Cushing’s syndrome (CS) results from sustained pathologic hypercortisolism. The clinical features are variable and the most specific features for CS include abnormal fat distribution, particularly in the supraclavicular and temporal fossae, proximal muscle weakness, wide purple striae, and decreased linear growth with continued weight gain in a child. Clinical presentation of CS can be florid and in this case the diagnosis is usually straightforward. However, the diagnosis can be difficult particularly in states of mild or cyclical or periodical hypercortisolism. Several tests based on the understanding of the physiologic characteristics of the hypothalamic–pituitary–adrenal axis have been used extensively to confirm the diagnosis of Cushing’s syndrome, but none has proven fully capable of distinguishing all cases of CS from normal and/or pseudo-Cushing individuals. Three first-line diagnostic tests are currently used to screen for CS: measurement of free cortisol in 24-hour urine (UFC), cortisol suppressibility by low doses of dexamethasone (DST), and assessment of cortisol circadian rhythm using late-night serum and/or salivary cortisol. This paper discusses the effectiveness regarding best cut-off values, the sensitivity and the specificity of these tests to screen for CS. Late-night salivary cortisol appears to be the most useful screening test. UFC and DST should be performed to provide further confirmation of the diagnosis.

Keywords: Cushing’s syndrome; Hypercortisolism; Salivary cortisol; Urinary free cortisol; Dexamethasone suppression; Circadian rhythm
Cushing’s Syndrome (CS) results from lengthy and inappropriate exposure to excessive concentrations of circulating free glucocorticoids. CS may be caused by excess ACTH production (80–85%), usually by a pituitary corticotroph adenoma — Cushing’s disease (CD), less frequently by an extrapituitary tumor (ectopic ACTH syndrome), or very rarely by a tumor secreting CRH (ectopic CRH syndrome). CS can also be ACTH-independent (15–20%) when it results from excess secretion of cortisol by unilateral adrenocortical tumors, either benign or malignant, or by bilateral adrenal hyperplasia or dysplasia (1,2).

However, it is important to point out that the most common cause of Cushing’s syndrome is use of supraphysiological amounts of exogenous glucocorticoids, including topical or inhaled corticosteroids (iatrogenic Cushing’s syndrome). Therefore, the diagnosis of endogenous CS should begin with a careful case history and a thorough physical examination, looking for the characteristic features while excluding the use of oral, parenteral, inhaled, or topical corticosteroids.

The suspicion of CS in a patient arises in the presence of central obesity with supraclavicular fat accumulation, a cervical fat pad, thinned skin, purple striae, proximal muscle weakness, fatigue, high blood pressure, glucose intolerance, acne, hirsutism, and menstrual irregularity. Neuropsychological disturbances including depression, emotional irritability, sleep disturbances, and cognitive deficits are also frequently observed. Muscular atrophy and purple striae are particularly helpful stigmata in adults, whereas in children growth retardation is frequently present (1,2).

Clinical presentation of CS can be florid and in this case the diagnosis is usually straightforward. However, the diagnosis can be difficult particularly in states of mild or cyclical or periodical hypercortisolism (3-5). CS diagnosis suspicion should also arise with a less complete picture, particularly if concomitant recent weight gain, impaired glucose tolerance, and high blood pressure are present (6). The epidemic of obesity and metabolic syndrome increased the number of patients with the Cushing’s phenotype, such as hypertensive patients and men with unexplained osteoporosis (12). Therefore, the diagnosis of CS is often a challenge for clinicians due to the variable pattern and the nonspecificity of clinical manifestations.

Primary care physicians may suspect CS and proceed to the initial biochemical screening tests. However, due to the potential complexity of investigation, further evaluation and treatment of this syndrome should be conducted in specialized endocrinology referral centers. The choice of optimal laboratory screening procedures for patients in whom the suspicion of CS has arisen is not firmly established. In addition, in cases where the diagnosis of CS is suspected clinically but initial screening tests are normal, the patient should be reevaluated at a later date, and invasive procedures should be postponed.

