Salivary cortisol response to ACTH stimulation is a reliable alternative to serum cortisol in evaluating hypoadrenalism.

Nadia El-Farhan[1], Sarah Tennant[2], Seren E Rees[3], Carol Evans[2], D Aled Rees[4].

[1]Department of Medical Biochemistry, Royal Gwent Hospital, Newport, NP20 2UB UK

[2]Department of Medical Biochemistry, Immunology and Toxicology, University Hospital of Wales, Cardiff, CF14 4XW UK

[3]Birmingham Medical School, University of Birmingham, Birmingham, B15 2TT UK

[4]Neuroscience and Mental Health Innovation Institute, School of Medicine, Cardiff University, Cardiff, CF14 4XN UK.

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Corresponding author and address for reprints: Aled Rees, Neuroscience and Mental Health Research Institute, School of Medicine, Cardiff University, Heath Park, Cardiff CF24 4HQ, United Kingdom. ORCID ID: 0000-0002-1165-9092

Fax: +44 (0)2920 744671

Telephone: +44 (0)2920 742305

Email: reesda@cf.ac.uk

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Abstract

Context The serum total cortisol response to the ACTH stimulation test is widely used to assess adrenocortical function but is affected by changes in cortisol-binding globulin (CBG) concentration. Salivary cortisol reflects free cortisol concentrations and may offer a reliable alternative.

Objectives 1. To establish the salivary cortisol response to ACTH stimulation in healthy volunteers and patients with altered CBG concentrations. 2. To evaluate the performance of a lower reference limit (LRL) determined in healthy volunteers in patients with suspected hypoadrenalism (SH-patients).

Design A 250 µg-ACTH stimulation test was undertaken in 139 healthy volunteers, 24 women taking an estradiol-containing oral contraceptive pill (OCP-females), 10 patients with low serum protein concentration (LP-patients) and 30 SH-patients. Salivary cortisol was measured by liquid chromatography-tandem mass spectrometry. Mean and LRL of the 30-minute salivary cortisol response (mean - 1.96 standard deviation) were derived from log-transformed concentrations. The LRL was applied as a diagnostic cut-off in SH-patients, with comparison to the serum response.

Results Mean CBG concentrations [range] were 58 [42-81] mg/L, 64 [43-95] mg/L, 41 [28-60] mg/L and 116 [84-159] mg/L in males, females, LP-patients and OCP-females, respectively. The mean 30-minute salivary cortisol concentration was 19.3 [2.5^{th}-97.5^{th} percentile 10.3-36.2] nmol/l in healthy volunteers. Corresponding values were not different in OCP-females (19.7 [9.5-41.2] nmol/l; p=0.59) or LP-patients (19.0 [7.7-46.9] nmol/l; p=0.97). Overall diagnostic agreement between salivary and serum responses in SH-patients was 79%.

Conclusions Salivary cortisol response to ACTH stimulation offers a reliable alternative to serum and may be especially useful in conditions of altered CBG concentration.

Introduction

The adrenocorticotropin (ACTH) stimulation test (synthetic (1-24) ACTH [synacthen®]), is the most widely used test of adrenal glucocorticoid reserve.[1,2] Most commonly, the test uses a 250-microgram dose to stimulate a cortisol response, with measurement of serum cortisol values at baseline and 30 minutes after intravenous injection. Applying diagnostic thresholds allows reliable discrimination of hypoadrenalism from normative responses although we and others have shown that such cut-offs are highly method-dependent.[3-5] Difficulties remain, however, in assessing hypoadrenalism in patients with disorders of protein concentration,[6,7] where total serum cortisol concentrations are affected by changes in carrier protein (cortisol-binding globulin [CBG] and albumin) synthesis, leading to potential misdiagnosis. A variety of conditions may affect protein synthesis: cirrhosis, nephrotic syndrome, malnutrition and critical illness may all reduce it, whereas estrogen (e.g. in pregnancy or in combined oral contraceptives) increases it. Clinicians are thus faced with challenges in making an accurate diagnosis of hypoadrenalism in such circumstances, whilst patients taking estrogen therapy may be faced with the inconvenience of discontinuing treatment for several weeks in order for a reliable assessment of adrenal reserve to be made.

Free cortisol represents the biologically active unbound fraction and accounts for 5-10% of total serum cortisol. Analysis of free cortisol has been shown to overcome the challenges presented by conditions of altered protein synthesis in the diagnosis of hypoadrenalism,[8] but direct measurement is labour-intensive, time-consuming and expensive, limiting its utility in the routine laboratory setting. Calculated free cortisol measurement using validated equations has also been proposed but may be unreliable in critical illness.[9] Salivary cortisol measurement is an attractive alternative as it is unbound and in equilibrium with circulating free cortisol.[10] Previous studies have assessed salivary cortisol responses to ACTH stimulation in healthy volunteers and patients[11-19] yet few studies have analysed the utility of salivary measurement in patients with altered protein concentrations and many have been limited by relatively small sample sizes. Furthermore, only a few studies have reported CBG

concentrations.[19-22] We therefore sought to evaluate salivary and serum cortisol responses to the high-dose ACTH stimulation test in a large sample of healthy volunteers, in addition to comparing responses in patients with disordered protein synthesis and patients with confirmed or suspected hypoadrenalism.

Subjects and Methods

Subjects

One hundred and thirty-nine healthy volunteers (60 male, 79 female; mean age (range) 37.1 (22-62) years and 40.7 (20-66) years, respectively) were recruited from staff at the University Hospital of Wales and Cardiff University. Exclusion criteria included pregnancy and breastfeeding, use of estrogen-containing medication, significant intercurrent disease, a history of thyroid or other autoimmune disease, previous sensitivity to ACTH testing, asthma or an allergic disorder, and treatment with corticosteroids. An additional 24 healthy female volunteers (28.7 (21-40) years) taking an estrogen-containing oral contraceptive pill (OCP), containing between 20 and 35 micrograms of ethinyloestradiol, were recruited, along with 10 patients (7 male, 3 female; 57.4 (42-78) years) with recently diagnosed, untreated nephrotic syndrome (n=1) or established liver cirrhosis (n=9) (mean albumin concentration 30.3 g/L (range 29 – 34)). Thirty patients with established or suspected adrenal insufficiency (13 male, 17 female; 52.4 (23 – 82) years) were recruited from Endocrine clinics at the University Hospital of Wales. Patients were stratified into high, low or intermediate likelihood of hypoadrenalism, based on our clinical judgement and derived from risk factors for hypoadrenalism identified in their medical and medication history. These included: pre-existing Addison's disease or pan-hypopituitarism, adrenalectomy, pituitary adenoma with or without partial hypopituitarism, symptoms of hypoadrenalism, other autoimmune disease, hydrocortisone and/or fludrocortisone replacement, oral or inhaled glucocorticoids and other medication known to affect the hypothalamic-pituitary-adrenal axis. The presence of multiple different risk factors was also taken into account when assigning risk category.

