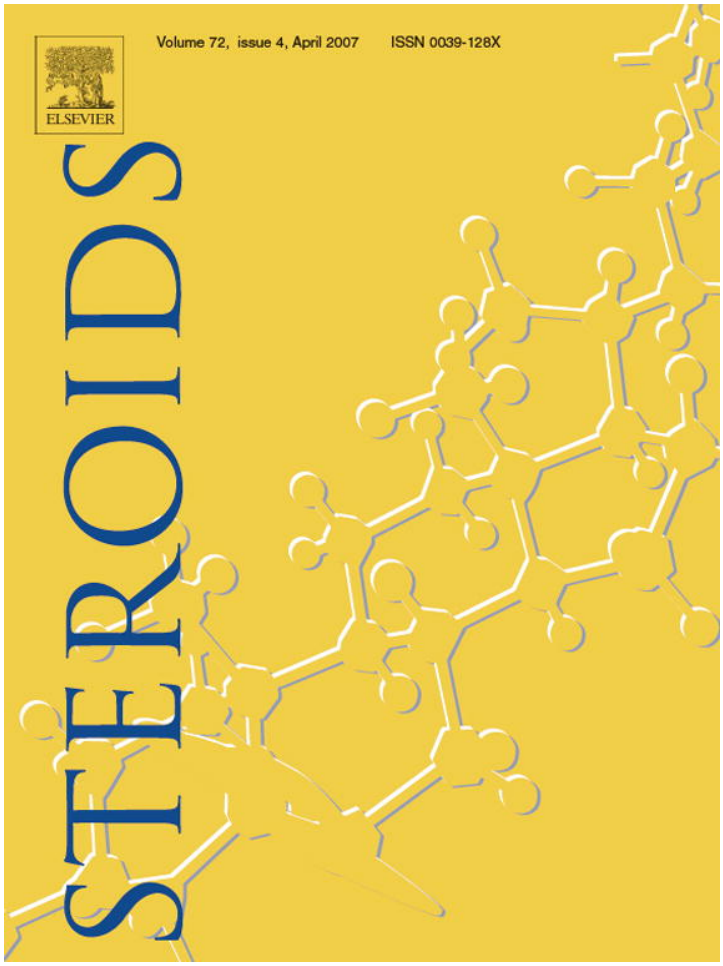

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Assessment of adrenal function by measurement of salivary steroids in response to corticotrophin in patients infected with human immunodeficiency virus

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ABSTRACT

Objective: Adrenal insufficiency has been reported among critically ill HIV-infected patients. This is the first study that attempts to detect subclinical hypoadrenal states in non-critical HIV patients through salivary steroids in response to intramuscular low-dose ACTH injection.

Patients and methods: We studied 21 ambulatory adult HIV-infected patients without specific clinical signs or symptoms of adrenal insufficiency. Normal salivary flow-rate and salivary α-amylase activity confirmed adequate salivary gland function. Salivary cortisol (SAF) and salivary aldosterone (SAL) were obtained at baseline and 30 min after the injection of 25 μg of ACTH in the deltoid muscle (LDT_s). Assessment of salivary steroids after stimulation with 250 μg of intramuscular ACTH (HDT_s) was performed on those who hyporesponded to LDT_s. Basal blood samples were drawn for steroids, renin and ACTH measurements.

Results: At baseline SAF and SAL correlated significantly ($p = 0.0001$) with basal serum cortisol and aldosterone ($r = 0.70$ and 0.91, respectively). Plasma ACTH and renin concentrations were within the normal range in all patients. Eight of the twenty-one HIV^+ patients were LDT_s hyporesponders in either SAF (n:1) or SAL (n:7). LDT_s repeated in six cases after a year reconfirmed the impairment of aldosterone secretion. LDT_s hyporesponders had normal steroid responses to HDT_s.

Conclusions: LDT_s is a simple, safe, well-accepted and non-invasive approach to assess adrenal function in HIV-infected ambulatory patients. It revealed subnormal cortisol (5%) and aldosterone responses (33%) when HDT_s results were normal.