**BIOCHEMICAL DIAGNOSIS OF HYPERCORTISOLEMIA**

Several tests based on the understanding of the physiologic characteristics of the hypothalamic–pituitary–adrenal axis have been used extensively to confirm the diagnosis of the Cushing syndrome, but none has proven fully capable of distinguishing all cases of CS from normal and/or pseudo-Cushing individuals. Among these, four diagnostic tests are currently used to screen for CS: measurement of free cortisol in 24-hour urine (UFC), cortisol suppressibility by low doses of dexamethasone, and assessment of cortisol circadian rhythm using late-night serum and salivary cortisol levels (6) and the dexamethasone-CRH test. Table 1 shows the diagnostic criteria and sensitivities and specificities for these tests.

**Measurement of free cortisol in 24-hour urine**

UFC is an integrated index of blood circulating free cortisol during 24-hour period. In contrast to plasma cortisol levels, which measure total cortisol, it is not affected by corticosteroid-binding globulin (CBG) levels (13). Urinary creatinine may also be measured with UFC; if glomerular filtration rate is less than 30
The reference range for cortisol levels depends on the type of assay used (15). Measurement of urinary cortisol by immunoassays (RIA or immunometric assays; normal range: < 80–120 µg/24 h or < 220–330 nmol/24 h) is influenced by various metabolites of cortisol and some synthetic glucocorticoids, whereas measurements using high-performance liquid chromatography (HPLC) allow the separation of various urinary glucocorticoids and metabolites (normal range: < 50 µg/24 h or < 138 nmol/24 h). Thus, HPLC or gas chromatography coupled with mass spectrometry provides the best specificity for measuring UFC (16,17) and is replacing older immunoassay methods because of its higher specificity. Differences in assay methodology may make interpretation of the data very challenging. Although HPLC has a high sensitivity and specificity, occasionally interfering substances, such as carbamazepine and digoxin, can also coelute with cortisol and produce false elevations of the UFC (18). The sensitivity of UFC for Cushing’s syndrome may be only 45–71% at 100% specificity (19,20). Furthermore, pseudo-Cushing’s states may have false-positive UFC testing (21,22). Therefore, although UFC may be useful to confirm Cushing’s syndrome, its sensitivity and specificity are not optimal as an initial screening test and it cannot be considered as a universal single screening test for the detection of CS (6,23).

### Low-dose dexamethasone suppression tests (DST)

Two dexamethasone-suppression tests (DST) are used for screen CS, the overnight and the 48-h DST. These tests depend on the concept that dexamethasone will suppress ACTH and cortisol release in normal subjects, while patients with corticotroph adenomas will not suppress below a specified cut-off. The overnight low-dose (1 mg) DST consists of the oral intake of 1 mg dexamethasone between 2300 and 2400 h, followed by measurement of fasting plasma cortisol between 0800 and 0900 h in the following morning. In the 48-h test, dexamethasone is given at the dose of 0.5 mg every 6 h for 2 days at 0900 h, 1500 h, 2100 h, and 0300 h with measurements of cortisol in serum at 0900 h at the start and end of the test. The original criterion for normal level of suppression was a plasma cortisol level below 5 µg/dL (138 nmol/liter) (24).

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### Table 1. Screening tests for Cushing’s syndrome: diagnostic criteria, sensitivities and specificities for each test.

<table>
<thead>
<tr>
<th>Screening test</th>
<th>Criteria</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFC (3 samples)</td>
<td>3-fold basal values</td>
<td>90–98%</td>
<td>45–95%</td>
</tr>
<tr>
<td>Plasma cortisol levels at 2300 h</td>
<td>&lt; 1.8 µg/dL</td>
<td>93–100%</td>
<td>20–26%</td>
</tr>
<tr>
<td>Salivary cortisol levels at 2300 h</td>
<td>&lt; 250 ng/dL*</td>
<td>90–96%</td>
<td>96–100%</td>
</tr>
<tr>
<td>Salivary cortisol after 1 mg DST</td>
<td>&lt; 1.8 µg/dL</td>
<td>91–97%</td>
<td>87–94%</td>
</tr>
<tr>
<td>Plasma cortisol after 2 mg DST/2 days</td>
<td>&lt; 390 ng/dL</td>
<td>93–98%</td>
<td>94%</td>
</tr>
<tr>
<td>UFC after 2 mg DST/2 days</td>
<td>&lt; 10 µg/24h</td>
<td>97–100%</td>
<td>90–100%</td>
</tr>
<tr>
<td>Plasma cortisol after low dose DST/CRH</td>
<td>&lt; 1.4 µg/dL</td>
<td>100%</td>
<td>67–100%</td>
</tr>
<tr>
<td>DDAVP</td>
<td>ACTH increase &gt; 27 pg/mL or &gt; 6 pmol/L</td>
<td>86.8%</td>
<td>90.7%</td>
</tr>
</tbody>
</table>