The study protocol was approved by the South East Wales Research Ethics Committee, Cardiff University (study sponsor) and the Medicines and Healthcare Products Regulatory Authority. All subjects provided written informed consent before study commencement.

Sample collection and handling

The Synacthen® tests were undertaken between 08.30 and 11.30 h. Subjects were not required to fast overnight, but were restricted from eating, drinking or smoking for 30 minutes before the test. There were no restrictions on prior physical exercise but participants were asked to rest in a sitting position for 15 minutes beforehand and for the duration of the test. Once informed consent had been obtained, subjects were asked to collect a 5 mL saliva sample by passive drooling into a Universal container (Sterilin^{TM} polystyrene 30mL; Thermo Fisher Scientific Ltd, Loughborough, UK). An indwelling catheter was inserted into a superficial antecubital vein and 20 mL of blood was collected. A 250 µg bolus of synthetic $ACTH_{1-24}$ (Tetracosactide) (Synacthen, Alliance Pharmaceuticals Ltd, Wiltshire) was then administered intravenously. Thirty minutes later a further 20 mL of blood was collected and subjects were asked to collect a second 5 mL saliva sample. Further details of simultaneous blood collection, serum handling and analysis have been reported previously.[3]

Analytical methods

Cortisol binding globulin was measured using a manual solid-phase, competitive binding radioimmunoassay in accordance with the manufacturer's instructions (DiaSource, Nivelles, Belgium) (Catalog # KIP1809, RRID:AB_3064898). The intra- and inter-assay CVs were 7.6% and 12.8% respectively at a concentration of 30 mg/L, and 3.1% and 8.7% respectively at a concentration of 110 mg/L. Serum cortisol was measured by GC-MS and the Abbott Architect immunoassay (Abbott Laboratories, Chicago, IL, USA) (Catalog # 8D15, RRID:AB_2783639) as described previously.[3] Salivary cortisol was measured using an in-house LC-MS/MS method. A 250 µL aliquot of saliva, containing 5 nmol/L deuterated cortisol was extracted with 2 mL of dichloromethane. The tubes were centrifuged for 5 mins at 4000 rpm and the top

aqueous layer was discarded. The solvent phase was evaporated under a gentle stream of nitrogen and the dried extract was reconstituted with 250 µl of mobile phase. A 20 µl volume of this extract was injected into the LC-MS/MS instrument for analysis. The LC-MS/MS instrument was a Premier XE triple quadrupole tandem mass spectrometer (Micromass MS Technologies, Manchester, UK) with an Acquity ultra-performance liquid chromatography (UPLC) system comprising a binary pump and auto-sampler (Waters Ltd, California, USA). The LC column was a silica-based reverse-phase C18 (1.7µm, 2.1x50 mm) column (Waters Ltd) and the chromatographic mobile phases were composed of two solutions; (A) deionised water containing 2 mmol/L ammonium acetate and 0.1% v/v formic acid and (B) methanol containing 2 mmol/L ammonium acetate and 0.1% v/v formic acid. The mobile phase was delivered at a flow rate of 0.40 mL/min. The retention time for cortisol and *d4*-cortisol was 0.95 min and the analysis time for each sample was 4.5 min. The MS/MS was operated with electrospray ionisation (ESI) source and Z-spray interface and selected reaction monitoring mode, monitoring at a mass to charge ratio (*m/z)* of 363.3 transitioning to 121.1 (363.3>121.2) for cortisol and 365.3 to 121.2 (365.3>121.2) for *d2*-cortisol. Data acquisition and quantitation of cortisol levels were achieved using MassLynx NT and QuanLynx (Waters Ltd.) software, respectively. The limit of quantitation was 1 nmol/L. The intra- and inter-assay CVs were 5.6% and 6.0% respectively at a concentration of 1.2 nmol/L, 2.3% and 5.8% respectively at 5.4 nmol/L and 3.0% and 3.8% respectively at 15.1 nmol/L.

Statistical analysis

Statistical analyses were performed using SPSS versions 16.0, 19.0 and 23.0 (SPSS Inc., Chicago, Illinois and IBM Corporation, New York). The Kolmogorov-Smirnov test was used to determine whether data were normally distributed. Since the distributional form was found to vary by time-point and gender, all data were log-transformed before analysis. A mean salivary cortisol concentration was determined at each time point, and a lower reference limit calculated from the mean cortisol concentration at 30 min as the 2.5^{th} percentile. These values

were then back-transformed to generate the geometric mean, 2.5th and 97.5th centile values and lower reference limits presented here. Comparisons between means were made using paired and unpaired t-tests, or the Mann-Whitney U test where data remained non-parametric following log transformation. Results from patients with known Addison's disease (and undetectable serum cortisol) were excluded from calculations of the mean to avoid introducing negative bias to comparisons between patients with suspected hypoadrenalism and healthy volunteers. In all cases, differences were considered to be significant when P <0.05.

Results

Baseline salivary cortisol

Baseline salivary cortisol was not normally distributed in male or female volunteers nor in women taking an estrogen-containing oral contraceptive pill (OCP-females) but was normally distributed in patients with low protein concentrations (LP-patients) (data not shown). There was no significant concentration difference between male and female volunteers (table 1) and no age effect (p=0.43).

The concentration range of the untransformed data was wide in all groups: 0.6 to 12.0 nmol/L in men, 0.8 to 9.2 nmol/L in women, 1.5 to 12.4 nmol/L in OCP-females and 1.5 to 16.9 nmol/L in LP-patients. Mean baseline concentrations, calculated after log-transformation, were significantly higher in OCP-females and LP-patients than in healthy volunteers (respectively 5.1 nmol/L, 5.3 nmol/L and 2.9 nmol/L; both p<0.01) (table 2; figure 1).

