# The utility of salivary cortisone in the overnight dexamethasone suppression test in adrenal incidentalomas.

Basil George Issa. Department of Endocrinology and Diabetes, Manchester University NHS Foundation Trust

Fahmy WF Hanna. Department of Endocrinology and Diabetes, University Hospitals of North Staffordshire NHS Trust.

Anthony A Fryer. Impact Accelerator Unit, School of Medicine, Keele University

Grace Ensah. Department of Endocrinology and Diabetes, Manchester University Foundation Trust.

Ikenna Ebere, Department of Clinical Biochemistry, Manchester University Foundation Trust.

David Marshall. Department of Clinical Biochemistry, Manchester University Foundation Trust.

Brian Keevil. Department of Clinical Biochemistry, Manchester University Foundation Trust.

Keywords: overnight dexamethasone suppression test, serum cortisol, salivary cortisol, salivary cortisone

Correspondence: Dr Basil Issa, Department of Endocrinology and Diabetes, Manchester University Foundation Trust

basil.issa@mft.nhs.uk

https://orcid.org/0000-0001-5681-2698

Disclosure Statement: The authors have nothing to disclose

**Abstract**

Background: Guidelines recommend the assessment of cortisol secretion in patients with adrenal incidentalomas (AI) using the overnight dexamethasone suppression test (ONDST). This requires attendance to a health care facility and venepuncture. Alternatively, the ONDST can be done by measuring salivary cortisol and cortisone which can be collected at home. We aimed to assess the utility of these measurements in patients with AI.

Materials and Methods: A retrospective analysis of data from 173 patients with AI who underwent an ONDST and salivary cortisol/cortisone diurnal studies. Serum and salivary cortisol and salivary cortisone were collected at 09:00, late night and at 09:00 post dexamethasone. Dexamethasone levels were measured in the post dexamethasone samples. Serum and salivary samples were analysed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Stata.

Results: We identified a strong correlation between salivary cortisone and serum cortisol post 1 mg dexamethasone (r= 0.95). Stepwise multivariate regression showed that post-dexamethasone salivary cortisone, baseline serum cortisol, salivary cortisone suppression (pre: post-dexamethasone ratio) and sex as the only significant or near significant independent variables. Performance of predictive indices using these four parameters (sensitivity=88.5%, specificity=91.2%; kappa 0.80) and post-dexamethasone salivary cortisone alone (sensitivity=85.3%, specificity=91.7%; kappa 0.77) were comparable when used to predict an ONDST serum cortisol of $\leq$50 nmol/L.

No correlation was observed with any of the other measured parameters.

Conclusion: In AI patients, post dexamethasone, salivary cortisone correlates very strongly with serum cortisol in the ONDST and could therefore be used as an alternative sampling method which does not require venepuncture or attendance to hospital.

## Introduction

AI are adrenal masses that are incidentally discovered on imaging that was not performed for suspected adrenal disease. These are being detected more frequently in an ageing population[1] through the increased use of computed tomography (CT) (e.g. CT urogram, or colonoscopy and magnetic resonance imaging (MR) scans with enhanced resolution[2] with the overall prevalence being 4.2%[3], increasing to around 10% in patients over the age of 70[4,5]. The majority of AI are benign and do not secrete excessive amounts of adrenal hormones that would result in the development of a well-defined endocrine pathology. Approximately 1-1.2% are aldosterone producing tumours and 5.1-5.6% pheochromocytomas[1,2,4,6]. Excess cortisol secretion without clinical features of Cushing's syndrome is demonstrated however, in up to 30% of these patients[7], and is referred to as autonomous cortisol secretion, subclinical hypercortisolism, subclinical Cushing's, pre-clinical Cushing's or mild cortisol excess. To screen for Cushing's syndrome the Endocrine Society guidelines recommend one of the following tests; urinary free cortisol, ONDST or late-night salivary cortisol. If positive, confirmation of the diagnosis by another test is recommended[9]. The European Society of Endocrinology/European Network for the Study of Adrenal Tumours guidelines recommend that all patients with AI should have an 1 mg ONDST and suggest two diagnostic categories based on cortisol concentrations post dexamethasone; possible autonomous cortisol secretion defined as a cortisol of between 50-138 mmol/l and autonomous cortisol secretion, defined as a cortisol of >138 mmol/l[5]. These patients have a predominantly high nocturnal cortisol exposure[10] and do not have any overt

clinical features of Cushing's syndrome. There is, however, good evidence that they are at risk of increased cardiometabolic complications including hypertension[11], insulin resistance[12], type 2 diabetes mellitus[13], obesity[14], metabolic syndrome[12], and increased mortality[15]. Furthermore, a recent large cross-sectional study showed that mild autonomous cortisol secretion is a cardiometabolic risk condition predominantly affecting women and is associated with increased risk of type 2 diabetes and hypertension[16]. The ONDST is not without its caveats. One of the factors that may affect the specificity of the test is that measurement of serum cortisol may be affected by concentrations of cortisol binding globulin (CBG). Dexamethasone bioavailability may differ between individuals and some drugs can increase its metabolism leading to potentially low dexamethasone concentrations which may influence the interpretation of the test results. Furthermore, the ONDST requires attendance to a healthcare facility, venepuncture and sometimes overnight stay in a hospital if there is concern about the reliability of the patient taking dexamethasone at the correct time. Other methods for assessing cortisol over secretion also have their drawbacks. Urinary free cortisol measurements require 24- hour sample collections which is often inaccurate and therefore 2 or more samples are required. Despite the good evidence supporting the use of LNSC as a highly sensitive screening test for Cushing's syndrome, this has not been shown to be the case in patients with AI[16,17,18].

