (F) in 29 hypotensive end stage renal disease patients with normal (N) or impaired adrenal function (AI) to LDTs. SAF (●); F (□).

3.4. *Clinical follow up*

Two patients with diagnosis of primary adrenal insufficiency (Table 1, # 24 and 25) were treated with replacement doses of hydrocortisone (30 and 20 mg/day, respectively) and fludrocortisone (0.2 and 0.1 mg/day, respectively) achieving systolic blood pressure levels ≥100 mm/Hg with improvement in dialysis adequacy and life quality. Patient 26 had a stroke, passing away before beginning treatment.

Patient 27 received substitutive daily doses of hydrocortisone with monthly tapering during 9 months. Recovery of normal adrenal function was confirmed by a salivary cortisol level of 25 nM, 30 min after ACTH stimulus. Currently, her mean systolic blood pressure is 110 mm/Hg rendering to adequate hemodialysis. Patients 28 and 29 are still on hydro-

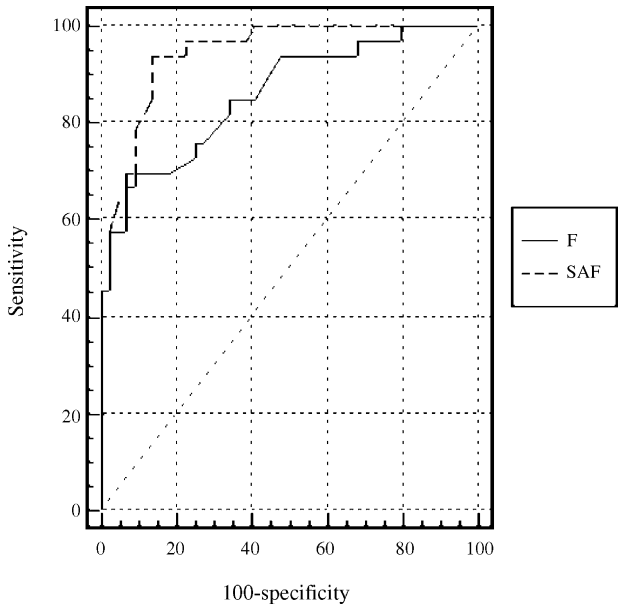

Fig. 4 – ROC curves for morning baseline salivary cortisol (SAF) and total serum cortisol (F) obtained from subjects with normal and impaired adrenal responses to LDTs. The areas below the ROC curve were 0.945 and 0.860 for SAF and F, respectively. A cutoff of 4.4 nM for SAF and 232.0 nM for F showed the best prediction accuracy for adrenal insufficiency with sensitivities of 93.9% and 69.7% and specificities of 86.4% and 91.0%, respectively.

cortisone tapering. Unfortunately these patients had to be hospitalized several times for inter-currencies that required the administration of higher doses of glucocorticoids.

4. Discussion

As previously reported in healthy subjects, a positive correlation between basal salivary cortisol and total serum cortisol was confirmed in ESRD patients. Salivary cortisol responses to low-dose ACTH challenge led to the detection of adrenal insufficiency in 21% of hypotensive ESRD patients. Appropriate salivary aldosterone responses helped to differentiate patients with primary ($n = 3$) from those with secondary ($n = 3$) adrenal insufficiency. Baseline salivary cortisol levels were able to predict cortisol hypo-responsiveness with higher sensitivity (93%) than baseline serum cortisol (69.7%). Cutoff values for basal salivary cortisol $\leq$4.4 nM and basal serum cortisol $\leq$232.0 nM suggest adrenal insufficiency (specificities: 86.4% and 91.0%, respectively).

During the last 20 years there has been much debate on which should be the most appropriate test for the detection of adrenal hypo-function. The short Synacthen test remains the most popular because it is safer, less expensive and unpleasant for the patients than other diagnostic procedures [19]. Measuring salivary steroids in response to intramuscular low-ACTH dose (LDTs) has shown to be practical and less invasive to detect either complete or selective adrenal insufficiency [7,17]. Based on our results, a clear separation between ESRD patients with normal and impaired adrenal function could be obtained through salivary cortisol levels 30 min after ACTH stimulation. Twenty-three (79.0%) subjects had adequate adrenal responses to low-ACTH dose reaching salivary cortisol $\geq$20.0 nM at 30 min. After excluding adrenal insufficiency, hypotension might be ascribed to other factors such as autonomic dysfunction, decreased vascular reactivity to vasopressor agents, over-production of vasodilators substances (nitric oxide and adrenomedulin) or cardiac dysfunction [11].

In the two ESRD cases in which primary adrenal insufficiency was confirmed, steroid replacement therapy improved blood pressure and life quality. Treatment of patients with secondary adrenal insufficiency was a major challenge. Tapering glucocorticoid doses was successful in only one of three cases, taking longer than usually to achieve normal adrenal function [20].

Among ESRD mean baseline salivary cortisol concentrations were lower in those with impaired response to LDTs than in those who achieve adequate responses. These data agree with Perlman et al. [8] who found the lowest salivary cortisol levels in hypo-adrenal patients. On the other hand, in ESRD, mean baseline total serum cortisol levels were lower in hypo-responders but not significantly different from normal-responders. As described by Erturk et al. [21] baseline morning total serum cortisol has very limited power in differentiating normal and impaired hypothalamic–pituitary–adrenal axis (HPA) activity. Yet, morning levels of total serum cortisol higher than 400.0 nM are predictive of an intact HPA axis [19] and those below 100.0 nM strongly suggest hypo-function [22].

ROC analysis demonstrated that basal salivary cortisol levels $\leq$4.4 nM and serum cortisol levels $\leq$232.0 nM suggest adrenal impairment, although adrenal insufficiency cannot be ruled out by any of both cutoff values with 100% sensitivity.

In conclusion adrenal function could be easily investigated through basal and stimulated salivary cortisol measurements in ESRD. Our findings suggest that salivary cortisol is a reliable biomarker that should be included in the detection of adrenal hypo-function in ESRD patients who develop sustained hypotension resistant to conventional medical therapy.

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