

THE 10:00-11:00 PM URINE CORTISOL/CREATININE RATIO. AN ALTERNATIVE TO LATE-NIGHT SALIVARY CORTISOL IN THE DIAGNOSIS OF CUSHING'S SYNDROME

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Abstract The aim of this study was to investigate interchangeability of two tests to diagnose Cushing's syndrome. We compared 10:00-11:00 PM urinary free cortisol/creatinine ratio (UFC/Cr) with late night 11:00 PM salivary cortisol (LNSC) in normal and obese controls vs. patients with Cushing's syndrome. Mean UFC/Cr did not differ between 69 normal and 62 obese controls (9.9 ± 7.9 vs. 9.7 ± 9.3) whereas 116 Cushing's patients had significantly higher values (277.0 ± 318.0 ; z : -11.1 and -10.2, respectively; $p < 0.001$). LNSC was 1.9 ± 1.2 nmol/l in 44 normal and 2.5 ± 1.6 in 45 obese subjects with no differences between them, but was significantly higher in 47 Cushing's patients (24.8 ± 23.3 ; z : -7.22 and -6.96, respectively, $p < 0.001$). Comparison of UFC/Cr and LNSC in samples obtained simultaneously showed that UFC/Cr was 12.0 ± 8.7 ng cortisol/mg creatinine in 34 normal, 12.3 ± 8.9 in 40 obese and 319.5 ± 333.4 in 35 CS subjects ($p < 0.001$ vs. normal and obese), whereas LNSC was 1.8 ± 1.2 nmol/l in normal, 2.6 ± 1.7 in obese and 24.6 ± 17.4 in CS patients ($p < 0.001$ vs. normal and obese); ROC curves showed comparable high sensitivity and specificity figures for the diagnosis of CS. We concluded that UFC/Cr test is easy to perform, readily available in routine laboratories, has high sensitivity and specificity, and offers a valuable alternative to LNSC in the study of Cushing's syndrome.

Keywords: Cushing's syndrome, urinary cortisol, salivary cortisol

Resumen *Relación cortisol/creatinina en orina emitida entre 22 y 23 h. Una alternativa a la determinación de cortisol en saliva nocturna para el diagnóstico del síndrome de Cushing.* Investigamos la equivalencia de dos tests para el diagnóstico de síndrome de Cushing (SC). Comparamos la determinación de la relación cortisol/creatinina (UFC/Cr) en orina emitida entre 22-23 h con la medición de cortisol en saliva a las 23 h (LNSC). La media de UFC/Cr no fue diferente entre 69 sujetos controles normales y 62 obesos (9.9 ± 7.9 vs. 9.7 ± 9.3), en tanto que 116 pacientes con SC presentaron valores significativamente mayores (277.0 ± 318.0 ; z : -11.1 y -10.2, respectivamente; $p < 0.001$). LNSC fue 1.9 ± 1.2 nmol/l en 44 normales y 2.5 ± 1.6 en 45 obesos, sin diferencias entre ellos, pero fue significativamente más elevado en 47 pacientes con SC (24.8 ± 23.3 ; z : -7.22 y -6.96, respectivamente, $p < 0.001$). La comparación del UFC/Cr y LNSC en muestras simultáneas evidenció valores de UFC/Cr de 12.0 ± 8.7 ng cortisol/mg creatinina en 34 normales, 12.3 ± 8.9 en 40 obesos y 319.5 ± 333.4 en 35 sujetos con SC ($p < 0.001$ vs. normales y obesos), mientras que el LNSC fue 1.8 ± 1.2 nmol/l en normales, 2.6 ± 1.7 en obesos y 24.6 ± 17.4 en pacientes con SC ($p < 0.001$ vs. normales y obesos); las curvas ROC mostraron alta sensibilidad y especificidad en el diagnóstico del SC, para ambas determinaciones. Concluimos que el test de UFC/Cr es de realización simple, presenta altos valores de sensibilidad y especificidad y constituye una alternativa válida en el estudio del síndrome de Cushing.