The availability of LC-MS/MS also enables the measurement of other glucocorticoid analytes. In the saliva, cortisone is present at a higher concentration than cortisol, with the salivary cortisone to cortisol ratio being greater than 4:1 due to conversion of cortisol to cortisone by the enzyme 11 beta-hydroxysteroid dehydrogenase 2 (11 $\beta$ -HSD2)[19,20]. This makes it easier to measure than salivary cortisol; when salivary cortisol is undetectable salivary cortisone is always measureable[20]. Salivary cortisone has been shown by some investigators to have a better and more linear relationship with serum total cortisol and serum free cortisol than salivary cortisol[19,20,21].

We therefore hypothesised that an alternative method of conducting the ONDST is by measuring cortisol and/or cortisone in a salivary sample which is non-invasive, stress free[17,18] and would negate the need for venepuncture. Furthermore, because salivary samples are stable, they can be collected at home and can be posted to the laboratory, thereby reducing the need to attend hospital. Cortisol/cortisone in the saliva is not bound to any proteins and therefore false positive and negative results due to variations in CBG are negated.

## Materials and Methods

### Patients

Manchester Foundation NHS Trust's Research and Innovation department advised that, as this was a secondary analysis of pseudonymised data collected as part of a clinical audit of the service, the study did not require approval from an NHS Research Ethics Committee.

We retrospectively studied patients with AI who attended the investigation unit at Wythenshawe Hospital, part of Manchester University Hospitals NHS Foundation Trust. Adrenal lesions were discovered incidentally on CT or MR scans performed for reasons other than suspicion of adrenal disease. None of the patients had overt features of Cushing's syndrome. They underwent an ONDST and studies of diurnal variation of serum and salivary cortisol and salivary cortisone measurements.

As per the local department protocol, patients were asked to stop any oral oestrogen at least 6 weeks prior to the test. Transdermal oestrogen was allowed to continue. Patients were admitted to the investigation unit at around 8 am and had 9 a.m. samples for serum and salivary cortisol, salivary cortisone, ACTH and sex steroids. Serum and salivary cortisol and salivary cortisone were collected late night (11 p.m-midnight). Patients were then given 1 mg dexamethasone. Serum cortisol, dexamethasone and salivary cortisol and cortisone were collected the following morning at ~ 9 a.m.

All patients also had plasma metanephrines to exclude pheochromocytoma and recumbent aldosterone plasma renin activity ratio to exclude primary aldosteronism when indicated.

Assays

As described previously, serum cortisol[22], saliva cortisol/cortisone[23] and dexamathsone[24] were measured by electrospray positive ion mode liquid chromatography tandem mass spectrometry. Lower limit of quantification was 0.46 nmol/L for salivary cortisol and 0.42 nmol/L for salivary cortisone. Between batch imprecision for cortisol showed coefficient variations (CV) of 13.4% to 2.7% across a range of concentrations from 4.2 to 118 nmol/L. Between batch imprecision for salivary cortisone showed CV of 8.6% to 2.3% across a range of concentrations from 5.0 to 130.9 nmol/L. Recovery was 93% and 96% for cortisol and cortisone respectively. 20 alpha and 20 beta dihydrocortisone showed baseline separation with cortisone and did not interfere in the assay.

ACTH was measured by two-site chemiluminescent immunometric assay on Siemens IMMULITE 2000 platform.

Statistical analysis

All statistical analyses were performed using the Stata software package (version 17; Stata Corporation, College Station, TX). Linear regression was used to examine the association of biochemical parameters with post-dexamethasone serum cortisol concentrations expressed as a continuous variable. Stepwise logistic regression was used to examine associations with a binary endpoint (i.e. post-dexamethasone serum cortisol value of >50 nmol/L vs. ≤50 nmol/L), starting with an empty model and using an inclusion p value of <0.1. Receiver operating characteristic (ROC) analysis of these models were used to assess the area under the curve. Model cross-validation was

performed using the K-fold internal cross-validation technique utilising the *cvauroc* command in Stata[25]. Predictive indices were generated using the coefficients derived from these models. Kappa analysis was used to assess agreement.

## Results

There were 173 patients in the AI group whose baseline characteristics are shown in table 1. 103/165 suppressed salivary cortisone to ≤2.7, 100/172 suppressed serum cortisol to ≤50 and 90/164 patients suppressed both serum cortisol ≤50 and salivary cortisone to ≤2.7.

### Relationship between ONDST post-dexamethasone serum cortisol concentration and other biochemical parameters

Using linear regression, we examined the association of biochemical parameters with post-dexamethasone serum cortisol concentrations. Post-dexamethasone salivary cortisone demonstrated the strongest correlation (r=0.95). Other biochemical parameters demonstrating statistically significant associations (Table 2), in order of decreasing strength of correlations, were: post-dexamethasone salivary cortisol (r=0.74), midnight salivary cortisone (r=0.51), midnight serum cortisol (r=0.47), baseline salivary cortisol (r=0.41) and midnight salivary cortisol (r=0.33). (Table 2).

Scatterplots showing the correlations between post-dexamethasone serum cortisol, salivary cortisol and salivary cortisone levels are shown in Supplementary Figure[26].

When post-dexamethasone serum cortisol values were dichotomised into ≤50 nmol/L and >50 nmol/L, those with a value of ≤50 nmol/L had a median (range) salivary cortisol of <0.3 (<0.3-0.9 nmol/L) and salivary cortisone of 1.8 (0.7-4.3 nmol/L). In those with post-dexamethasone serum

cortisol levels of >50 nmol/L, equivalent values for salivary cortisol and cortisone concentrations were 0.5 (<0.3-17.4 nmol/L) and 3.5 (1.9-45.5 nmol/L).