Post-ACTH salivary cortisol

Post-ACTH salivary cortisol was not normally distributed in healthy volunteers and in OCP-females, whilst LP-patient values remained normally distributed. Following ACTH stimulation, there was no significant difference in mean salivary cortisol concentration between male and

female volunteers (19.1 *vs* 19.6 nmol/L; p=0.44; table 1). The wide concentration range of the untransformed data persisted, ranging from 10.5 to 39.7 nmol/L in male volunteers, 10.1 to 34.8 nmol/L in females, 9.0 to 44.2 nmol/L in OCP-females and 8.0 to 36.0 nmol/L in LP-patients.

In contrast to baseline values, mean post-ACTH salivary cortisol concentrations (calculated after log-transformation) in OCP-females and LP-patients did not differ significantly from healthy volunteers (19.7 nmol/L, 19.0 nmol/L and 19.3 nmol/L, respectively) (table 2; figure 1).

The 2.5th percentile of the combined male and female healthy volunteer response, 10.3 nmol/L, was subsequently taken forward as a cut-off to differentiate between an adequate salivary cortisol response to ACTH stimulation and adrenal insufficiency.

Serum vs salivary cortisol responses to ACTH

In contrast to salivary cortisol, baseline and post-ACTH serum cortisol concentrations were normally distributed in male volunteers, OCP-females and LP-patients, but not in female volunteers. There was no significant difference between baseline serum cortisol concentrations in male and female volunteers with the GC-MS assay (p=0.19), but the slightly lower concentrations in female volunteers were statistically significant when measured by immunoassay (p=0.02). Baseline concentrations in LP-patients were not significantly different to those in healthy volunteers, when measured by either GC-MS or immunoassay (p=0.11, p=0.43, respectively), but were significantly higher in OCP-females (p<0.01) (table 3, figure 1).

Differences in CBG concentrations are likely to explain some of the observed differences in serum cortisol concentration. As anticipated, mean CBG concentration was lowest in LP-patients (41 [28-60] mg/L; p<0.01 vs male volunteers) followed by male volunteers (58 [42-81] mg/L), female volunteers (64 mg/L [43-95]; p<0.01 vs male volunteers) and OCP-females (116 [84-159] mg/L; p<0.01 vs male volunteers). There was no significant effect of age on CBG

concentration and no difference between CBG concentrations at baseline and post-ACTH (p=0.49).

Following ACTH stimulation, mean serum cortisol concentrations were not significantly different between male and female volunteers (p=0.91) or LP-patients (p=0.85), when measured by GC-MS, although mean concentrations in male volunteers were marginally higher than in female volunteers and LP-patients when measured by immunoassay (P=0.01; p=0.03, respectively) (table 3). In contrast, mean serum cortisol concentration was significantly higher in OCP-females (p<0.01) than healthy volunteers, whether assessed by GC-MS or immunoassay (p<0.01) (table 3, figure 1).

Comparison between baseline salivary and serum cortisol concentrations (all subjects) measured by GC-MS and immunoassay (figure 2) showed a moderately-positive correlation overall (R^2=0.42 and 0.53, respectively). This relationship was lost post-ACTH stimulation, with little correlation between salivary and serum concentrations when measured by either GC-MS or immunoassay (R^2=0.08 and 0.14, respectively).

Salivary cortisol lower reference limit as a diagnostic cut-off in patients with suspected hypoadrenalism

The validity of the proposed cut-off in defining adequate adrenal function was explored in a group of patients undergoing ACTH stimulation tests as part of their routine clinical care to explore possible hypoadrenalism (suspected hypoadrenalism [SH] patients) (table 4). Each patient was assigned a high, low or intermediate pre-test likelihood of adrenal insufficiency based on our clinical judgement, in addition to undergoing both serum and salivary ACTH tests.

Nine of the ten patients with a high pre-test likelihood of adrenal insufficiency failed the serum ACTH stimulation test; eight of these also failed the salivary test and one patient was unable to produce sufficient saliva for cortisol measurement. One patient (patient 3) had a high pre-

test likelihood of adrenal insufficiency but passed both the serum and salivary ACTH stimulation tests. There was 100% agreement between serum and salivary outcomes in this group and 90% agreement with pre-test likelihood of disease.

Twelve of the fifteen patients with a low pre-test likelihood of adrenal insufficiency passed both salivary and serum tests. Patient 21 passed the serum test, with a cortisol concentration of 502 nmol/L (cut-off 430 nmol/L), but marginally failed the salivary test, with a concentration of 9.9 nmol/L (cut-off 10.3 nmol/L). Two patients (22 and 23) marginally failed the serum test, with cortisol concentrations of 406 nmol/L and 396 nmol/L, respectively, but convincingly passed the salivary test, with concentrations of 16.3 nmol/L and 15.6 nmol/L. Overall agreement between the two tests in this group was 80%, with 87% agreement between the serum test and pre-test likelihood of disease, and 93% agreement with pre-test likelihood for the salivary test.

Five patients were classed as being at intermediate likelihood of adrenal insufficiency. Two passed both the serum and salivary ACTH stimulation tests, two passed the serum test, but failed the salivary test, and one patient failed the serum test, but passed the salivary test. Agreement between serum and salivary tests in this group was only 40%; although in each of the three discordant cases both results were relatively close to the LRL (patient 11: serum cortisol 451 nmol/L, salivary cortisol 8.7 nmol/L; patient 12: serum cortisol 379 nmol/L, salivary cortisol 10.9 nmol/L and patient 13: serum cortisol 468 nmol/L; salivary cortisol 8.6 nmol/L). The overall pass rate for the serum test was 80% and 60% for the salivary test.

Overall agreement between serum and salivary ACTH stimulation tests in the entire group was 79% (23/29), with 22 of 25 (88%) serum Synacthen tests and 22 of 24 (91.7%) salivary tests showing agreement with pre-test likelihood of disease.

Discussion

In this large study of healthy volunteers, including participants with altered CBG concentration, we demonstrate the potential utility of salivary cortisol response to the high dose ACTH stimulation test in the biochemical evaluation of patients with suspected hypoadrenalism. We confirmed that salivary cortisol responses to ACTH stimulation were unaffected by estrogen treatment, in contrast to corresponding serum values. Furthermore, agreement between salivary and serum diagnostic cut-offs in patients undergoing clinical evaluation for possible hypoadrenalism was high, especially in patients with high- or low- pre-test likelihood of disease. Our observations are consistent with previous studies of salivary cortisol responses to ACTH stimulation[11-19] which have shown excellent diagnostic sensitivity and specificity. We have added to the information available by including a large sample of healthy volunteers, inclusion of participants with altered CBG concentration and a comparison of diagnostic performance in patients undergoing evaluation for potential adrenal insufficiency in a healthcare setting.