Palabras clave: síndrome de Cushing, cortisol urinario, cortisol salival

The biochemical screening of Cushing's syndrome (CS) usually relies on the demonstration of high excretion of urinary cortisol in 24-h samples, abnormal suppression of morning serum cortisol after overnight 1-mg dexametha-

some test and/or higher than normal values of late-night salivary cortisol¹. Among these, midnight or late-night salivary cortisol determination is considered by some authors to be the best choice because of its elevated sensibility and specificity values and the advantage of being ambulatory and of easy sampling^{2,3}. However, independently of the technique employed⁴, this determination is not available in most routine laboratories, at least in Argentina, and probably in many other countries all over the world. For many

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years, our group has been using an alternative test based on the measurement of the urinary free cortisol/creatinine ratio in spot 10:00-11:00 PM samples (UFC/Cr). Here we aimed to analyze and compare this test against the reference method, the late-night 11:00 PM determination of salivary cortisol (LNSC) in normal and obese people and in Cushing's patients, and to evaluate its comparative efficacy in the diagnosis of Cushing's syndrome.

Materials and Methods

This descriptive cross sectional study was performed over a total of 256 individuals from the Buenos Aires area: 121 (99 females, 22 males) were patients with CS (64 pituitary-dependent, 28 adrenal, 7 ectopic and 22 of undetermined etiology), aged 13 to 77 years, with a mean body mass index (BMI) of 32.0 kg/m² (18.1-77.6), who were included in the study after informing them of the objectives of the diagnostic procedures to be undertaken. One hundred and forty five were control individuals without CS who gave their written consent to participate in the study, according to IRB approval: 77 pertaining to the medical staff (55 females, 22 males), aged 19-63 years with a mean BMI of 23.1 (19.0-27.3) and 68 obese subjects from the outpatient clinic (63 females, 5 males), aged 18-78 years with a mean BMI of 37.1 (26.8-59.2). We performed specific tests to rule out CS in some obese patients with suspicion signs and results of the tests were normal. All individuals had normal renal function.

Patients with CS were diagnosed on clear clinical basis, through the demonstration of high values of 24-h urinary cortisol (> 90 µg /24h) and abnormal 1 mg-dexamethasone suppression (1mg DEX) tests (> 1.8 µg/dl) in all but two patients, who lacked 1 mg DEX suppression tests but in whom diagnosis of pituitary-dependent CS was certified after results of transphenoidal surgery (remission) and pathology tests. Plasma ACTH concentrations were measured in all patients. Appropriate imaging studies and petrosal sinus catheterization were performed, when indicated. The etiological diagnosis was confirmed by the results of surgery (subnormal cortisol values), and/or pathology evaluation (evidence of corticotrophic pituitary adenomas, adrenal adenoma or carcinoma, and ectopic tumors including bronchial carcinoid, medullary thyroid carcinoma and bronchial carcinoma). Undetermined patients were those with clinically and biochemically proven ACTH-dependent CS but in whom definite etiological diagnosis had not yet been made at the time of the study due to different reasons: lost to follow up, indication of urgent adrenalectomy in severe cases, or impossibility to perform further studies because of lack of coverage or death during evaluation. In these 22 patients with undetermined etiology, the diagnosis of CS was confirmed on the basis of clinical signs, 24-h UFC and 1mg-DEX suppression tests.

For the sake of clarity, we can divide the study into two parts:

A) Urinary free cortisol in 10:00-11:00 PM spot samples was measured in 116 CS, 69 normal controls and 62 obese patients, whereas salivary cortisol was measured in 47 CS, 44 normal controls and 45 obese patients.

B) From the original groups of subjects, we also compared the UFC/Cr and LNSC in samples simultaneously obtained in thirty five patients with CS (23 females, 12 males; 17-65 year-old; 17 pituitary-dependent, 3 adrenal, 15 undetermined etiology); 34 normal volunteers (23 females, 11 males; 23-64 year-old) and 40 obese patients (35 females, 5 males; 18-78 year-old).