Predictors of a post-dexamethasone serum cortisol value of >50 nmol/L

To assess the potential association of these parameters as predictors of a post-dexamethasone serum cortisol value of >50 nmol/L, in the presence of each other and other covariates, we then performed a stepwise multivariate logistic regression analysis. The model included: serum dexamethasone concentration, pre- and post-dexamethasone salivary cortisol concentrations, pre- and post-dexamethasone salivary cortisone concentrations, midnight serum cortisol concentration, midnight salivary cortisol concentration, midnight salivary cortisone concentration, salivary cortisone suppression (ratio), salivary cortisol suppression (ratio), BMI, sex, age, type 2 diabetes status, presence of hypertension, presence of osteoporosis and presence of bilateral adrenal incidentaloma (vs. unilateral). This identified post-dexamethasone salivary cortisone, baseline serum cortisol, salivary cortisone suppression (ratio) and sex as the only significant or near significant independent variables (Table 3).

ROC analysis of this model showed an area under the curve of 0.96 (Figure 1a), with a sensitivity of 83.0%, specificity of 95.5% and accuracy of 90.5%. Cross-validation of the model gave a mean area under the curve of 0.95 with a standard deviation of 0.055.

To simplify this model, we then took the coefficients of this model and developed a predictive index as shown below:

Predictive index score = (Post-dexamethasone salivary cortisone*2.9) + (Baseline serum cortisol/80) + (female sex*1.4) - (Salivary cortisone suppression [ratio]/7)

Using the optimum cut-off for this score (>9.4), it was possible to obtain a model with a sensitivity of 88.5%, specificity of 91.2%, positive predictive value of 87.1%, negative predictive value of 92.2% and accuracy of 90.1% (Table 4).

Using kappa analysis to assess agreement between this predictive index score and a post-dexamethasone serum cortisol of >50 nmol/L, this showed an agreement of 90.1% (kappa 0.80, p<0.0001).

Potential of post-dexamethasone salivary cortisone as a marker of adequate dexamethasone suppression

It appeared that post-dexamethasone salivary cortisone was the strongest of these predictors. When considered alone, salivary cortisone gave an area under the ROC curve of 0.95 (Figure 1b). We therefore examined this further and identified that, using a cut-off of ≥2.7 nmol/L (defined in a study by Backlund et. al.[27]), it was possible to obtain a sensitivity of 85.3%, specificity of 91.7%, positive predictive value of 87.9% and negative predictive value of 89.8% in predicting a post-dexamethasone serum cortisol of >50 nmol/L (Table 4).

We then used kappa analysis to assess agreement between post-dexamethasone salivary cortisone using a cut-off of ≥2.7 nmol/L, with post-dexamethasone serum cortisol using a cut-off of >50 nmol/L, this showed an agreement of 89.0% (p<0.0001, kappa 0.77, where a kappa value of 0.61–0.80 indicates a substantial agreement). Hence, this marker alone performed well as a marker of adequate dexamethasone suppression in comparison to the 4-parameter predictive index (Table 4).

Comparison of post-dexamethasone serum cortisol and salivary cortisone

Using the post-dexamethasone salivary cortisone cut-off of ≥2.7 nmol/L, there were 18 discrepant cases compared to using the post-dexamethasone serum cortisol value of >50 nmol/L, as a marker of

inadequate adrenal suppression. These comprised 10 cases that had post-dexamethasone serum cortisol values of >50 nmol/L but post-dexamethasone salivary cortisone concentrations of <2.7 nmol/L (potential false negatives) and 8 cases with post-dexamethasone serum cortisol values of ≤50 nmol/L but post-dexamethasone salivary cortisone concentrations of ≥2.7 nmol/L (potential false positives). We therefore examined these discrepant cases in more detail.

Table 5 shows the biochemical characteristics of these cases. In the 10 potential false negative cases, all post-dexamethasone serum cortisol values were less than 70 nmol/L (mean 57.2 nmol/L) and, with the exception of patient 1, the reduction in serum cortisol following dexamethasone was at least 200 nmol/L. Similarly, excepting case 1, the salivary cortisone in the remaining 9 cases fell by a mean of 38 nmol/L and salivary cortisol by a mean of at least 13.7 nmol/L following dexamethasone administration. These data suggest that the salivary cortisone response may reflect better the adequacy of adrenal suppression than the serum cortisol. These observations are supported by ACTH (≥10 nmol/L) and/or DHEAS (≥1.0 nmol/L) values in cases 4,6,8,9 and 10, but not in cases 3 and 7.

In the 8 potential false positive cases, post-dexamethasone serum cortisol values were ≥30 nmol/L, with the exception of case 4 where the level was just detectable at 22 nmol/L (Table 5). In these 8 cases, the reduction in serum cortisol ranged from 112 to 299 nmol/L (mean 210 nmol/L). The decrease in salivary cortisone ranged from 6.4-50.8 nmol/L (mean 24.6 nmol/L), possibly indicating adequate suppression (in line with serum cortisol results), with the possible exception of case 7 which gave borderline results for both serum cortisol and salivary cortisone suppression. Salivary cortisol results also supported the view of adequate suppression in these cases; the mean difference between pre- and post-dexamethasone was at least 6.4 nmol/L.

## Discussion

In this retrospective study, we have shown that there is a strong correlation between post ONDST salivary cortisone and serum cortisol with an r value of 0.95 ($p<0.001$) with a sensitivity of 83.3%, specificity of 91.4% and accuracy of 88.2%. We have also shown, using a kappa analysis, that there is strong agreement between post-dexamethasone salivary cortisone and a predictive index score which comprised of post-dexamethasone salivary cortisone, baseline serum cortisol, midnight serum cortisol and sex as the strongest independent variables associated with a post dexamethasone serum cortisol of ≤50 mmol/l. Furthermore, we identified that, using a cut-off of <2.7 nmol/L for salivary cortisone alone, it was possible to obtain a sensitivity of 85.3% and specificity of 91.7% in predicting a post-dexamethasone serum cortisol of ≤50 nmol/L.