UFC (Urinary free cortisol): normal range: <80–120 µg/24 h or <220–330 nmol/24h (RIA); <50 µg/24 h or <138 nmol/24h (HPLC).

* Cut-off value (mean) based on several available studies (95%CI 153–346 ng/dL).

DST: dexamethasone suppression test, CRH: corticotrophin releasing hormone, DDAVP: Desmopressin.

To convert plasma cortisol from µg/dl to nmol/L multiply by 27.6.

To convert salivary cortisol from ng/dl to nmol/L multiply by 0.028.
More recently, this cut-off level has been reduced to less than 1.8 µg/dL (50 nmol/liter) (2), greatly enhancing the sensitivity of the overnight DST, especially in patients with mild hypercortisolism. However, the cortisol assay must have a high sensitivity (1 µg/dL or 27.6 nmol/liter). There are many interfering conditions in DST: decreased dexamethasone absorption, drugs enhancing hepatic dexamethasone metabolism, inducing the cytochrome P450-related enzymes (barbiturates, phenytoin, carbamazepine, rifampicin, meprobamate, aminogluthethimide, methaqualone), increased concentration of CBG (estrogen treatment, pregnancy) and pseudo-Cushing states (6). The 48-h test, although more cumbersome than the overnight test, is more specific. Because the 1 mg overnight DST is easy to perform and has low cost, it is used as a first-line screening test in outpatient screening.

Some patients with Cushing’s disease (3–8%) retain sensitivity to dexamethasone and show suppression of serum cortisol (25,26). Additionally, DST has a significant false positive rate, especially in chronically ill (23%) or obese patients (13%) and in patients with major depression (43%) or other psychiatric disorders (8–41%). A false-positive rate of up to 30% has been reported in other admitted patients and healthy individuals (27-29). Therefore, because of many interfering conditions and the significant variability of the biological behavior of corticotroph adenomas, neither the overnight nor 2-day DST test appears to be able to reliably rule out Cushing’s syndrome using standard cut-offs for serum cortisol (12).

Late-night plasma cortisol

Physiological cortisol secretion is characterized by circadian rhythmicity. Serum cortisol concentration reaches its zenith in the morning (0600–0800 h) and its nadir in the night during the first half of normal sleep. Normal circadian rhythm of cortisol secretion is lost in patients with Cushing’s syndrome and elevated late-night cortisol serum level has been reported to be the earliest and most sensitive marker of the disease (18). Thus, midnight serum cortisol has been demonstrated to be very effective in recognizing patients with mild signs and symptoms.

Newell-Price et al. (30) were the first authors to propose this screening test. They collected blood sample in 150 patients with CS and in 20 healthy subjects, in a sleeping state. The test achieved 100% sensitivity using a cut-off value of 1.8 µg/dL (50 nmol/L) but the specificity was not tested. Other authors also assessed patients, but in a nonsleeping state, and found higher cut-off values (7.5 µg/dL or 207 nmol/L) with good sensitivity (90.2–96%) and high specificity (96.5–100%) (21,31). Therefore, a single sleeping midnight plasma cortisol concentration of less than 1.8 µg/dL effectively excludes CS. Concentrations of more than 1.8 µg/dL are noted in individuals with CS and in patients with acute illness. An awake midnight concentration of cortisol in plasma of more than 7.5 µg/dL obtained in a nonsleeping state had 97% sensitivity to distinguish between CS and pseudo-CS in the largest series up to now reported but can miss mild disease diagnosis in about 7% of cases (21). More recently, Reimondo et al., (32) obtained 100% sensitivity with a threshold value of 1.5 µg/dL, but the specificity was only 68.2% at that level. The best cut-off value found with ROC analysis was 4.0 µg/dL, which is remarkably greater than that proposed by the recent consensus statement (6). Recently, in a large series of Cushing’s syndrome suspects, urinary free cortisol and cortisol suppression after 1 mg dexamethasone achieved satisfactory specificity. Conversely, serum cortisol concentrations at midnight should be interpreted with caution, especially with the 1.8 µg/dL cut-off (33). Besides controversial cut-off values, sleeping midnight blood sample collection for serum cortisol is not a stress-free sample and is not realistic under normal conditions of clinical practice, which make this test suboptimal for most clinicians (32).