For saliva sampling, subjects were instructed to have dinner at 8:00 PM, drink water normally, not to smoke or brush their teeth and to collect saliva samples at 10:30 PM into a centrifuge plastic tube. As for the 1-hour urine collection, they were instructed to have dinner at 8:00 PM, drink water normally, void urine at 10:00 PM and collect it at 11:00 PM into a plastic recipient containing 50 mg sodium borate as preservative. Both saliva and urine samples were kept at 4° C until delivered to the laboratory the next day. Salivary cortisol was measured by a DPC-Siemens Coat-A-Count solid-phase RIA using a standard curve adjusted to read values between 0.69 and 138 nmol/l; intra and inter assay coefficients of variation were 5.0 and 11.0% respectively. Results of measured cortisol were expressed in nmol/l (to transform in µg/dl divide by 27.59). After extraction with dichloromethane, urinary cortisol was measured by using a DPC-Siemens Coat-A-Count solid-phase RIA, with a sensitivity of 0.5 µg/dl, and creatinine was determined by the method of Jaffe; results were expressed in ng cortisol/mg creatinine. When cortisol readings were below 0.5 µg/dl, the lowest UFC/Cr ratio was considered to be 1.0. The intra and inter assay coefficients of variations were 7 and 14%, respectively.

Results are presented as means ± SD or medians and range, unless otherwise indicated. Differences between groups were calculated by using the non-parametric Mann Whitney U-test. To assess interchangeability, UFC/Cr and LNSC were measured simultaneously in 35 CS, 34 normal weight controls and 40 obese patients and a linear regression analysis was made with the obtained data. Correlation between UFC/Cr test and LNSC method was evaluated by concordance analysis and a Bland-Altman plot was performed. P-values < 0.05 were considered to be significant. To determine the tests' sensitivity, specificity and cut-off values, the receiver operating characteristic (ROC) curves of sensitivity against 1-specificity were plotted and areas under the curve (AUC) were calculated⁵. An Excel® 2010 for Windows® (Microsoft Corp) spreadsheet programmed *ad hoc* was used to perform the statistical procedures.

Results

All patients with CS presented clinical signs suggesting hypercortisolism which was confirmed by biochemical testing. Results of 24 h UFC and of overnight 1 mg dexamethasone test are shown in Table 1. No correlation was found between UFC/Cr values with 24 h-UFC (r: 0.420, p: 0.16; n = 115) nor with 1 mg dexamethasone test (r: 0.311, p: 0.22; n = 112). All patients in the group with undetermined aetiology had abnormal values in both tests

TABLE 1.- Results of 24-h urinary cortisol (24-UFC) and of overnight 1 mg dexamethasone suppression test at diagnosis in 121 patients with Cushing's syndrome

Test	Mean ± SD	Median (range)
UFC, µg/24 h	449 ± 376	354 (97-2337)
Serum cortisol post 1 mg-Dex, µg/dl*	20.2 ± 10.4	18.7 (2.3-47.8)

UFC: urinary free cortisol; Dex: dexamethasone; * n = 119 (see text)

(UFC: $284 \pm 299 \mu\text{g}/24 \text{ h}$ - mean \pm SD, range 127-292; nocturnal 1-mg dexamethasone test: $23.1 \pm 20.3 \mu\text{g}/\text{dl}$ - mean \pm SD, range 11.4-23.2; $n = 22$).

A) Results of 10:00-11:00 PM urine cortisol (Fig. 1) did not differ significantly between normal and obese controls (9.9 ± 7.9 vs. $9.7 \pm 9.3 \text{ ng}/\text{mg}$ creatinine, respectively; means \pm SD) whereas Cushing's patients had significantly higher values ($277 \pm 318 \text{ ng}/\text{mg}$ creatinine vs. normal and obese controls; z : -11.1 and -10.2, respectively; $p < 0.001$); the cut-off line shown was derived from ROC curves (see below).