In a retrospective study to evaluate the accuracy of different tests used for the assessment of cortisol secretion and co-morbidities associated with autonomous cortisol secretion in patients with AI, the authors found that the reliability of UFC, ACTH, LNSC and DHEAS was low (kappa index <0.3) when the 1 mg ONDST was used as a gold standard[28]. The diagnostic performance for these tests for co-morbidities possibly related to autonomous cortisol secretion was also poor. It is likely therefore that ONDST will continue to be the most reliable test for assessing autonomous cortisol secretion in patients with AI. Modifying its methodology to make it more convenient for the patient and negating some of the factors which would lead to spurious results would be advantageous.

<u>Previous research showing utility of salivary cortisol/cortisone post 1 mg dexamethasone in AI/Cushing's syndrome</u>

Unlike the widely accepted cut-off values for serum cortisol response to dexamethasone in patients with AI, salivary cortisol and cortisone agreed cut-offs, with their sensitivity/specificity and predictive values have not been yet established. In a study by Backlund et al., using samples taken from 155

reference subjects and 22 patients with Cushing's syndrome the reference intervals for salivary cortisol and cortisone post 1 mg dexamethasone were 0.25-0.79 nmol/l and 0.59-3.5 nmol/l respectively. These findings confirmed a high diagnostic accuracy of both salivary measurements which were not inferior to serum cortisol measurement. The cohort of study participants, however, did not knowingly include patients with AI and the authors advocated that the utility of salivary cortisol and cortisone should be tested separately in this group of patients[27].

Ng et al studied 99 subjects (52 with adrenal incidentalomas) and found a strong correlation between post-dexamethasone serum cortisol and salivary cortisone (r= 0.94). They found that the optimal (where the Youden's index, i.e. "sensitivity + specificity-1", is maximal) cut-off value for salivary cortisone post dexamethasone was 7.45 nmol/l, and when setting the sensitivity at 95% the cut off value was 3.25 nmol/l, a slightly higher value than in our study[29]. A possible explanation for this discrepancy is the difference in the population studied (patients with AI in addition to patients investigated for hypertension/diabetes/Cushing's syndrome and a pituitary mass in this study as compared to only patients with AI in our study).

Serum vs. salivary sampling

The ONDST measuring serum cortisol is a well-established and validated test in people with symptoms and signs Cushing's syndrome. However, it does require attendance to a healthcare facility, venepuncture and occasionally overnight admission to hospital. This may be inconvenient and require much pre-organisation particularly for some elderly and infirm individuals. Furthermore, the experience with the recent Covid-19 pandemic has discouraged hospital attendance particularly for immunocompromised and some elderly subjects. Therefore, the availability of an alternative test that can be conducted at home with minimal inconvenience would be advantageous.

Measuring salivary as opposed to serum cortisol/cortisone also confers the advantage of avoiding false serum readings because of variation in binding proteins concentrations. For example, women on oestrogen containing oral contraceptives, will have increased CBG[30]. In a study involving 30 healthy volunteers, CBG levels varied significantly within- and between-individuals, resulting in alterations in the total serum cortisol. These changes were significant enough to potentially affect the outcome of tests of the hypothalamic-pituitary-adrenal axis. As an example, posture change from standing to lying, resulted in a drop of the CBG from 51±3.4 to 43± 3.2 ($P<0.0001$)[31]. This confounder is avoided when salivary cortisol/cortisone is measured.

Salivary cortisone vs. cortisol

In the saliva, most of the cortisol is converted into cortisone by the enzyme 11β-HSD2 which is present in abundance in the salivary glands and therefore, cortisone and cortisol are present in a ratio of > 4:1, compared to the 1:4 ratio in serum[32]. Salivary cortisone has been shown to closely reflect cortisol exposure under physiological conditions[20] and free serum cortisol after adrenal stimulation and hydrocortisone administration and was unaffected by CBG changes [20,33]. A recent study investigated the utility of salivary cortisone and cortisol as alternatives to serum cortisol in a prospective cross-over design in 14 healthy volunteers (median age 28, IQR 25-36). Under physiological conditions, salivary cortisol was undetectable whilst salivary cortisone correlated strongly with serum cortisol (r= 0.91; 95% confidence interval, 0.89 – 0.93; P=0.001). Following oral or IV hydrocortisone, salivary cortisone correlated strongly with serum cortisol (r= 0.91; 95% confidence interval, 0.89 – 0.92; P=0.001), whilst salivary cortisol produced spurious results due to contamination. A mixed-effects model showed that, in this cohort, 94% of the variation in salivary cortisone could be predicted from serum cortisol. These findings indicated that salivary cortisone better reflects serum cortisol and provides a non-invasive alternative to serum cortisol. Salivary cortisol was inferior to salivary cortisone being frequently undetectable and contaminated by oral

hydrocortisone[20]. The same group also showed a good relationship between serum cortisol and salivary cortisone in patients with AI and autonomous cortisol secretion (r=0.91). Furthermore, they showed that three equi-spaced samples of salivary cortisone provide a good estimate for 24-hour cortisol exposure therefore providing an alternative method of assessing cortisol secretion in these patients[34]. The superiority of salivary cortisone over salivary cortisol as a diagnostic test has also been recently demonstrated in the diagnosis of patients with adrenal insufficiency[35].

The major drawback for salivary cortisol is potential contamination from topical creams containing cortisol, generally available without prescription. These preparations can markedly increase the cortisol in saliva samples whereas cortisone is not affected[36].