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1. Introduction

Human immunodeficiency virus infection (HIV+) and the acquired immune deficiency syndrome (AIDS) are associated with endocrine abnormalities, particularly of the hypothalamic-pituitary-adrenal axis. Before the institution of retroviral drugs the adrenal gland was reported to be a common site of pathological involvement. Autopsies have demonstrated that more than 50% of adrenal glands from patients with AIDS had evidence of *Cytomegalovirus*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Pneumocystis carinii*, *Cryptococcus neoformans*, *Toxoplasma gondii* and infiltration with Kaposi's sarcoma. Although the prognosis and surveillance of HIV-infected and AIDS patients improved over time with the advance of medical therapy, the adrenal gland remains an important target organ among AIDS patients. Thus, in Brazil, 99.2% of 128 autopsied patients with AIDS during 1989–1998 were found to have adrenal involvement. The main pathological findings were inflammatory infiltrates. Necrosis, fibrosis, hemorrhages, neoplasias and calcification of the adrenal central vein were also observed. *Cytomegalovirus* was the most frequent infectious agent (48.4% of the cases) followed by *Trypanosome cruzi* and *Balamuthia mandrillaris* [1,2].

Studies of the hypothalamic-pituitary-adrenal function in HIV-infected individuals have yielded variable results although all report, puzzling abnormalities that are not clearly understood [3–15].

It is well known that the intravenous injection of 1 μg of ACTH is useful in detecting adrenal insufficiency in subclinical states [16–18]. However, there are few reports using this test in ambulatory HIV-infected patients and critically ill AIDS patients. In addition the prevalence of adrenal insufficiency described varies widely between these limited series [19–21].

Salivary neutral steroids, such as cortisol (SAF) and aldosterone (SAL), reflect the free-circulating steroid that is bioavailable to the target tissues [22]. In addition, saliva collection is easy to perform, less stressful than blood sampling and useful when sequential samples are needed. We have demonstrated that adrenal function could be accurately investigated through the measurement of either circulating or salivary steroids following an intramuscular injection of 25 μg (low dose) or 250 μg (high dose) of ACTH. The salivary low-dose ACTH test showed to be more useful than salivary high-dose ACTH test in the evaluation of adrenal function in patients suspected for subclinical primary or secondary adrenal insufficiency [23]. The usefulness of assessing salivary cortisol as an alternative to serum cortisol after intravenous ACTH stimulation (1 μg) has been reported in patients with endocrine disorders [24].

Xerostomia and swelling of the major salivary glands have been described in some HIV-infected patients [25]. Therefore, investigation of salivary gland functions such as measurement of salivary secretion rate (sialometry) and analysis of salivary composition (sialochemistry) should be employed to insure reliable assessment of salivary steroids in these patients.

This study aims at assessing the integrity of the adrenal cortex in non-critically HIV-infected outpatients by measuring simultaneously salivary cortisol and salivary aldosterone in response to intramuscular low-dose ACTH stimulus (LDT_s) in an attempt to detect subclinical potentially life-threatening adrenal abnormalities.

2. Experimental

2.1. Patients

This study included 21 HIV+-1-infected outpatients (16 men and 5 women, aged 27–64 years) recruited from infectious disease practices (Hospital E. Tornú). The inclusion criteria were: seropositivity confirmed within at least 1 year before participation in the study; age <65 years; having normoalbuminemia, normonatremia, and normokalemia; and showing absence of azotemia. They were classified using Center Disease Control criteria [26] as asymptomatic (four in stage A_1, seven in A_2 and two in A_3), symptomatic (one in stage B_2) and with AIDS-defining conditions (one in stage C_1, five in C_2 and one in C_3). Clinical characteristics and associated diseases are shown in Table 1. All patients were on normal sodium diets for at least 3 months prior to the study. Assessment of total urinary sodium excretion that ranged from 132 to 150 mequiv./day was confirmed. The subjects had received retroviral drugs for more than 6 months, had no clinical evidence of endocrine disease and were at least 3 months off drugs that may interfere with adrenocortical functions.