Salivary cortisol (Fig. 2) was $1.9 \pm 1.2 \text{ nmol}/\text{l}$ (mean \pm SD) in 44 normal and 2.5 ± 1.6 in 45 obese, with no significant differences between them. Conversely, it was significantly higher in 47 Cushing's patients ($24.8 \pm 23.3 \text{ nmol}/\text{l}$) than in normal and obese controls (z : -7.22 and -6.96, respectively, $p < 0.001$); likewise, the cut-off line shown was derived from ROC curves (see below).

B) Results of measurements of UFC/Cr and LNSC in samples obtained simultaneously in subjects of the three groups studied are shown in Table 2.

Urinary free cortisol/creatinine ratio was $12.0 \pm 8.7 \text{ ng}$ cortisol/mg creatinine in normal, 12.3 ± 8.9 in obese and 319 ± 333 in CS ($p < 0.001$ vs. normal and obese controls). On the other hand, late night salivary cortisol values were $1.8 \pm 1.2 \text{ nmol}/\text{l}$ in normal, 2.6 ± 1.7 in obese and 24.6 ± 17.4 in CS subjects ($p < 0.001$ vs. normal and obese). Fig. 3 shows the Bland-Altman and the correlation plots between the two methods for all data obtained. The difference between their means is $0.798^* \log$ in favor of UFC/Cr. There is a positive and significant correlation ($r = 0.866$; $p < 0.001$; $n = 109$) and the calculated concord-

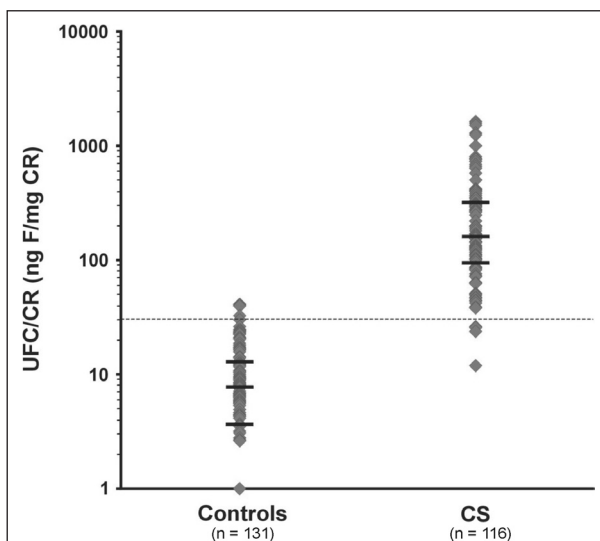


Fig. 1.- Results of UFC/CR method, obtained from control (69 normal and 62 obese pooled) and 116 CS individuals, expressed in semi logarithmic plot to avoid the large dispersion of them. The lines among the values indicate the 1st; 2nd (median) and 3rd quartiles. The dotted line sets the cut off value calculated from the corresponding ROC.

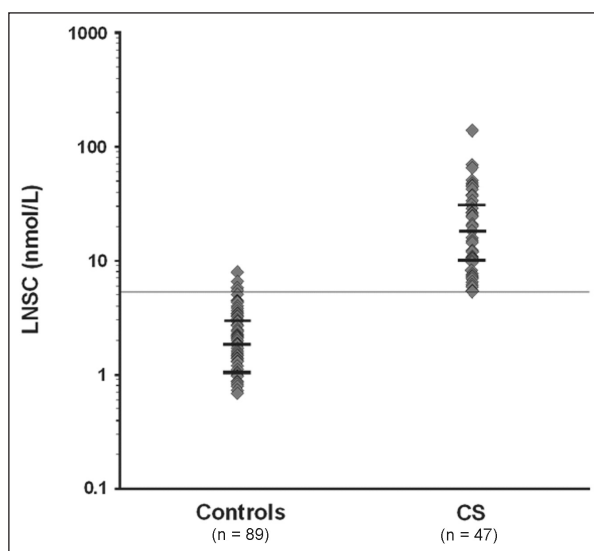


Fig. 2.- Results of LNSC method, obtained from control (44 normal and 45 obese pooled) and 47 CS individuals, expressed in semi logarithmic plot to avoid the large dispersion of them. The lines among the values indicate the 1st; 2nd (median) and 3rd quartiles. The dotted line sets the cut off value calculated from the corresponding ROC; the lower three values observed in CS patients (5.5, 5.5, 5.4 nmol/l) are on the cut off line and were considered as false negatives.