In addition to topical hydrocortisone, liquorice and blood contamination may both cause elevated salivary cortisol concentrations, whereas salivary cortisone is essentially unaffected by the different preanalytical confounders[37].

The few discrepancies that we noted between serum cortisol and salivary cortisone post dexamethasone were almost inevitable as the two were compared against each other rather than against another independent gold standard test. However, our aim was to establish the high level of agreement between salivary cortisone and the widely accepted serum cortisol.

Our study includes a good number of patients with AI who underwent standardised tests in an investigation unit of a large university hospital and with all biochemical analysis undertaken in a laboratory well-established in steroid assays in general and LC-MS/MS in particular. The study has several limitations in that our data was analysed retrospectively and some data about the smoking status and work time patterns of our subjects was missing.

In conclusion, the measurement of salivary cortisone provides an accurate and convenient marker for cortisol secretion in the ONDST using a cut off of 2.7 nmol/l in AI patients.

Data availability: Data are available from the corresponding author on reasonable request.

References

1. Young WF Jr. Management approaches to adrenal incidentalomas. A view from Rochester, Minnesota. Endocrinol Metab Clin North Am 2000;29:159-185.

2. Terzolo M, Stigliano A, Chiodini I, et al. Italian Association of Clinical Endocrinologists. AME position statement on adrenal incidentaloma. Eur J Endocrinol 2011;164:851-870.

3. Bovio S, Cataldi A, Reimondo G, et al. Prevalence of adrenal incidentaloma in a contemporary computerized tomography series. J Endocrinol Invest 2006;29:298-302.

4. Young WF. Clinical practice. The incidentally discovered adrenal mass. N Engl J Med 2007;356:601-610.

5. Fassnacht M, Arlt W, Bancos I, et al. Management of adrenal incidentalomas: European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors. Eur J Endocrinol 2016;175:G1-34.

6. Barzon L, Sonino N, Fallo F, Palu G, Boscaro M. Prevalence and natural history of adrenal incidentalomas. Eur J Endocrinol 2003;149:273-285.

7. Terzolo M, Pia A, Reimondo G. Subclinical Cushing's syndrome: definition and management. Clin Endocrinol (Oxf) 2012;76:12-18.

9. Nieman LK, Biller BM, Findling JW, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2008;93(5):1526-1540.

10. Debono M, Harrison RF, Chadarevian R, Gueroult C, Abitbol JL, Newell-Price J. Resetting the abnormal circadian cortisol rhythm in adrenal incidentaloma patients with mild autonomous cortisol secretion. J Clin Endocrinol Metab. 2017;102(9):3461–3469

11. Terzolo M, Bovio S, Reimondo G, et al. Subclinical Cushing's syndrome in adrenal incidentalomas. Endocrinol Metab Clin North Am. 2005;34(2):423-439.

12. Reincke M, Fassnacht M, Vath S, Mora P, Allolio B. Adrenal incidentalomas: a manifestation of the metabolic syndrome? Endocr Res. 1996;22(4):757-761.

13. Fernandez-Real JM, Engel WR, Simo R, Salinas I, Webb SM. Study of glucose tolerance in consecutive patients harbouring incidental adrenal tumours. Study Group of Incidental Adrenal Adenoma. Clin Endocrinol (Oxf). 1998;49(1):53-61.

14. Tauchmanova L, Rossi R, Biondi B, et al. Patients with subclinical Cushing's syndrome due to adrenal adenoma have increased cardiovascular risk. J Clin Endocrinol Metab. 2002;87(11):4872-4878.

15. Debono M, Bradburn M, Bull M, Harrison B, Ross RJ, Newell-Price J. Cortisol as a marker for increased mortality in patients with incidental adrenocortical adenomas. J Clin Endocrinol Metab. 2014;99(12):4462-4470.

16. Alessandro Prete, MD et. al. Cardiometabolic Disease Burden and Steroid Excretion in Benign Adrenal Tumors. A Cross-Sectional Multicenter Study. Ann Intern Med. 2022;175(3):325-334.

17. Viardot A, Huber P, Puder JJ, Zulewski H, Keller U, MullerB. Reproducibility of nighttime salivary cortisol and its use in the diagnosis of hypercortisolism compared with urinary free cortisol and overnight dexamethasone suppression test. J Clin Endocrinol Metab. 2005;90:5730-5736.

18. Graham UM, Hunter SJ, McDonnell M, Mullan KR, Atkinson AB. A comparison of the use of urinary cortisol to creatinine ratios and nocturnal salivary cortisol in the evaluation of cyclicity in patients with Cushing's syndrome. J Clin Endocrinol Metab. 2013;98:E72-76.

16. B. Masserini, V. Morelli, S. Bergamaschi, F. Ermetici, C. Eller- Vainicher, A.M. Barbieri, M.A. Maffini, A. Scillitani, B. Ambrosi P. Beck-Peccoz, I. Chiodini, The limited role of midnight salivary cortisol levels in the diagnosis of subclinical hypercortisolism in patients with adrenal incidentaloma. Eur. J. Endocrinol. 2009;160: 87–92.

17. S. Palmieri, V. Morelli, E. Polledri, S. Fustinoni, R. Mercadante, L. Olgiati, C. Eller Vainicher, E. Cairoli, V.V. Zhukouskaya, P. Beck-Peccoz, I. Chiodini. The role of salivary cortisol measured by liquid chromatography-tandem mass spectrometry in the diagnosis of subclinical hypercortisolism. Eur. J. Endocrinol. 2013;168:289–296.

18. Filippo Ceccato, Mattia Barbot, Nora Albiger, Giorgia Antonelli, Marialuisa Zilio, Marco Todeschini, Daniela Regazzo, Mario Plebani, Carmelo Lacognata, Maurizio Iacobone, Franco Mantero, Marco Boscaro, Carla Scaroni. Daily salivary cortisol and cortisone rhythm in patients with adrenal incidentalomas. Endocrine 2018;59:510–519.