Late-night salivary cortisol

Cortisol concentrations in saliva are independent of flow rate and transcortin fluctuations and reflect those in the free fraction of plasma (34,35). Salivary samples are obtained by noninvasive stress-free procedures, easier to collect, has stability at room temperature, and may be collected many times a day (36). The importance of salivary cortisol in the evaluation of the pituitary-adrenal function, specifically in CS, has been demonstrated more than two decades ago using a competitive assay (37,38). Because of the continuing need for improved noninvasive means of distinguishing pseudo-Cushing from CS, salivary cortisol is a highly suitable screening procedure for outpatient assessment. Indeed, it has now been convincingly demonstrated in more than 10 studies in adults (20,22,31, 32,39-42) and children (40,43,44) that an elevated late-night salivary cortisol sampling is an excellent substitute for increased midnight serum cortisol in the diagnosis of Cushing’s syndrome. It could be particularly useful in investigating patients with cyclical CS with repeated evening measurements over time. Late-night salivary cortisol has also been shown to be useful to identify patients with pseudo-Cushing’s syndrome.
that is the presence of the cushingoid habitus associated with alcohol intake, type 2 diabetes, and major depression (22,31). However, there are several other conditions such as hypertension, advanced age, and psychiatric diagnoses that may lead to false-positive late-night salivary cortisol results (45).

Diagnostic ranges vary between reports because of the different assays and the comparison groups used to set cut-off points. Table 2 shows an overview of late-night salivary cortisol studies conducted in adult controls and CS patients. In the majority of previous papers, salivary cortisol was measured by radioimmunoassay (RIA). Recently the U.S. Food and Drug Administration approved a new enzyme-linked immunosorbent assay (EIA) developed by Diagnostic Systems Laboratories (DSL) for salivary cortisol (46). Using this assay, Raff et al. (47) compared in the same sample the measurement of salivary cortisol obtained by this method and also by RIA. Saliva samples, collected at 2300 h, were sent to their laboratory to screen patients for Cushing’s syndrome. Other set of samples was obtained at 2300 h from healthy elderly and from apparently healthy individuals. The important finding was that EIA and RIA gave different results on the same sample. The EIA showed consistently higher salivary cortisol values than the RIA. The RIA results were much closer to the expected value of an independently created cortisol stock solution diluted in saliva. These data suggest that salivary cortisol measured by RIA reflects more accurately the free level of circulating cortisol.

Another immunoassay has been commercialized from ROCHE Elecsys immunoassay system (Salimetrics EIA) (48). The salivary cortisol measurement using this assay was also compared with the measurement by the DPC RIA (46). The authors observed that Salimetrics EIA yielded results very close to those obtained with the DPC RIA, particularly in the critical diagnostic range of 0.3–10.0 nmol/L (11–357 ng/dL). These results are in contrast to those previously obtained by EIA from DSL method (47). The only relevant disadvantage of the Salimetrics EIA was that the highest calibrator concentration (49.7 nmol/L) is considerably lower and frequent dilution of high-concentration samples from patients with Cushing’s syndrome are necessary. The advantages of the Salimetrics EIA compared with the DSL EIA are: it produces results not different from those obtained with the DPC RIA in the clinically important range, and it is FDA-cleared for in vitro diagnostic use.

More recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement of salivary cortisol has been also available commercially (49,50). RIA and LC-MS/MS measurement of salivary cortisol and their accuracy of reference ranges were compared, in a cross-sectional prospective study of outpatients, in healthy volunteers and obese subjects with cushingoid features (49). This study demonstrated an important rate of abnormal bedtime salivary cortisol, measured in two different commercial assays and evaluated with laboratory provided normative ranges. The authors suggested that although a normal bedtime salivary cortisol result is useful to exclude Cushing’s syndrome, an abnormal salivary cortisol value should not be used alone to establish the diagnosis of Cushing’s syndrome. In a recent paper, Liu et al. (45) evaluated salivary cortisol in 206 male veterans with an enzyme immunoassay whose normal range was based on 73 healthy lean subjects. In that report, 20% of all participants and 40% of diabetic, hypertensive

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**Table 2.** Overview of studies conducted in control subjects and Cushing’s syndrome patients using late-night (2300 h) salivary cortisol.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cortisol assay</th>
<th>Detection limit (ng/dL)</th>
<th>Control subjects</th>
<th>Cushing’s syndrome</th>
<th>Cut-off values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laudat et al. (1988)</td>
<td>CPB assay</td>
<td>57</td>
<td>78–146</td>
<td>1278 ± 178</td>
<td>328</td>
</tr>
<tr>
<td>Raff et al. (1998)</td>
<td>RIA</td>
<td>14</td>
<td>43 ± 3.8</td>
<td>821 ± 150</td>
<td>130</td>
</tr>
<tr>
<td>Castro et al. (1999)</td>
<td>RIA</td>
<td>62</td>
<td>95 ± 8</td>
<td>924 ± 94</td>
<td>280</td>
</tr>
<tr>
<td>Papanicolaou et al. (2002)</td>
<td>RIA</td>
<td>100</td>
<td>260 ± 20</td>
<td>630</td>
<td>415</td>
</tr>
<tr>
<td>Raff et al. (2003)</td>
<td>EIA</td>
<td>10.7</td>
<td>43 ± 3.6</td>
<td>728 ± 464</td>
<td>153</td>
</tr>
<tr>
<td>Pultignano et al. 2003</td>
<td>RIA</td>
<td>50</td>
<td>180 ± 20</td>
<td>970 ± 130</td>
<td>350</td>
</tr>
<tr>
<td>Yaneva et al. (2004)</td>
<td>RIA</td>
<td>30</td>
<td>79 ± 59</td>
<td>1210 ± 2031</td>
<td>200</td>
</tr>
<tr>
<td>Viardot et al. (2005)</td>
<td>RIA</td>
<td>29</td>
<td>75 (29–153)</td>
<td>674 (250–1824)</td>
<td>220</td>
</tr>
<tr>
<td>Vogeser et al. (2006)</td>
<td>EIA</td>
<td>36</td>
<td>178 (50–596)</td>
<td>NA</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>LC-MS/MS</td>
<td>20</td>
<td>65–157</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Baid et al. (2007)</td>
<td>RIA</td>
<td>50</td>
<td>142 ± 218</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LC-MS/MS</td>
<td>4</td>
<td>46 ± 70</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

CBP: competitive binding assay, RIA: radioimmunoassay, EIA: enzyme immunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry; NA: not available.

Salivary cortisol levels (ng/dl) are presented as mean ± SD or range, in control subjects and Cushing’s syndrome patients.
Subjects at least 60 yr of age had an abnormal 2300-h salivary cortisol level. At the time of publication, no participant had been diagnosed with Cushing’s syndrome. Substantially lower concentrations were found with isotopic dilution LC-MS/MS compared to RIA. Similar results were also observed comparing LC-MS/MS and the Roche Cobas cortisol immunoassay results (49,50).

Because salivary cortisol measurement is increasing in popularity, it is important to be aware of the different results generated by the commercially available methods and to interpret the published reference intervals appropriately. Therefore, the normal reference ranges are assay-dependent and should be validated for each laboratory. Nevertheless, late-night salivary cortisol measurement can be recommended as a first-line diagnostic test for CS in both low-risk and high-risk (pseudo-Cushing states) patients. Given its convenience and diagnostic accuracy (table 1), with sensitivity and specificity of between 95% and 98%, this test may profitably be added to traditional screening procedures, such as UFC and 1 mg DST.