TABLE 2.- Results of measurements of LNSC (nmol/l) and UFC/Cr (ng cortisol/mg creatinine) in samples obtained simultaneously in normal, obese and Cushing's patients

Test	Normal (n = 34)	Obese (n = 40)	Cushing's (n = 35)
UFC/CR 10-11 PM	12.0 ± 8.7	12.3 ± 8.9	$319 \pm 333^*$
LNSC 11 PM	1.8 ± 1.2	2.6 ± 1.7	$24.6 \pm 17.4^*$

Values are means \pm SD; * $p < 0.001$ vs. Normal and Obese

ance correlation coefficient is 0.829 (CI95: 0.667-0.992). The obtained linear model is: $\log y = 0.701 + 1.165 * \log x$.

The receiver operating characteristic (ROC) curves were calculated in 89 control subjects and 47 Cushing patients and in 131 controls and 116 Cushing patients for LNSC and UFC/Cr, respectively. They showed a close relationship between both methods with comparable high sensitivity and specificity figures for the diagnosis of CS, for salivary as well as for urinary cortisol (97/94% and 97/97%, respectively). The area under the curve (AUC) values was also very similar (0.9874 and 0.9973 for LNSC and UFC/Cr, respectively) (Fig. 4).

Discussion

One of the key alterations in Cushing's syndrome is the loss of the normal circadian rhythm, which can be put in

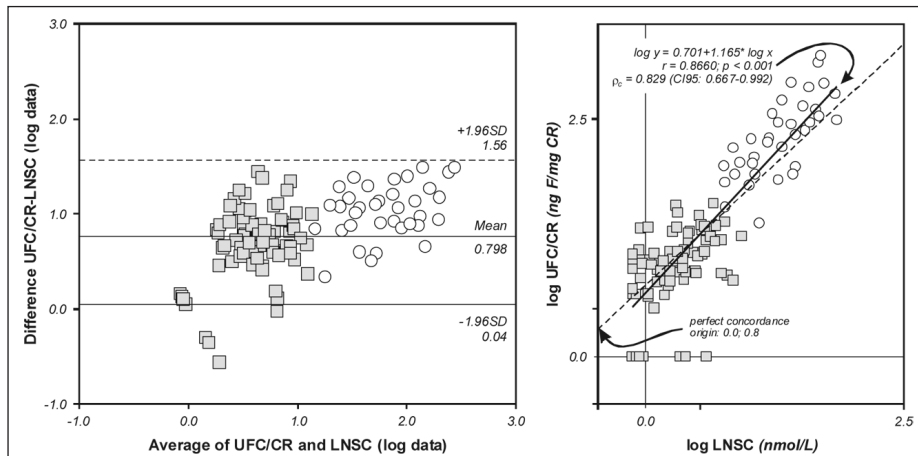


Fig. 3.– Left: Bland-Altman plot of concordance between two methods: UFC/Cr and LNSC; filled squares, control subjects ($n = 74$); open circles, CS patients ($n = 35$). It shows the mean and 95% upper - lower concordance limits of all values. Right: Linear regression between values with concordance correlation coefficient (ρ_c).