19.Blair J, Adaway J, Keevil B, Ross R. Salivary cortisol and cortisone in the clinical setting. Curr Opin Endocrinol Diabetes Obes. 2017; 24(3):161–168.

20. Debono M, Harrison RF, Whitaker MJ, Eckland D, Arlt W, Keevil BG, Ross RJ. Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. J Clin Endocrinol Metab. 2016;101(4):1469–1477.

21. Katz FH, Shannon IL. Parotid fluid cortisol and cortisone. J Clin Invest 1969; 48:848–855.

22. Owen LJ, Adaway JE, Davies S, Neale S, El-Farhan N, Ducroq D, Evans C, Rees DA, MacKenzie F, Keevil BG. Development of a rapid assay for the analysis of serum cortisol and its implementation into a routine service laboratory. Ann Clin Biochem. 2013;50:345-352.

23. Jones RL, Owen LJ, Adaway JE, Keevil BG. Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;15:881-882.

24. Hawley JM, Owen LJ, Debono M, Newell-Price J, Keevil BG. Development of a rapid liquid chromatography tandem mass spectrometry method for the quantitation of serum dexamethasone and its clinical verification. Ann Clin Biochem. 2018;55:665–672

25. Luque-Fernandez, M. A., Redondo-Sánchez, D., & Maringe, C. cvauroc: Command to compute cross-validated area under the curve for ROC analysis after predictive modeling for binary outcomes. The Stata Journal 2019;19:615–625

26. Issa, Basil, 2023, "The utility of salivary cortisone in the overnight dexamethasone suppression test in adrenal incidentalomas (supplementary figures)", https://doi.org/10.7910/DVN/7LDRJH, Harvard Dataverse

27. Bäcklund N, Brattsand G, Israelsson M, Ragnarsson O, Burman P, Edén Engström B, Høybye C, Berinder K, Wahlberg J, Olsson T, Dahlqvist P. Reference intervals of salivary cortisol and cortisone and their diagnostic accuracy in Cushing's syndrome. Eur J Endocrinol. 2020;182(6):569-582. 27. Ng CM, Lam TK, Au Yeung YC, Choi CH, Iu YP, Shek CC, Tiu SC. Clinical utility of late-night and post-overnight dexamethasone suppression salivary cortisone for the investigation of Cushing's syndrome. Hong Kong Med J 2017;23:570–578

28. Araujo-Castro M, CanoAG, MendiguchíaLJ, Escobar-Morreale HF,Pablo Valderrábano. Diagnostic accuracy of the different hormonal tests used for the diagnosis of autonomous cortisol secretion. Scientific Reports 2021;11:20539.

29. Ng CM, Lam TK, Au Yeung YC, Choi CH, Iu YP, Shek CC, Tiu SC. Clinical utility of late-night and post-overnight dexamethasone suppression salivary cortisone for the investigation of Cushing's syndrome. Hong Kong Med J 2017;23:570–578

30. Nickelsen T, Lissner W, Schoffling K. The dexamethasone suppression test and long-term contraceptive treatment: measurement of ACTH or salivary cortisol does not improve the reliability of the test. Exp Clin Endocrinol. 1989;94:275–280

31. Dhillo WS, Kong WM, Le Roux CW, Alaghband-Zadeh J, Jones J, Carter G, Mendoza N, Meeran K, O'Shea D. Cortisol-binding globulin is important in the interpretation of dynamic tests of the hypothalamic--pituitary--adrenal axis. Eur J Endocrinol. 2002;146(2):231-235

32. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG. Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:3771–3775.

33. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker for serum free cortisol. J Clin Endocrinol Metab. 2010;95:4951– 4958

34. Robert F Harrison, Miguel Debono, Martin J Whitaker, Brian G Keevil, John Newell-Price, and Richard J Ross. Salivary Cortisone to Estimate Cortisol Exposure and Sampling Frequency Required Based on Serum Cortisol Measurements. J Clin Endocrinol Metab. 2019; 104(3):765–772

35. Debono M, Elder CJ, Lewis J, et al. Home waking salivary cortisone to screen for adrenal insufficiency. NEJM Evid. 2023;2(2). DOI: 10.1056/EVIDoa2200182.

36. Raff H, Singh RJ. Measurement of late-night salivary cortisol and cortisone by LC-MS/MS to assess preanalytical sample contamination with topical hydrocortisone. Clin Chem 2012;58:947-948. DOI: 10.1373/clinchem.2012.182717. NEJM EVIDENCE 2

37. Imamovic M, Bäcklund N, Lundstedt S, Brattsand G, Aardal E, Olsson T, Dahlqvist P. Confounding effects of liquorice, hydrocortisone, and blood contamination on salivary cortisol but not cortisone. Endocr Connect. 2022 Dec 15;12(1):e220324. doi: 10.1530/EC-22-0324.