The protocol was approved by the Human Research Ethics Committee of the School of Medicine, University of Buenos Aires and all patients gave their written informed consent to participate in the study. The ambulatory patients were studied and confidentiality of their identity and tests results was maintained during the investigation.

2.2. Salivary analyses

From 8.00 to 9.00 a.m. and after an overnight fast, whole saliva was collected from all HIV+ individuals and 50 healthy

Table 1 – Symptoms, signs, and associated diseases in 21 HIV+ patients

Symptoms/incidence
Asthenia: 43%
Depression: 33%
Dizziness: 19%
Nausea: 10%
Anorexia: 5%
Signs
Hyperhidrosis: 24%
Hypotension: 14%
Weight loss: 5%
Associated diseases
Pneumocystis carinii: 9%
Hystoplasma capsulatum: 5%
Cytomegalovirus: 5%
Herpes Zoster: 5%
Herpes Zoster + cervix carcinoma: 5%
Cryptoccocosis + Pneumocystis carinii + Kaposi sarcoma: 5%
TBC + Pneumocystis carinii + Toxoplasmosis + Atypical mycobacterium: 5%

subjects. The collection was done through a soft plastic catheter located at the floor of the mouth connected to a suction device. Initial salivary flow-rate was calculated as described [27]. An aliquot of the basal saliva sample was stored at −20 °C until determination of α-amylase activity, then saliva production was stimulated with three filter paper disks of 18 mm diameter soaked with citric acid (2%). The saliva was collected for 3 min to calculate the reflex salivary flow-rate.

After confirming that salivary characteristics did not differ from those found in controls the low-dose ACTH salivary test (LDT_s) was performed.

2.3. ACTH-stimulation tests

The following day, a rapid low-dose intramuscular ACTH stimulation (LDT_s) was performed on all patients. From 8.00 to 9.00 a.m., after being upright for at least 2 h, the patients were instructed to collect 3.5 ml of whole saliva by directly spitting into polypropylene tubes and a simultaneous blood sample was drawn in each case. A dose of 25 μg of synthetic human $\beta^{1\text{-}24}$ACTH (Synacthen; Novartis Pharma AG, Basle, Switzerland; provided through Novartis SA, Argentina), prepared as previously described [23], was directly injected into the deltoid muscle. Salivary samples were obtained 30 min after intramuscular ACTH stimulation. The supernatant obtained after centrifugation of saliva (1000 × *g*, 10 min) was kept at −20 °C for later SAF and SAL measurements. Basal plasma (for ACTH) and serum samples (for renin, cortisol and aldosterone) were frozen at −20 °C until assayed. When SAF did not increase normally after LDT_s a further evaluation by injecting 250 μg of ACTH intramuscularly (HDT_s) was performed after a 1-week interval [28]. Patients with subnormal SAL concentrations after LST_s^1 were closely followed and reevaluated (LST_s^2 followed by HDT_s) 12 months after the initial test.

2.4. Salivary steroid measurements

SAF and SAL were measured by RIA (Diagnostic Products Corporation, Los Angels, CA, USA) in saliva samples as previously described [29]. SAF was expressed as nmol/l and the minimal detectable SAF concentration was 0.5 nmol/l. SAF intra and interassay coefficients of variation (CVs) were less than 6% and 13%, respectively. SAL was expressed as pmol/l and the minimal detectable dose was 13.0 pmol/l. The intra and interassay CVs were less than 8% and 12%, respectively. Salivary amylase activity was measured as described [27] and expressed as units (one unit equals the milligrams of maltose liberated in 3 min at 20 °C by 1 ml of amylase).

2.5. Blood assessments

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

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

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

2.6. Normal hormonal values (Endocrine Research Laboratory)

Basal values (from 8 to 9 a.m., in upright position). Serum cortisol: 139–500 nmol/l, serum aldosterone: 138–500 pmol/l, salivary cortisol: 2.5–18.0 nmol/l; salivary aldosterone: 20–70 pmol/l; plasma ACTH: 5–50 pg/ml; serum renin: 0.55–3.10 pmol/l.