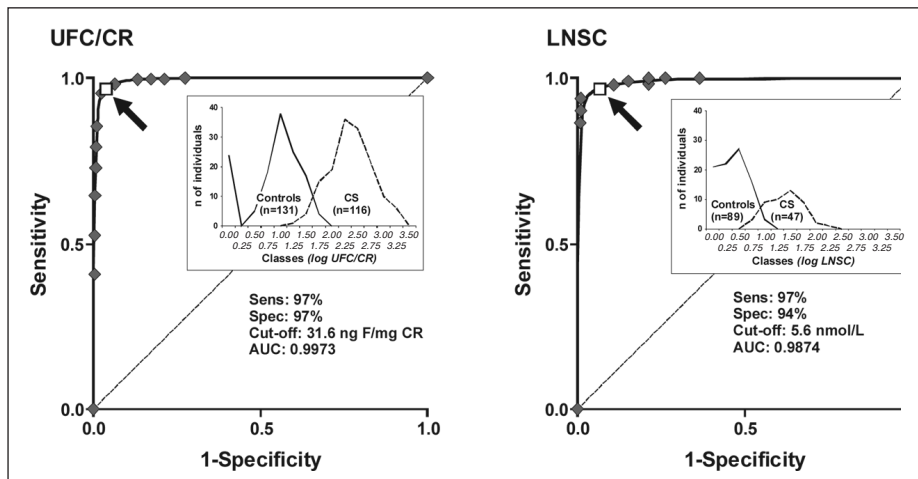


Fig. 4.– ROC curves for the two methods studied: Left, UFC/Cr method; right, LNSC method. Inset, the log normal distribution of the samples employed to make the curves.

evidence through the evaluation of cortisol concentrations in nocturnal serum⁶ or saliva⁷ samples. The measurement of late-night salivary cortisol has been shown to be a highly sensitive and specific test for the ambulatory screening of CS. It presents several advantages over serum samples because it is stress-free, does not require hospitalization and it is not influenced by factors such as estrogen administration since it represents free, non-protein bound cortisol. In addition, saliva samples are stable at room temperature for several days and can be sent to the laboratory by simple mail⁸. In a meta-analysis of seven studies comprising 339 patients with CS, pooled data revealed a sensitivity of 92% and specificity of 96%⁹. This test is considered by many endocrinologists to be the first line for screening and diagnosing Cushing's syndrome⁷.

Salivary cortisol can be measured by several methods⁴. Although less specific than liquid chromatography/tandem mass spectrometry, radioimmunoanalytic (RIA) techniques have been frequently used because of their simplicity, adaptability to routine laboratories and availability. However, the measurement by RIA of the small concentrations of free cortisol usually present in saliva requires setting up a special standard curve, different from that employed to measure cortisol in serum or urine, which implies some operational difficulties in most laboratories of medium or small complexity. This difficulty can be overcome if platform immunoassays are used but, again, this methodology has not been always widely available. Enzymeimmunoassay¹⁰ could also be a good technical alternative but it has to be validated in large groups of patients. Since

the diagnostic screening for Cushing's syndrome is mainly made by physicians other than endocrinologists¹¹ working at places frequently far away from sophisticated medical centers, it is of the outmost importance that they may have access to simple, readily available, sensitive and specific tests to accomplish that goal.

In 1986, Contreras et al.¹² described the utility of spot urinary determinations of cortisol in the assessment of pituitary-adrenal function. They showed that evening values of urine cortisol were elevated in each one of a small group of 14 patients with Cushing's syndrome and that there was no diurnal variation and no overlap with normal subjects. Corcuff et al. reported in 1998¹³ good sensitivity and specificity values in 30 patients with CS by measuring cortisol in 12 h-nocturnal urine collections. In a study including 27 patients with CS, Sakihara et al.¹⁴ concluded that late-night urinary cortisol levels provided weak information because of overlapping results between Cushing's syndrome and controls, but they only specified that urinary samples were taken at 11:00 PM without giving information about the length of time of collection (which could have been quite variable amongst subjects). More recently, Burch¹⁵ investigated the 11:00 PM urine cortisol concentrations in 30-60 minute urine collections of 11 patients with CS and found significant differences against normal controls. We are aware of the fact that the ideal method for measuring urinary cortisol is not RIA after extraction but tandem mass spectrometry or HPLC instead¹⁶⁻¹⁸.