Legend for Figure 1

**Figure 1 Receiver operating characteristic (ROC) curves showing the association between post-dexamethasone serum cortisol of >50 nmol/L and; (a) the 4-parameter stepwise model and (b) post-dexamethasone salivary cortisone alone.**

**Table 1 Patient baseline characteristics**

| Parameter | Mean (±SD) |
|---|---|
| Age (years) | 64.2±11.3 |
| BMI | 29.7±6.4 |
| | Proportion (%) |
| Sex (female) | 55.5 |
| Hypertension | 54.1 |
| Smokers (none/ex/current) | 47.6/21.7/30.7 |
| T2DM | 23.8 |
| Osteoporosis/osteopenia | 4.1/1.1 |
| AI location (left/right/bilateral) | 58.3/17.9/23.8 |
| Patients with post dexamethasone cortisol ≤50 | 58 (number: 100) |
| Patients with post dexamethasone cortisol 51-138 | 34 (number: 59) |
| Patients with post dexamethasone cortisol >138 | 8 (number: 13) |

**Table 2 Univariate linear regression showing associations of biochemical and demographic parameters with post-dexamethasone serum cortisol concentration.**

| Biochemical parameter | coefficient | p value | adj $R^2$ | r |
|---|---|---|---|---|
| Serum cortisol midnight | 0.30 | <0.001 | 0.2164 | 0.47 |
| Serum cortisol baseline | 0.05 | 0.118 | 0.0087 | 0.09 |
| Serum cortisol suppression (ratio)* | -6.55 | <0.001 | 0.2701 | 0.52 |
| Serum cortisol suppression (difference)* | -0.13 | <0.001 | 0.0922 | 0.30 |
| Salivary cortisol midnight | 8.25 | <0.001 | 0.1089 | 0.33 |
| Salivary cortisol baseline | 2.65 | <0.001 | 0.1676 | 0.41 |
| Salivary cortisol post-dexamethasone | 30.75 | <0.001 | 0.5524 | 0.74 |
| Salivary cortisol suppression (ratio) | -0.80 | 0.001 | 0.0603 | 0.25 |
| Salivary cortisol suppression (difference) | 2.42 | <0.001 | 0.1109 | 0.33 |
| Salivary cortisone post-dexamethasone | 10.90 | <0.001 | 0.8950 | 0.95 |
| Salivary cortisone midnight | 4.54 | <0.001 | 0.2631 | 0.51 |
| Salivary cortisone baseline | 0.39 | 0.289 | 0.0001 | 0.01 |
| Salivary cortisone suppression (ratio) | -3.21 | <0.001 | 0.2300 | 0.48 |
| Salivary cortisone suppression (difference) | -1.26 | <0.001 | 0.0810 | 0.28 |
| Dexamethasone concentration | 0.57 | 0.686 | -0.0059 | 0.08 |
| ACTH concentration | -1.08 | 0.038 | 0.0233 | 0.15 |
| DHEAS concentration | -8.19 | 0.108 | 0.0112 | 0.11 |
| BMI | 10.90 | <0.001 | 0.0073 | 0.09 |
| Sex | -23.27 | 0.011 | 0.0317 | 0.18 |
| Age (years) | 0.95 | 0.018 | 0.0269 | 0.16 |
| Presence of type 2 diabetes | -9.24 | 0.397 | -0.0016 | 0.04 |

| | | | | | |
|---|---|---|---|---|---|
| Presence of hypertension | 9.27 | 0.317 | <0.0001 | <0.01 |
| Presence of osteoporosis | 11.96 | 0.622 | -0.0045 | 0.07 |
| Presence of bilateral AI | 11.14 | 0.037 | 0.0204 | 0.14 |

*note: these parameters incorporate the post-dexamethasone serum cortisol value and hence will, by definition, have a strong association

**Table 3 Stepwise multivariate logistic regression analysis of factors associated with a post-dexamethasone serum cortisol of >50 nmol/L**

| Variable | coefficient | p value |
|---|---|---|
| Post-dexamethasone salivary cortisone | 2.90 | <0.001 |
| Female sex | 1.42 | 0.034 |
| Baseline serum cortisol | 0.012 | 0.005 |
| Salivary cortisone suppression [ratio] | -0.14 | 0.057 |

**Table 4** **Comparison of the performance of the 4-parameter model predictive index with post-dexamethasone salivary cortisone alone**

| | Predictive index | Post-dexamethasone salivary cortisone alone |
|---|---|---|
| **Model area under the curve (ROC)** | 0.96 | 0.95 |
| **Cut-off used** | >9.4 | ≥2.7 nmol/L |
| **Sensitivity** | 88.5% | 85.3% |
| **Specificity** | 91.2% | 91.7% |
| **Positive predictive value** | 87.1% | 87.9% |
| **Negative predictive value** | 92.2% | 89.8% |
| **Accuracy** | 90.1% | 89.0% |
| **Kappa** | 0.80 | 0.77 |

**Table 5** **Biochemical characteristics of discrepant cases between post-dexamethasone serum cortisol and salivary cortisone:** a) false negatives and b) false positives.

**a) False negatives**

| Patient | Dexamethasone | Serum cortisol | | | Salivary cortisol | | | Salivary cortisone | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | pre | post | difference | pre | post | difference | pre | post | difference |
| 1 | 6.1 | 152 | 51 | 101 | 0.8 | <0.8 | - | 2.4 | 2.2 | 0.2 |
| 2 | 10.1 | 268 | 63 | 205 | 3.6 | 0.5 | 3.1 | 15.2 | 2.2 | 13.0 |
| 3 | 6.8 | 441 | 54 | 387 | 5.6 | <0.3 | >5.3 | 25.0 | 2.0 | 23.0 |
| 4 | 16.0 | 425 | 60 | 365 | 10.0 | 0.4 | 9.6 | 29.9 | 2.0 | 27.9 |
| 5 | 6.5 | 382 | 55 | 327 | 11.5 | <0.3 | >11.2 | 48.9 | 2.4 | 46.5 |
| 6 | 6.6 | 352 | 51 | 301 | - | 0.3 | - | - | 2.5 | - |
| 7 | 11.4 | 528 | 55 | 473 | 9.8 | <0.3 | >9.5 | 41.8 | 2.3 | 39.5 |
| 8 | 11.7 | 502 | 63 | 439 | 14.8 | 0.4 | 14.4 | 36.3 | 2.4 | 33.9 |
| 9 | 9.3 | 318 | 52 | 266 | 4.5 | <0.3 | >4.2 | 20.5 | 1.9 | 18.6 |
| 10 | 9.5 | 615 | 68 | 547 | 52.9 | 0.3 | 52.6 | 106.2 | 2.5 | 103.7 |
| **Mean** | **9.4** | **398.3** | **57.2** | **341.1** | **12.6** | **<0.4** | **>13.7** | **36.2** | **2.2** | **34.0** |