Salivary cortisol measurement offers many advantages over serum measurement, including convenience, non-invasive collection and avoidance of venepuncture (albeit that Synacthen still needs to be administered intravenously). Samples are stable at room temperature for many weeks[23] and cortisol concentration is independent of salivary flow rate.[10] Salivary cortisol also offers the significant benefits of close correlation with unbound (free) serum cortisol and is independent of serum CBG concentration.[10,24] Furthermore, specific measurement of salivary cortisol concentration by LC-MS/MS circumvents the problem of cross-reactivity with other steroids that is commonly observed with immunoassays. We would thus recommend mass spectrometry as the measurement method of choice, accepting that this may be less generally available than immunoassay and more labour-intensive.

Previous studies have suggested that the correlation between salivary and serum cortisol may be non-linear, with an exponential model best explaining this relationship.[12] Our observations of a linear association are not inconsistent with these findings, given the relatively weak correlation of 0.42 and 0.53, with GC-MS and immunoassay cortisol, respectively. This is

likely best explained by the saturation of CBG binding capacity when total cortisol exceeds 500 nmol/l.[12,25,26] In agreement, the correlation we observed between serum total cortisol and salivary cortisol at baseline was lost post-ACTH (figure 2). In addition, previous reports have shown a poor correlation in the early dynamic phase of the Synacthen® test,[15] perhaps due to the difficulties of obtaining contemporaneous paired samples.

In contrast to studies in healthy volunteers, only a few studies have examined salivary cortisol responses in patients with altered protein concentration, in whom measurement of total serum cortisol may be unreliable because of disrupted CBG production. Albert *et al* established reference values for salivary cortisol at 0, 30-, 60- and 90-minutes post 250 µg intravenous ACTH in 39 subjects with decompensated cirrhosis, finding similar mean concentrations and increments from baseline with healthy volunteers.[20] Mean salivary cortisol values at baseline (19.9 nmol/l) and at 30 minutes (40 nmol/l) in patients with cirrhosis were higher than in our study (5.3 and 19 nmol/l respectively), likely due to measurement by immunoassay rather than mass spectrometry. Their patient group had similar modest reductions in albumin concentration to ours (mean 30 g/l) but CBG levels were not measured. In this context, it's noteworthy that CBG levels were also only modestly reduced in our low-protein population, suggesting that more profound reductions may be needed before differences in serum cortisol concentrations become clinically apparent. Indeed, Fede *et al* demonstrated that CBG levels correlated with Child-Pugh cirrhosis severity score, and accounted for the overestimation of adrenal insufficiency based on measurement of total (serum) cortisol.[21] Thevenot *et al* similarly found a correlation between low CBG and low serum cortisol in their study of 95 patients with non-septic cirrhosis, with baseline serum cortisol concentrations being significantly lower in patients with CBG concentrations of <35 mg/L compared to those with normal CBG values.[22] Similarly, subnormal serum cortisol responses to high dose ACTH stimulation were associated with low CBG levels.[22] Salivary cortisol concentrations, as anticipated, were unaffected by CBG status. Perogamvros *et al* also found a similar discordance in salivary and serum cortisol responses in two patients with CBG deficiency,[12] although salivary measurement is likely to

find much wider clinical application in common disorders of altered CBG production such as cirrhosis, nephrotic syndrome, sepsis and critical illness than this rare genetic disorder.

Estrogen exerts a profound stimulatory effect on CBG production.[27,28] Early morning serum cortisol values in women using ethinyl estradiol contraception (reference interval: 284-994 nmol/l) are thus significantly greater than in non-users (159-569 nmol/l).[27] Similarly, we found a marked elevation in mean serum cortisol among estrogen users in our study, likely as a result of the anticipated increase in CBG concentrations. In contrast, as others have also demonstrated,[29,30] stimulated salivary cortisol values were not different in OCP users and non-users. These observations have potentially significant clinical value since patients are currently advised to discontinue estrogen therapy for up to 6 weeks in order to obtain a reliable assessment of serum cortisol responses to dynamic testing. We did find a significant elevation in basal salivary cortisol values in women taking estrogen, although this contrasts with previous studies[27,28] and is unlikely to be of clinical significance.

To our knowledge, very few previous studies have tested the performance of salivary cortisol responses to ACTH stimulation in a cohort of patients undergoing evaluation for potential adrenal insufficiency in a routine clinical setting. Applying the 2.5th percentile for salivary cortisol responses to establish a cut-off, we compared the diagnostic utility of salivary and serum responses using immunoassay serum cortisol 'cut-offs' that we had established previously.[3] We found excellent diagnostic performance of salivary cortisol, especially in patients with high or low pre-test probability of adrenal insufficiency. Even in the intermediate probability group, discordance in serum and salivary measures was largely due to minor differences around the respective lower reference limits, some of which could be explained by assay precision (with coefficients of variation of 5.4% for the Abbott assay at a cortisol concentration of 549 nmol/L and 3.0% for salivary cortisol at a concentration of 15.1 nmol/L). Perogamvros and colleagues similarly confirmed excellent sensitivity and specificity of salivary cortisol responses to high dose ACTH stimulation in their study of 78 patients undergoing dynamic testing,[12] albeit that key differences from our study were a significantly higher pre-

test probability of disease (since testing was largely confined to patients who had undergone pituitary or adrenal surgery, had congenital adrenal hyperplasia or a history of previous glucocorticoid exposure) and establishment of a normative salivary response based on serum responses in their patient population rather than in healthy volunteers. They also measured serum cortisol by immunoassay and defined an adequate serum cortisol response as a concentration of >500 nmol/l at 30 minutes, a value which we have shown is heavily assay-dependent and significantly higher than when measured by either mass spectrometry or contemporary immunoassays.[3]