So far, the majority of bedtime salivary cortisol measurement data were obtained by RIA. A critical analysis of these RIA data (table 2) indicates that among several available studies the cut-off value of late-night salivary cortisol levels, reliable to segregate pseudo Cushing from CS, was 250 ± 104 ng/dL (mean ± SD, ranging from 130 to 415, 95% CI 153–346 ng/dL). Therefore, in the presence of bedtime salivary cortisol levels higher than 350 ng/dL, the diagnosis of CS is quite confident. On the other hand, in the presence of values lower than 150 ng/dL, the CS diagnosis is unlikely. However, if the bedtime salivary cortisol levels are in the gray area (> 150 and < 350 ng/dL), it is suggested that salivary cortisol sampling and other screening tests should be repeated. Until widely acceptable thresholds are generated, we suggest that each center should establish its own reference range and cut-off points (12,23,31).

**Other second-line diagnostic tests to screen for CS**

The dexamethasone/corticotropin-releasing-hormone (DST-CRH) test was designed to take advantage of the physiologic impact of glucocorticoid-negative feedback on the hypothalamic-pituitary-adrenal axis, as well as the sensitivity of the pituitary adrenal system to hypothalamic stimulation with CRH. There are occasional patients in whom the screening tests described above are equivocal (figure 1). The combined DST-CRH test has been used in these conditions. The DST-CRH test was first shown by the NIH center to be highly accurate in distinguishing CS from pseudo-CS (51), mainly mild Cushing’s disease from normal physiology (52).

The test is performed by giving dexamethasone orally 0.5 mg every 6 h for 48 h, starting at 1200 h, and then administering ovine-sequence CRH (1 µg/kg) iv at 0800 h (2 h after the last dose of dexamethasone). The plasma cortisol value 15 min after CRH is greater than 1.4 µg/dL (38 nmol/liter) in patients with CS, but remains suppressed in normal individuals and in patients with pseudo-CS (51). Criteria for a normal ACTH response have not been firmly established. The DST-CRH test is expensive and time-consuming. Caution is needed in interpreting this test since it requires highly sensitive cortisol assays and the precision of the majority of cortisol assays in routine use at the this cut-off level is poor. Furthermore, abnormal DST-CRH testing has been found in patients with anorexia nervosa (53) suggesting that non-Cushing’s related increases in hypothalamic-pituitary adrenal axis activity will lead to false positive results.

Because desmopressin (1-deamino-8D-arginine vasopressin, DDAVP), a vasopressin analogue, stimulates ACTH release in patients with CS but not in the majority of normal, obese, and depressed subjects, it has been used to investigate its ability to discriminate CS from pseudo-Cushing states (54,55). In these studies, a plasma peak ACTH increase equal to or greater than 6 pmol/L (27 pg/mL), indicated by ROC analysis, allowed the best discrimination between patients with CS and the other groups, with a sensitivity of 86.8%, a specificity of 90.7%, and a diagnostic accuracy of 89%. Therefore, when doubt remains about diagnosis, the DST-CRH test and the

![Figure 1. Algorithm for establishing the diagnosis of Cushing's syndrome. DST: dexamethasone suppression test, UFC: urinary free cortisol, DST-CRH: dexamethasone-corticotropin-releasing-hormone test.](image-url)
Screening Tests in Cushing’s Syndrome
Castro & Moreira

desmopressin test can be used. However, their diagnostic accuracy needs further validation.

In conclusion, different approaches have been developed over the years, but all of them have demonstrated some limitations, and none of the proposed screening tests can detect all cases of CS. The three commonly performed diagnostic tests for CS — UFC, low doses DST, and late-night plasma or salivary cortisol — are complementary. Figure 1 summarizes a suggested approach to screening for Cushing’s syndrome. In patients with mild or intermittent hypercortisolism, any of these tests may yield “normal” results and be misleading. Because of the high sensitivity and ease with which repeated measurements can be performed, late-night salivary cortisol appears to be the most useful screening test. UFC and DST should be performed to provide further confirmation of the diagnosis.

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