| **b) False positives** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.0 | 322 | 40 | 282 | 6.3 | 0.5 | 5.8 | 21.9 | 2.8 | 19.1 |
| 2 | 9.6 | 314 | 36 | 278 | 10.6 | 0.4 | 10.2 | 54.7 | 3.9 | 50.8 |
| 3 | - | 202 | 30 | 172 | 4.3 | <0.3 | >4.0 | 27.8 | 2.7 | 25.1 |
| 4 | - | 213 | 22 | 191 | - | 0.8 | - | - | 4.3 | - |
| 5 | - | 230 | 46 | 184 | 4.1 | <0.3 | >3.8 | 23.9 | 2.7 | 21.2 |
| 6 | - | 342 | 43 | 299 | 15.4 | <0.3 | >15.1 | 37.6 | 3.4 | 34.2 |
| 7 | 7.1 | 161 | 49 | 112 | 2.6 | 0.3 | 2.3 | 9.3 | 2.9 | 6.4 |
| 8 | 8.5 | 208 | 47 | 161 | 3.8 | 0.4 | 3.4 | 18.9 | 3.2 | 15.7 |
| **Mean** | **7.6** | **249.0** | **39.1** | **209.9** | **6.7** | **<0.4** | **>6.4** | **27.7** | **3.2** | **24.6** |

| | | | | |
|---|---|---|---|---|
| | | | | |
| | | | | |

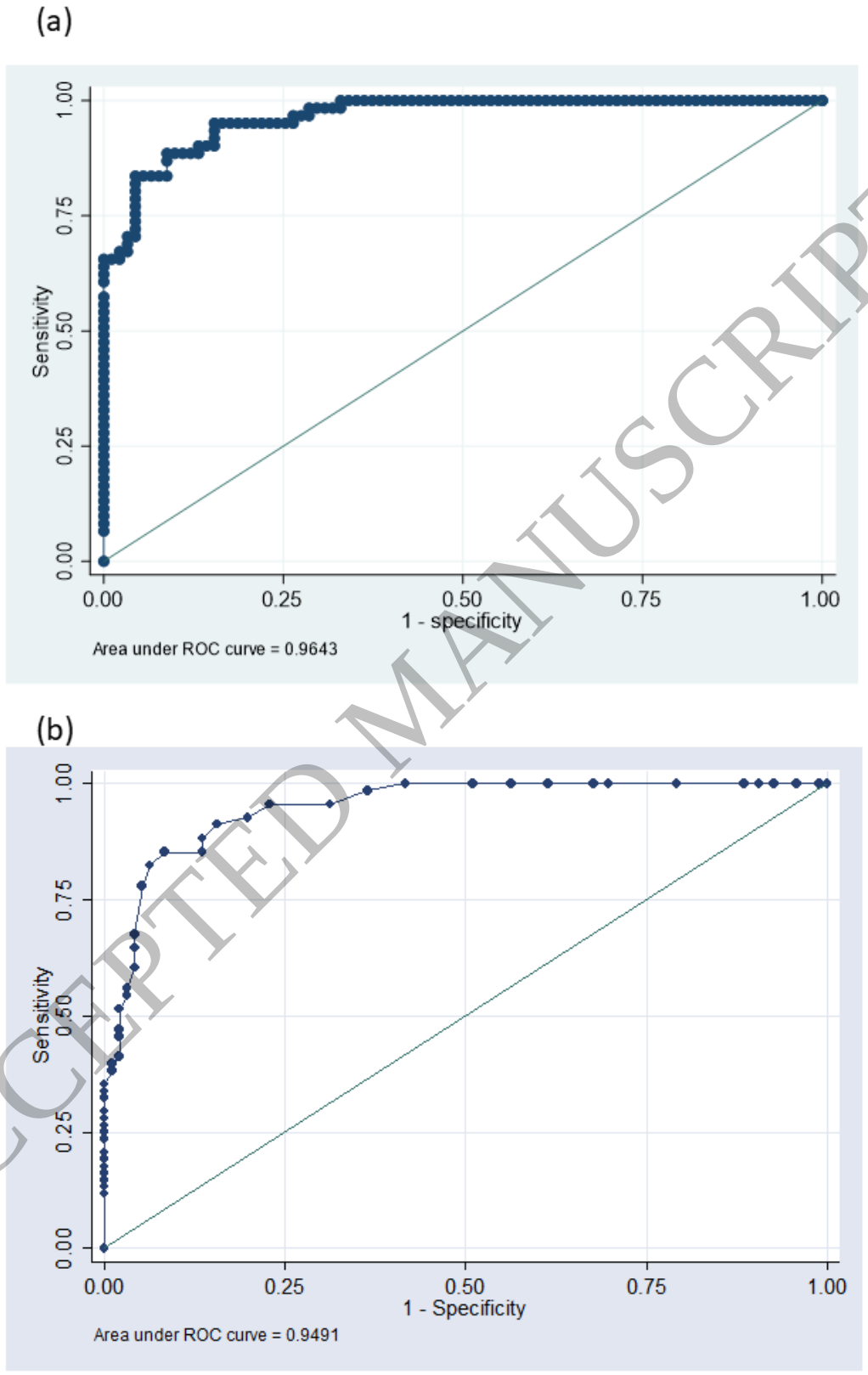


*Figure 1*
*190x275 mm ( x DPI)*