Criteria used to define a normal salivary steroid response 30 min after ACTH i.m. [23] and locally established reference values for SAF and SAL (5th and 95th percentiles) were 20–70 nmol/l and 100–340 pmol/l, respectively, for healthy subjects (*n*:50) after either 25 or 250 μg of ACTH.

2.7. Statistical analysis

Results are expressed as mean ± S.D. unless otherwise specified. Data were analyzed by analysis of variance (ANOVA) and non-parametric Mann–Whitney test. Correlations between serum and salivary steroids levels were evaluated by Spearman analysis. Statistical analysis was performed with PRIMER of biostatistics (Version 4.02, 1996, McGraw-Hill). *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Salivary flow-rate and salivary α-amylase activity

Basal (0.40 ± 0.21 ml/min) and stimulated (0.91 ± 0.42 ml/min) flow-rates in HIV^+ patients were not different from controls (0.32 ± 0.07 and 0.76 ± 0.18 ml/min, respectively; $p = 0.534$ and 0.425, respectively). In addition, salivary α-amylase activity in HIV^+ (175 ± 134 units) did not show significant differences than healthy subjects (174 ± 92 units; $p = 0.597$).

3.2. Baseline serum and salivary steroids in HIV^+ patients (Fig. 1)

All HIV^+ patients showed normal basal serum cortisol levels, ranging from 152 to 500 nmol/l. Basal serum aldosterone levels were normal (139–500 pmol/l) in all but one patient in whom a level below the normal range was detected (120 pmol/l). Simultaneous salivary cortisol (2.5–18.0 nmol/l) and salivary aldosterone (13.5–55.0 pmol/l) concentrations were within the range found in normal subjects.

A positive and significant correlation was found between baseline serum and salivary cortisol levels (*r*: 0.70, $p = 0.0001$) and baseline serum and salivary aldosterone concentrations (*r*: 0.91, $p = 0.0001$).

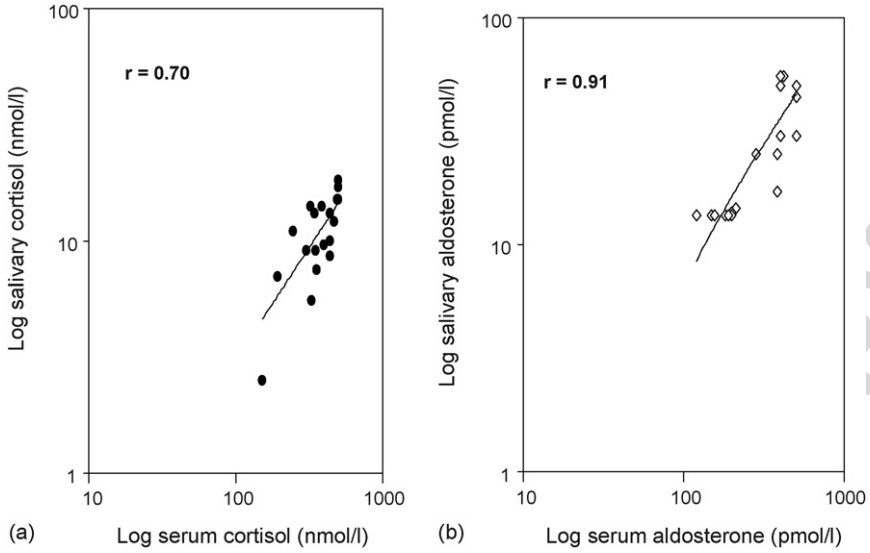

Fig. 1 – Significant correlation ($p = 0.0001$) between basal serum and salivary levels of cortisol (a) and aldosterone (b) was observed in 21 HIV-infected patients.

3.3. *First dynamic testing*

Thirteen of the twenty-one HIV⁺ patients achieved normal SAF and SAL concentrations 30 min after LDT_s, supporting adequate adrenal steroid function (Fig. 2). Plasma ACTH and renin concentrations were in the normal range. Normal responders were in the following CDC stages: A_1 ($n = 3$), A_2 ($n = 5$), A_3 ($n = 1$), B_2 ($n = 1$), C_1 ($n = 1$) and C_2 ($n = 2$).