Our study has several strengths and weaknesses. Strengths include the large number of subjects recruited, measurement of salivary cortisol by LC-MS/MS, measurement of serum cortisol concentration by GC-MS as well as immunoassay, evaluation of CBG concentration and an assessment of the performance of the lower reference limit for 30-minute salivary cortisol concentration as a diagnostic cut-off in a clinical population. Our study also has several limitations. Firstly, we confined post-stimulation measurement to a 30-minute value only. Others have shown that cortisol responses, including those in saliva, rise further at 60 minutes, and might potentially lead to misclassification of some patients with adrenal insufficiency if the 30-minute values alone are relied upon.[15,31] Elder *et al* demonstrated an ongoing rise in serum and salivary cortisol concentration at least up to 120 minutes after 250 micrograms ACTH. The time taken for cortisol to reach peak concentration (Tmax) was the same in both, consistent with very rapid transfer of free cortisol from serum to saliva.[15] However, adopting method-dependent lower reference limits improves the specificity of the adrenocorticotropin test,[32] and we showed similar discriminatory potential for serum and salivary cortisol measurements at 30 minutes when applied in our patient population with potential adrenal insufficiency. Further studies are thus needed to determine whether additional sampling at 60 minutes is necessary. Secondly, we didn't measure salivary cortisone in our study. Others have found that salivary cortisone reflects serum total and free cortisol better than salivary cortisol[33-36], not least because salivary cortisol is rapidly oxidised to inactive cortisone by 11β-

hydroxysteroid dehydrogenase type 2. Salivary cortisone is also more sensitive than salivary cortisol at low serum cortisol concentrations, potentially adding to its diagnostic utility in patients with adrenal insufficiency.[13,14,33,35] Thirdly, we only evaluated serum and salivary responses to high dose ACTH (250 micrograms). Many clinicians advocate a preference for a low-dose (1 microgram) test as it more closely reflects the physiological state, although meta-analyses have not shown a benefit of one over the other.[37] Finally, we acknowledge that our approach to the classification of patients as having a low, intermediate or high pre-test probability of adrenal insufficiency is unvalidated and based entirely on clinical judgement. Nevertheless, we were reassured to see a similar diagnostic performance of salivary and serum cortisol responses to ACTH stimulation across each of these categories. Further studies seeking to establish and validate a clinical rating scale for probability of adrenal insufficiency are needed, with the potential to guide clinicians in selecting patients for dynamic testing.

In conclusion, in this study comparing salivary and serum cortisol responses to high dose ACTH stimulation measured by mass spectrometry, we have established normal ranges of salivary cortisol in a large sample of healthy volunteers and confirmed the excellent diagnostic utility of salivary cortisol in patients undergoing evaluation for potential adrenal insufficiency. Salivary cortisol responses may be especially useful as an alternative to serum measurement in patients with diseases associated with reduced CBG production and in women taking estrogen therapy, in whom an inconvenient period of estrogen withdrawal may be avoided.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Bornstein SR, Allolio B, Arlt W, et al. Diagnosis and treatment of adrenal insufficiency: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2016;101:364-389.
2. Elder CJ, Sachdev P, Wright NP. The sort Synacthen test: a questionnaire survey of current usage. Arch Dis Child 2012;97:870-873.
3. El-Farhan N, Pickett A, Ducroq D, Bailey C, Mitchem K, Morgan N, Armston A, Jones L, Evans C, Rees DA. Method-specific serum cortisol responses to the adrenocorticotrophin test: comparison of gas chromatography-mass spectrometry and five automated immunoassays. Clin Endocrinol (Oxf) 2013;78:673-680.
4. Klose M, Lange M, Rasmussen AK, et al. Factors influencing the adrenocorticotropin test: role of contemporary cortisol assays, body composition and oral contraceptive agents. J Clin Endocrinol Metab 2007;92:1326-1333.
5. Clark PM, Neylon I, Raggatt PR, et al. Defining the normal cortisol response to the short synacthen test: implications for the investigation of hypothalamic-pituitary disorders. Clin Endocrinol (Oxf) 1998;49:287-292.
6. Suri D, Moran J, Hibbard U, Kasza K, Weiss RE. Assessment of adrenal reserve in pregnancy: defining the normal response to the adrenocorticotropin stimulation test. J Clin Endocrinol Metab 2006;91:3866-3872.
7. Raff H, Brock S, Findling JW. Cosyntropin-stimulated salivary cortisol in hospitalized patients with hypoproteinemia. Endocr Journal 2008;34:68-74.
8. Rauscheker M, Abraham SB, Abel S, et al. Cosyntropin-stimulated serum free cortisol in healthy, adrenally insufficient, and mildly cirrhotic populations. J Clin Endocrinol Metab 2016;101:1075-1081.
9. Molenaar N, Groeneveld ABJ, de Jong MFC. Three calculations of free cortisol versus measured values in the critically ill. Clinical Biochemistry 2015;48:1053-1058.

10. Vining RF, McGinley RA, et al. Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. Ann Clin Biochem 1983;20:329-335.
11. Arafah BM, Nishiyama FJ, Tlaygeh H, Hejal R. Measurement of salivary cortisol concentration in the assessment of adrenal function in critically ill subjects: a surrogate marker of the circulating free cortisol. J Clin Endocrinol Metab 2007;92:2965-2971.
12. Perogamvros I, Owen LJ, Keevil BG, Brabant G, Trainer PJ. Measurement of salivary cortisol with liquid chromatography-tandem mass spectrometry in patients undergoing dynamic endocrine testing. Clin Endocrinol 2010;72:17-21.
13. Cornes MP, Ashby HL, Khalid Y, Buch HN, Ford C, Gama R. Salivary cortisol and cortisone responses to tetracosactrin (synacthen). Ann Clin Biochem 2015;52:606-610.
14. Mak IYF, Au Yeung BYT, Ng YW, et al. Salivary cortisol and cortisone after low-dose corticotropin stimulation in the diagnosis of adrenal insufficiency. J Endocr Soc 2017;1:96-108.
15. Elder CJ, Harrison RF, Cross AS, Vilela R, Keevil BG, Wright NP, Ross RJ. Use of salivary cortisol and cortisone in the high- and low-dose synacthen test. Clin Endocrinol 2018;88:772-778.
16. Deutschbein T, Unger N, Mann K, Petersenn S. Diagnosis of secondary adrenal insufficiency in patients with hypothalamic-pituitary disease: comparison between serum and salivary cortisol during the high-dose short synacthen test. Eur J Endocrinol 2009;160:9-16.
17. Nolan BJ, Sorbello J, Brown N, Dimeski G, Inder WJ. Characterisation of the serum and salivary cortisol response to the intravenous 250 µg ACTH1-24 stimulation test. Endocr Journal 2018;59:520-528.
18. Contreras LN, Arregger AL, Persi GG, Gonzalez NS, Cardoso EM. A new less-invasive and more informative low-dose ACTH test: salivary steroids in response to intramuscular corticotrophin. Clin Endocrinol 2004;61:675-682.