The difficulties encountered in getting salivary cortisol analysis as a routine test in our country moved us to further explore the possibility of replacing it by free cortisol measurements in nocturnal 1-hour urine samples, obtained between 10:00 and 11:00 PM. We were able to extend the study to 116 patients with CS of different aetiologies and to compare them with groups of normal volunteers and obese people. The study showed significant differences between CS and the two other groups and the calculated values of sensitivity and specificity for diagnosing CS were highly satisfactory. Moreover, when values for LNSC and UFC/Cr were compared in samples obtained simultaneously, once again similar high figures of sensitivity and specificity were found as well as a very significant positive correlation between both parameters. This strong correlation had also been observed by Graham et al. who analysed the validity of LNSC as compared to early morning UFC/Cr ratio in evaluating patients with cyclical Cushing's syndrome¹⁹.

The tests we have done and that we show in this study performed very well, probably due to the fact that patients were clinically hypercortisolic which was corroborated by biochemical tests and results of surgery, pathology, etc. and that no cases of mild or doubtful Cushing's syndrome were included in the evaluation. Besides, we did not find

high values in our control populations, which could have also contributed to the high specificity observed.

The results obtained are clearly confirmatory of the utility of UFC/Cr to separate CS from normal and obese patients. There is, however, one limitation to its clinical applicability which is that the presence of even a small reduction in renal glomerular filtration rates might possibly produce misleading results²⁰. In these particular conditions, salivary cortisol would clearly be superior and should therefore be recommended as first choice until new information is provided. In conclusion, evening spot urine cortisol determination constitutes a valuable alternative to late night salivary cortisol in the study of CS. It is easy to perform, more readily available for routine laboratories and fulfills the necessary requirements of sensitivity and specificity for diagnosing Cushing's syndrome. The 10:00-11:00 PM UFC/Cr test described in the present study could be realized, after validation, by other less contaminating techniques such as chemiluminescence assays, which probably perform identically to the RIA assay.

Conflict of interest: None to declare

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LA TAPA

Sello postal de Alemania Federal (2005). Homenaje a personalidades. Conmemorativo a los 100 años de que a Robert Koch le fuera otorgado el premio Nobel de Medicina y Fisiología “por sus investigaciones y descubrimientos en relación con la tuberculosis”.

El sello está acompañado de bandeleta con la firma de Robert Koch. Catálogo *Ivert et Tellier* Nº 2321. Sello y fotografía: Dr. Claudio Zuckerberg.

Robert Koch nació en Clausthal, Baja Sajonia, Alemania. A los cinco años sorprendió a sus padres diciéndoles que había aprendido a leer solo, con los periódicos; de su padre, ingeniero de minas, heredó el gusto por los viajes. Estudió Medicina en la Universidad de Göttingen, fue influido por Henle, su profesor de Anatomía, y después en Berlín por Virchow. Ejerció un tiempo la medicina, después se dedicó a la salud pública y la bacteriología. Sus aportes fueron reconocidos, en el Imperio Alemán su carrera fue ascendente. En 1885 fue nombrado profesor de Higiene y director de Higiene de la Universidad de Berlín, en 1890 Cirujano General, Ciudadano de Honor de Berlín, y en 1891 profesor honorario de la Facultad de Medicina de Berlín y director del nuevo Instituto de Enfermedades Infecciosas. No le faltaron ni discípulos ni reconocimiento internacional, larga es la lista de honores que recibió antes de que se le otorgara el Premio Nobel. Koch murió en 1910 en Baden-Baden. (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1905/koch-bio.html).

Las diferencias de Koch y sus discípulos con Pasteur y los suyos son conocidas, no tanto las razones. Agnes Ulmann, profesora del *Institute Pasteur* de París, analiza esas razones con esmero e incluye, entre ellas, la mala traducción del francés al alemán de “*recueil* (selección, compilación) *allemand*” por “*orgueil* (orgullo, arrogancia) *allemand*,” en una reunión en Ginebra en 1882. Pasteur no sabía alemán, Koch no sabía francés. (Pasteur-Koch: Distinctive ways of thinking about infectious diseases. *Microbe* 2007; 2: 383-7)

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