After LDT_s one patient (stage C_3) failed to show normal increased SAF levels but had a normal rise in SAL (Fig. 2), suggesting selective hypocorticism reinforced by normal ACTH (20 pg/ml) and renin levels (3.0 pmol/l). After a 1-week interval 250 μg of ACTH (HDT_s) was given and the patient demonstrated a normal SAF level (25 nmol/l) at 30 min.

In seven patients SAF levels increased normally after LDT_s, whereas SAL concentrations were either blunted or subnormal (Fig. 2). These patients were classified (CDC criteria) in stages A_1 ($n = 1$), A_2 ($n = 2$), A_3 ($n = 1$) and C_2 ($n = 3$). HIV⁺ SAL hyporesponders demonstrated a significantly lower SAL increase (43 ± 31 pmol/l) after LDT_S than normoresponders (133 ± 65 pmol/l) and healthy subjects (134 ± 59 pmol/l) ($p = 0.003$). The measurement of renin levels after 2 h of deambulation revealed values within the normal range (0.8–2.0 pmol/l). In these seven patients, basal and stimulated SAF levels were compared with those found among the 13 HIV⁺ patients who showed normal responses; there were no significant differences between the groups ($p = 0.249$ and 0.632, respectively).

The abnormally low aldosterone response to LDT_s found in 7 of the 21 HIV⁺ patients associated with normal serum electrolytes and renin levels, led us to investigate, during the follow-up, if this was a permanent or transient state related

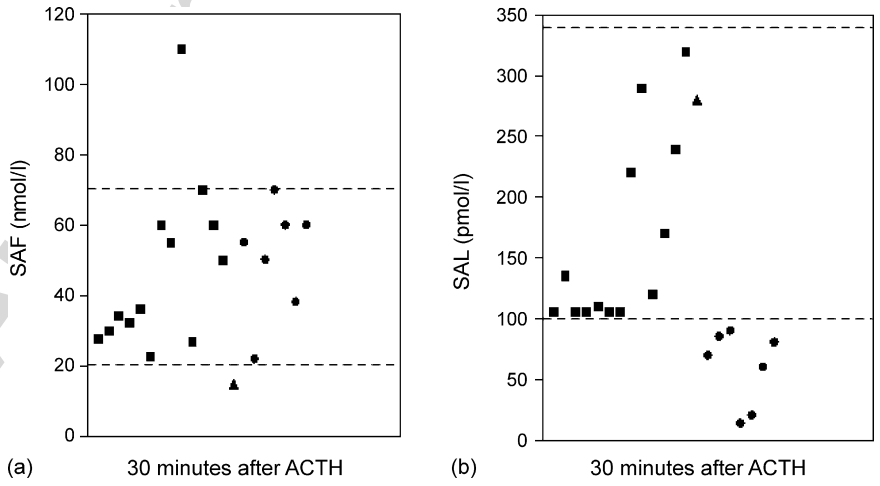

Fig. 2 – Salivary cortisol (a) and salivary aldosterone (b) levels after 30 min of 25 μg of ACTH i.m. (LDT_s) in 21 HIV-infected patients. (■) Normal responders in salivary cortisol and salivary aldosterone, (▲) hyporesponders in salivary cortisol, and (●) hyporesponders in salivary aldosterone. Dotted lines limit the normal range (5th–95th percentiles) of salivary cortisol (SAF) and salivary aldosterone (SAL) values obtained 30 min after LDT_s in 50 healthy subjects.