19. Marcus-Perlman Y, Tordjman K, Greenman Y, et al. Low-dose ACTH (1 mu g) salivary test: a potential alternative to the classical blood test. Clin Endocrinol 2006;64:215-218.
20. Albert L, Profitos J, Sanchez-Delgado J, Capel I, Gonzalez-Clemente JM, Subias D, et al. Salivary cortisol determination in ACTH stimulation test to diagnose adrenal insufficiency in patients with liver cirrhosis. Int J Endocrinol 2019:7251010. doi: 10.1155/2019/7251010
21. Fede G, Spadaro L, Tomaselli T, Privitera G, Scicali R, Vasianopoulou P, et al Comparison of total cortisol, free cortisol, and surrogate markers of free cortisol in diagnosis of adrenal insufficiency in patients with stable cirrhosis. Clin Gastroenterol Hepatol 2014;12:504-512.
22. Thevenot T, Borot S, Remy-Martin A, Sapin R, Cervoni J-P, Richou C, et al. Assessment of adrenal function in cirrhotic patients using concentration of serum-free and salivary cortisol. Liver Int 2011;31:425-433.
23. Toone RJ, Peacock OJ, Smith AA, Thompson D, Drawer S, Cook C, et al. Measurement of steroid hormones in saliva: effects of sample storage condition. Scand J Clin Lab Invest 2013;73:615-621.
24. Gozansky WS, Lynn JS, Laudenslager ML, Kohrt WM. Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic-pituitary-adrenal axis activity. Clin Endocrinol (Oxf) 2005;63:336-341.
25. Vogeser M, Briegel J, Zachoval R. Dialyzable free cortisol after stimulation with Synacthen. Clin Biochem 2002;35:539-543.
26. Tunn S, Möllmann H, Barth J, Derendorf H, Krieg M. Simultaneous measurement of cortisol in serum and saliva after different forms of cortisol administration. Clin Chem 1992;38:1491-1494.
27. Panton KK, Mikkelsen G, Irgens WO, Hovde AK, Killingmo MW, Øien MA, et al. New reference intervals for cortisol, cortisol binding globulin and free cortisol index in women using ethinyl estradiol. Scan J Clin Lab Invest 2019;79:314-319.

28. Liening SH, Stanton SJ, Saini EK, Salivary testosterone, cortisol, and progesterone: two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels. Physiol Behav 2010;99:8-16.
29. Marcus-Perlman Y, Tordjman K, Greenman Y et al. Low-dose ACTH (1 microg) salivary test: a potential alternative to the classical blood test. Clin Endocrinol (Oxf) 2006;64:215-218.
30. Simunková K, Hampl R, Hill M, Doucha J, Stárka L, Vondra K. Salivary cortisol in low dose (1 microg) ACTH test in healthy women: comparison with serum cortisol. Physio, Res 2007;56:449-453.
31. Chitale A, Musonda P, McGregor AM, et al. Determining the utility of the 60 min cortisol measurement in the short synacthen test. Clin Endocrinol (Oxf) 2013;79:14-19.
32. Evans C, El-Farhan N, Ducroq D, Rees DA. Response to 'Determining the utility of the 60-min cortisol measurement in the short Synacthen test'. Clin Endocrinol (Oxf) 2014;80:766-767.
33. Blair JC, Lancaster G, Titman A, Peak M, Newlands P, Collingwood C, et al. Early morning salivary cortisol and cortisone, and adrenal responses to a simplified low-dose short Synacthen test in children with asthma. Clin Endocrinol (Oxf) 2014;80:376-383.
34. Debono M, Harrison RF, Whitaker MJ, Eckland D, Arlt W, Keevil BG, et al. Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. J Clin Endocrinol Metab 2016;101:1469-1477.
35. 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.
36. Raff H. Measurement of salivary cortisone to assess the adequacy of hydrocortisone replacement. J Clin Endocrinol Metab 2016;101:1350-1352.
37. Ospina NS, Al Nofal A, Bancos I, et al. ACTH stimulation tests for the diagnosis of adrenal insufficiency: systematic review and meta-analysis. J Clin Endocrinol Metab 2016;101:427-434.

Table and Figures

Table 1. Geometric mean of baseline and post-ACTH stimulation salivary cortisol concentrations in male and female healthy volunteers.

	Salivary cortisol (nmol/L)			
	Male (n=60)	**Female (n=79)**	**P value***	**Combined (n=139)**
0 Minute	3.2 (0.8 – 12.0)	2.7 (1.0 – 7.5)	0.13	2.9 (0.9 – 9.2)
30 Minute	19.1 (9.8 – 37.3)	19.6 (10.9 – 36.2)	0.44	19.3 (10.3 – 36.2)

Results are expressed as geometric mean (2.5^{th} – 97.5^{th} percentile).

*P-value for differences between genders.

Table 2. Geometric mean of baseline and post-ACTH stimulation salivary cortisol concentrations in healthy volunteers, women taking a combined oral contraceptive pill and patients with low protein concentration.

	Salivary cortisol (nmol/L)		
	Healthy volunteers (n=139)	**OCP-Females (n=24)**	**Low protein patients (n=10)**
0 Minute	2.9 (0.9 – 9.2)	5.1 (1.9 – 14.0)*	5.3 (1.1 – 26.2)*
30 Minute	19.3 (10.3 – 36.2)	19.7 (9.5 – 41.2)	19.0 (7.7 – 46.9)

Results are expressed as geometric mean (2.5^{th} – 97.5^{th} percentile).

*Indicates a significant difference (P-value <0.05) when compared to concentrations in healthy volunteers at the same time point.

Table 3. Geometric mean of post-ACTH serum cortisol concentrations in male volunteers, female volunteers, low protein patients and females taking the oral contraceptive pill (OCP).