Table 2 – Salivary cortisol (SAF) and salivary aldosterone (SAL) concentrations in response to 25 μg (LDT_s) and 250 μg (HDT_s) of ACTH (i.m.) in six HIV-infected patients who showed subnormal salivary aldosterone response to LDT_s

	LDT_s^1 30 min after ACTH		LDT_s^2 30 min after ACTH		HDT_s^3 30 min after ACTH	
	SAF (nmol/l)	SAL (pmol/l)	SAF (nmol/l)	SAL (pmol/l)	SAF (nmol/l)	SAL (pmol/l)
HIV^+						
Mean ± S.D.	53 ± 16	60 ± 34	39 ± 14	56 ± 28	45 ± 8	167 ± 49[a]
Range	22–70	13.5–90	22–55	16–85	34–55	120–250

Values of SAF and SAL ranging from 20 to 70 nmol/l and 100 to 340 pmol/l, respectively, at 30 min after LDT or HDT define a normal response.
Abbreviations—HIV^+: patients infected with the human immunodeficiency virus; LDT_s: i.m. low-dose ACTH test; HDT_s: i.m. high-dose ACTH test; SAF: salivary cortisol; SAL: salivary aldosterone. LDT_s^1: low-dose salivary ACTH test performed at the beginning of the study; LDT_s^2: low-dose salivary ACTH test performed 12 months after the initial LDT_s; HDT_s^3: high-dose salivary ACTH test performed 1 week after LDT_s^2.
[a] $p = 0.003$; LDT_s *vs.* HDT_s.

to the primary infection and if the low aldosterone response was ACTH dose dependent.

3.4. *Second dynamic testing*

Six of the seven patients with subnormal aldosterone response to low-ACTH dose agreed to complete the study by performing a second LDT_s followed by HDT_s 12 months after the initial evaluation. In this study, salivary samples for SAL determinations were obtained at 15, 30, 45 and 60 min after LDT_s^2 [22].

Comparison of SAF levels (mean ± S.D.) at 30 min between LDT_s^1 (53 ± 16 nmol/l), LDT_s^2 (39 ± 14 nmol/l), and HDT_s (45 ± 8 nmol/l) was not significantly different ($p = 0.145$).

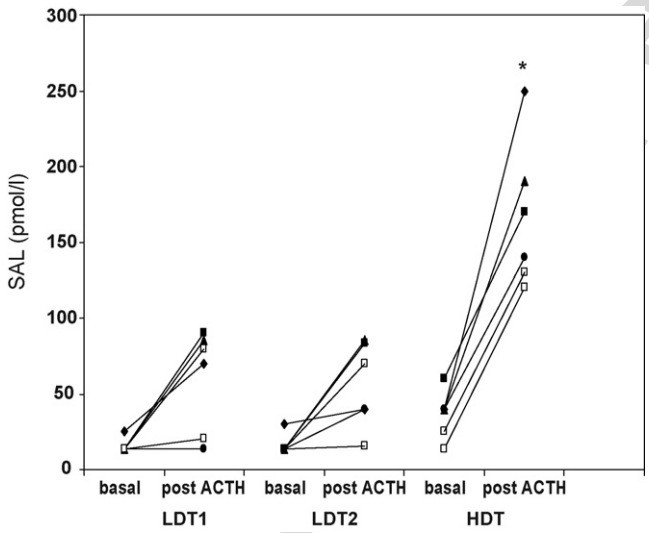

Fig. 3 – Individual levels of salivary aldosterone at baseline and 30 min after the intramuscular injection of ACTH = 25 μg (LDT^1; LDT^2) and 250 μg (HDT) in six HIV-infected patients with subnormal salivary aldosterone response to low- ACTH dose. Values of SAL ranging from 100 to 340 pmol/l at 30 min after LDT or HDT define a normal response. LDT^1 = low-dose salivary ACTH test performed at the beginning of the study; LDT^2 = low-dose salivary ACTH test performed 12 months after the initial LDT_1; HDT = high-dose salivary ACTH test performed 1 week after LDT_2. *SAL values after HDT *vs.* LDT^1 and LDT^2; $p = 0.003$.