	Serum cortisol (nmol/L)			
	Males	**Females**	**Low protein patients**	**OCP-females**
Baseline				
GC-MS	274 (131 - 575)	254 (139 - 463)	305 (173 – 537)	537 (315 - 914)†
Immunoassay	289 (151 - 556)	247 (134 - 455)*	282 (167 – 476)	465 (301 - 718)†
Post-ACTH				
GC-MS	563 (418 - 757)	555 (421 - 731)	552 (393 – 776)	869 (649 - 1162)†
Immunoassay	577 (430 - 773)	542 (416 - 707)*	514 (384 -688)**	747 (577 - 967)†

Results are expressed as geometric mean (2.5^{th} – 97.5^{th} percentile).

* Indicates a significant difference (P-value <0.05) when compared to concentrations in males at the same time point.

** Indicates a significant difference (P-value <0.05) when compared to concentrations in males at the same time point.

† Indicates a significant difference (P-value <0.05) when compared to concentrations in females at the same time point.

534 **Table 4**: Patients with suspected hypoadrenalism – characteristics, clinical presentation, pre-test likelihood of disease and ACTH test outcomes.

Patient	Gender	Age (years)	Clinical details	Pre-test likelihood	Post-Synacthen [serum] (nmol/L)	Serum outcome	Post-Synacthen [saliva] (nmol/L)	Saliva outcome
1	F	67	Addison's disease, hypothyroidism Medication - Hydrocortisone, Fludrocortisone, Thyroxine	High	<28	Fail	1.0	Fail
2	M	63	Addison's disease Medication - Hydrocortisone, Fludrocortisone	High	<28	Fail	0.2	Fail
3	F	57	Asthma, recurrent oral glucocorticoids, fatigue Medication - Seretide inhaler	High	515	Pass	17.5	Pass
4	M	62	Previous transsphenoidal resection of invasive pituitary adenoma Medication - Hydrocortisone, Thyroxine, Testosterone	High	279	Fail	1.3	Fail
5	M	64	Left adrenalectomy for autonomous cortisol secretion; ulcerative colitis, recent high dose glucocorticoids Medication - Hydrocortisone	High	414	Fail	6.2	Fail
6	F	40	Addison's disease, treated Graves' disease Medication - Hydrocortisone, Fludrocortisone	High	<28	Fail	0.3	Fail
7	F	81	Previously diagnosed adrenal suppression secondary to recurrent glucocorticoids Medication - Prednisolone	High	373	Fail	-	-
8	F	70	Previous transsphenoidal resection of non-functioning pituitary adenoma; transient diabetes insipidus; primary hypothyroidism	High	404	Fail	6.7	Fail
9	M	36	Type 1 diabetes mellitus, recurrent hypoglycemia; Medication - Hydrocortisone	High	201	Fail	0.5	Fail
10	F	28	Iatrogenic hypoadrenalism (prolonged glucocorticoid treatment for sarcoidosis); Medication - Hydrocortisone	High	396	Fail	8.5	Fail
11	***M***	***35***	***Previous resection of craniopharyngioma with partial hypopituitarism post-op; Medication - Thyroxine, Testosterone, Growth Hormone, Desmopressin***	***Intermediate***	***451***	***Pass***	***8.7***	***Fail***
12	***M***	***43***	***Type 1 diabetes mellitus, Recurrent hypoglycemia, weight loss***	***Intermediate***	***379***	***Fail***	***10.9***	***Pass***

13	***M***	***50***	***Previous surgical resection of non-functioning pituitary adenoma; isolated hypogonadotropic hypogonadism***	***Intermediate***	***468***	***Pass***	***8.6***	***Fail***
14	F	47	Autoimmune hypothyroidism; vitamin B12 deficiency; fatigue	Intermediate	478	Pass	11.7	Pass
15	F	43	Previous transsphenoidal resection of non-functioning pituitary adenoma; growth hormone deficiency Medication - Growth hormone	Intermediate	551	Pass	27.6	Pass
16	F	65	Pituitary macroadenoma -no pre-existing hormone deficit	Low	637	Pass	19.0	Pass
17	F	82	Previous resection of non-functioning pituitary macroadenoma – no pre-existing hormone deficit	Low	530	Pass	39.3	Pass
18	M	61	Non-functioning pituitary adenoma - no pre-existing hormone deficit	Low	431	Pass	17.1	Pass
19	M	74	Non-functioning pituitary adenoma - no pre-existing hormone deficit	Low	459	Pass	14.8	Pass
20	F	46	Fatigue, low energy	Low	490	Pass	17.5	Pass
21	***M***	***54***	***Isolated hypogonadotropic hypogonadism; normal pituitary MRI***	***Low***	***502***	***Pass***	***9.9***	***Fail***
22	***F***	***39***	***Dizziness, postural hypotension***	***Low***	***406***	***Fail***	***16.3***	***Pass***
23	***M***	***64***	***Crohn's disease; intermittent low-dose oral prednisolone***	***Low***	***396***	***Fail***	***15.6***	***Pass***
24	F	47	Non-functioning pituitary microadenoma; primary hypothyroidism Medication - Thyroxine	Low	524	Pass	22.3	Pass
25	M	46	Indeterminate random cortisol and isolated hypogonadotropic hypogonadism; normal pituitary MRI Medication - testosterone	Low	550	Pass	12.4	Pass
26	M	55	Isolated growth hormone deficiency; previous cranial radiotherapy	Low	554	Pass	13.0	Pass
27	F	23	Pituitary microadenoma	Low	622	Pass	22.7	Pass
28	F	46	Fatigue, low energy	Low	465	Pass	14.6	Pass
29	F	54	Generalized aches and pains, headaches, fatigue, low-mood	Low	762	Pass	21.7	Pass
30	F	29	Fatigue, dizziness	Low	495	Pass	11.1	Pass

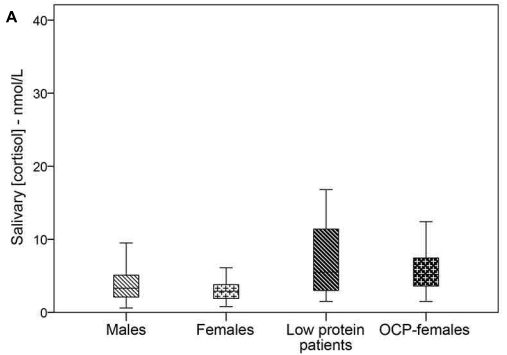
Patients ***11, 12, 13, 21, 22, 23 (highlighted in bold italics)*** showed discrepant serum and salivary test outcomes

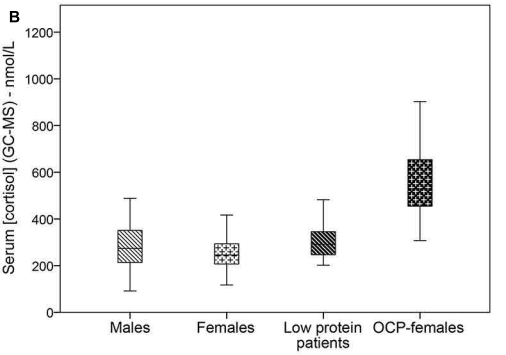
Figure 1: Mean salivary and serum cortisol concentrations in male and female volunteers, patients with low serum protein concentration and women taking an OCP at baseline and post-ACTH stimulation.