SAL levels 30 min after LDT_s^1 and LDT_s^2 were not different ($p = 0.614$, Table 2). However, a significant rise in SAL was found 30 min after HDT_s ($p = 0.003$, Table 2 and Fig. 3) reaching values similar to those found in normal responders. The comparison of SAL increments between the first and the second LDT_s did not show significant differences (40 ± 12 and 44 ± 34 pmol/l; $p = 0.803$). However, after HDT_s SAL increments (130 ± 43 pmol/l) were significantly higher than after both LDT_s^1 and LDT_s^2 ($p = 0.003$). The additional SAL samples obtained at 15, 45 and 60 min after LDT_s^2 demonstrated the absence of a significant steroid rise ($p \geq 0.061$).

Serum electrolytes were normal and renin concentrations were not different from those obtained during the first evaluation (range 0.77–2.4 pmol/l, $p = 0.673$).

4. Discussion

This study performed on non-critically ill HIV-infected outpatients with normal salivary gland function demonstrates that 38% were hyporesponders in either salivary cortisol (*n*:1) or salivary aldosterone (*n*:7) to intramuscular low-dose ACTH stimulation. Basal salivary steroid concentrations correlated positively with circulating levels. However, they were not helpful in defining adrenal hypofunction because they were in the normal range. All patients except one were followed-up after a year, and a reduced aldosterone response was reconfirmed during the LDT_s. Functional impairment of the adrenal gland expressed during the LDT_s was not recognizable after the HDT_s. Although subtle and non-specific clinical symptoms compatible with adrenal insufficiency were found in all HIV^+ patients, there were no evident clinical differences between those with normal ($n = 13$) and subnormal ($n = 8$) steroid responses to LDT_s.

We found a lower prevalence of subclinical selective hypocorticism (5%) than in literature descriptions found on critically ill and hospitalized HIV^+ patients [19–21]. This hypoadrenal state was masked during high-dose ACTH stimulation in agreement with other authors [20,21]. The etiology of the secondary form of partial adrenal insufficiency remained unclear. This patient had normal ACTH levels and pituitary MRI and refused to take the insuline tolerance test challenge. Replacement oral doses with hydrocortisone were indicated, but the patient refused them and left the HIV program.

Abnormalities in aldosterone secretory capacity seemed to be prevalent in this study. A defect in the 17-deoxy steroid pathway described by Membreno et al. [5] was found during LDT_s with preservation of 17-hydroxysteroid pathway. In vitro studies have suggested that elevated ACTH levels overtime induced 17 α-hydroxylase activity in glomerulosa cells thereby shifting steroid biosynthesis from an aldosterone- to a cortisol-producing pathway [30]. This model mimics the physiopathology of the hypoaldosteronism found in severely sick patients who demonstrated high ACTH and cortisol levels. However, this adaptive mechanism was not found in our patients. Neither potassium levels nor renin concentrations were sensitive indicators of glomerulosa hypofunction in this study. Flaws in renin measurements and abnormalities in systemic potassium equilibrium described in HIV patients [31,32] could contribute to hiding the aldosterone deficiency. The longitudinal evaluation in these patients reconfirmed the presence of subnormal aldosterone response to LDT 12 months later, the time suggested by other authors during the follow-up of adrenocortical function in HIV-infected patients [13]. However, mechanisms responsible for this abnormality remain unclear. Associated infections and pathologies found in three AIDS patients were *Cytomegalovirus* + cervix carcinoma ($n = 1$); *Pneumocystis carinii* + *TBC* + *Toxoplamosis* + *Atypical mycobacterium* ($n = 1$); and *Herpes Zoster* ($n = 1$). Two out of four HIV-infected patients had associated hepatitis A (one patient in stage A_2) and hepatitis B and C (one in stage A_3). The remaining two patients (A_1 and A_2) had no associated diseases. Patients were on abacavir, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, stavudine, and zidovudine therapy. None of these drugs has been reported to impair adrenal steroid biosynthesis. Thus, the subnormal aldosterone response to low-ACTH dose was not related to CDC stage, disease duration or previous medical therapy. However, such an impairment merits particular monitoring because it may be a harbinger of a progressive adrenal disorder.

The dynamic assessment of salivary cortisol and salivary aldosterone during the intramuscular low-dose ACTH stimulation is a useful non-invasive approach for early diagnosis of adrenal hypofunction in HIV-infected patients.

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