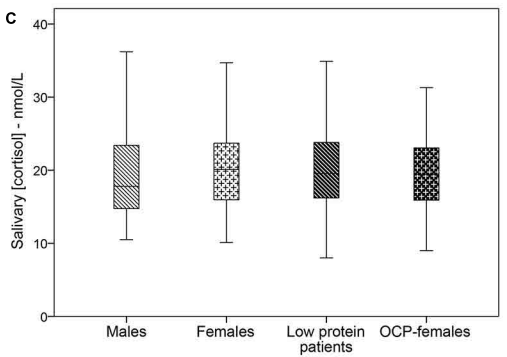
A) Baseline salivary cortisol concentrations, B) baseline serum cortisol concentrations, C) post-ACTH salivary cortisol concentrations, and D) post-ACTH serum cortisol concentrations.

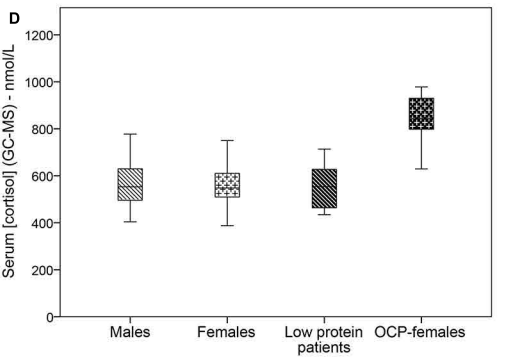
Figure 2: Correlation between salivary and serum cortisol measured by GC-MS and Immunoassay at baseline and post-ACTH stimulation.

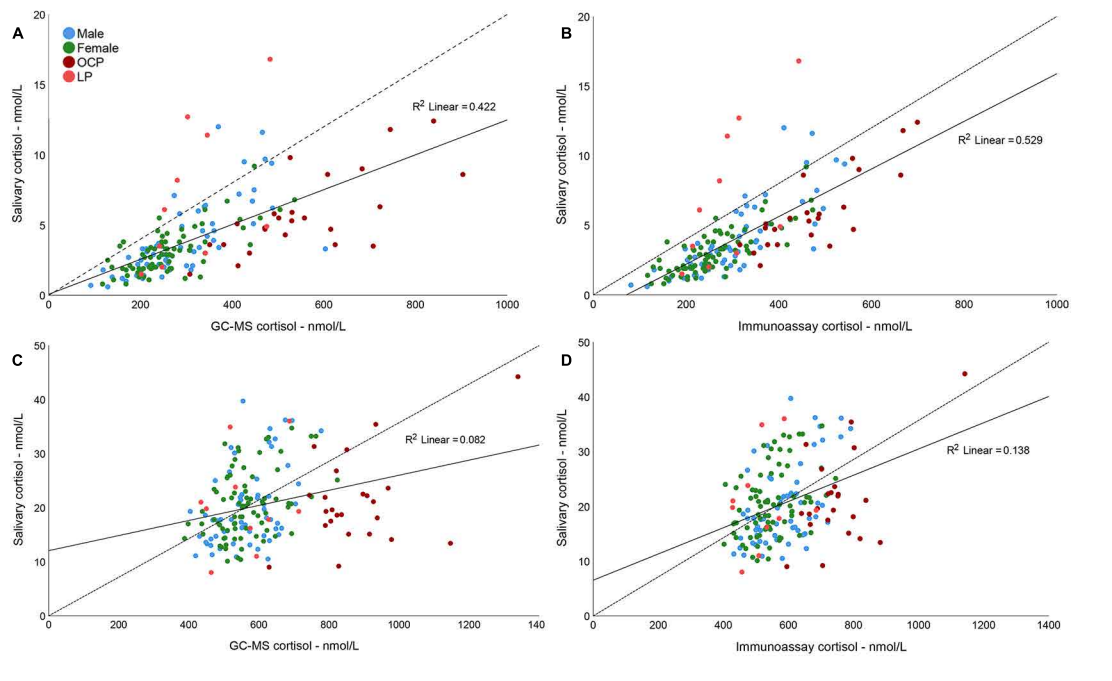
Plots A and C show correlation between salivary and serum cortisol measured by GC-MS at baseline and post-ACTH stimulation, respectively; plots B and D show correlation between salivary and serum cortisol measured by the Abbott Architect immunoassay at baseline and post-ACTH stimulation, respectively. Dotted black line (-------) indicates perfect correlation between salivary and serum cortisol; solid black line indicates actual correlation.


A
Salivary [cortisol] - nmol/L
0
10
20
30
40
Males
Females
Low protein patients
OCP-females


B
Serum [cortisol] (GC-MS) - nmol/L
0
200
400
600
800
1000
1200
Males
Females
Low protein patients
OCP-females


C
Salivary [cortisol] - nmol/L
0
10
20
30
40
Males
Females
Low protein patients
OCP-females


D
Serum [cortisol] (GC-MS) - nmol/L
0
200
400
600
800
1000
1200
Males
Females
Low protein patients
OCP-females


A
Male
Female
OCP
LP
R2 Linear = 0.422
Salivary cortisol - nmol/L
GC-MS cortisol - nmol/L
B
R2 Linear = 0.529
Salivary cortisol - nmol/L
Immunoassay cortisol - nmol/L
C
R2 Linear = 0.082
Salivary cortisol - nmol/L
GC-MS cortisol - nmol/L
D
R2 Linear = 0.138
Salivary cortisol - nmol/L
Immunoassay cortisol - nmol/L