# Renal tubular dysfunction in patients with American cutaneous leishmaniasis

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**Renal dysfunction seen in patients with American cutaneous leishmaniasis (ACL) has been attributed to the use of antimonials for treatment. To determine whether ACL itself causes tubular dysfunction, we measured renal function in 37 patients with ACL prior to their treatment and compared results to that in 10 healthy volunteers of similar mean age. None of the patients presented with glomerular dysfunction; however, 27 had a urinary concentrating defect. There was no statistical difference between groups in the pre- and post-desmopressin test of urine osmolality, but the post-test urine osmolality of the controls was significantly higher. Urinary AQP2 levels, determined by western blot of isolated exosomes, were found to be significantly lower in patients than in controls, whereas that of the cotransporter (NKCC2) was significantly higher. A urinary acidification defect (post-test pH greater than 5.50 following calcium chloride) was found in 15 patients. Pretest plasma bicarbonate was below normal in 12 patients as was the pretest plasma pH in 14. Expression of the Na/H exchanger (NHE3), $H^+$-ATPase, and pendrin were all significantly higher in patients with ACL than in controls. A combined urinary concentration and acidification defect was found in 12 patients. Thus, the urinary concentrating defect of ACL may be caused by decreased AQP2, with increased NKCC2 compensatory. Pendrin upregulation may be related to the urinary acidification defect with increased NHE3 and $H^+$-ATPase also compensatory. Hence, ACL can cause asymptomatic renal tubular dysfunction.**

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Leishmaniasis is an infectious, non-contagious, zoonotic disease caused by intra-macrophage protozoa of the genus *Leishmania*, which are transmitted by a vector belonging to the family *Psychodidae*.[1] The disease has a wide geographical distribution, occurring on nearly every continent. It is endemic in tropical and subtropical regions, such as North-east Africa and the Middle East, as well as parts of Europe, Central America, and South America.[2]

There are three clinical types of leishmaniasis: visceral (kala-azar), cutaneous, and mucosal. The parasite proliferates into the mononuclear phagocytic system, dermis, and nasopharyngeal mucosa, respectively.[1]

The cutaneous form is known as American cutaneous leishmaniasis (ACL). Eight countries account for ~90% of all cases of ACL: Afghanistan, Pakistan, Syria, Saudi Arabia, Nigeria, Iran, Brazil, and Peru.[3] Traditionally, ACL has been classified as Old-World leishmaniasis (species found in the Mediterranean region, the Middle East, and Africa) or New-World leishmaniasis (species found in Mexico, Central America, and South America), the virulence, pathogenicity, and clinical manifestations depending on the species involved.[4]

Studies of visceral leishmaniasis (VL) have demonstrated renal involvement ranging from nonspecific abnormalities on urinalysis to various types of glomerulonephritis.[5–8] In animal and human studies of VL, acute kidney injury (AKI) has been attributed to a number of mechanisms: interstitial nephritis secondary to the deposition of parasite antigens in the renal interstitium;[9] hypersensitivity to antimonials;[10,11] and deposition of immune complexes, leading to rapidly progressive glomerulonephritis.[12] In the presence of leishmaniasis, AKI increases mortality.[13] Renal tubular dysfunction has also been described in VL, manifesting as urinary concentrating defect, with or without urinary acidification defect. In a prospective study of 50 patients with VL, urinary concentrating defect was found in 68%, urinary acidification defect was found in 64%, and complete renal tubular acidosis was found in 30%.[14] Another prospective study of

VL patients demonstrated proximal tubular dysfunction, as determined by urinary levels of retinol-binding protein, in nearly half of the patients.[6]

In the literature, there are very few data regarding renal dysfunction in ACL, and the renal abnormalities described have been associated with the use of pentavalent antimonials.[10] Patients with ACL can develop AKI due to massive deposition of immune complexes that appear after the destruction of the parasites (Leishman–Donovan bodies), a phenomenon similar to the Herxheimer reaction.[15] Sampaio *et al.*,[16] in a prospective study of 11 patients with ACL, who received a high daily dose of an antimonial (40 mg/kg body weight (BW)) for 30 days, observed one case of AKI.[16] The authors found that, after 30 days of treatment, eight patients showed a reduction in the glomerular filtration rate (GFR), accompanied by tubular dysfunction, together with a reduction in urinary concentrating ability and an increase in the fractional excretion of sodium.[16]

When administered at low doses and for a short period, antimonials have low renal toxicity. However, in the treatment of ACL, it is often necessary to use higher doses for a longer period, which can increase toxicity. There are also reports of proteinuria and AKI in patients with ACL.[10,16–18] In an experimental animal study,[19] high doses of an antimonial were shown to result in impaired urinary concentrating ability without a reduction in GFR. This abnormality was attributed to inhibition of antidiuretic hormone secretion and to a direct effect of the drug on tubular cells. In some animals, high doses of the antimonial also caused a reduction in the GFR.[19]

To determine whether ACL itself causes tubular dysfunction, we studied the renal function of 37 ACL patients before antimonial treatment.

## RESULTS

All of the 59 patients recruited had previously been diagnosed with ACL on the basis of epidemiological, clinical, biochemical, and histopathological findings. Of those 59 patients, 22 were excluded: eight for testing negative on a new histopathological exam; nine for being under 15 years of age or over 60 years of age; two for subsequently declining to participate in the study; one for having hypertension; one for having diabetes mellitus; and one for having used an antimonial (meglumine antimoniate) within the last 30 days. Therefore, the study group comprised 37 ACL patients.

The mean age was 35.6 ± 12 years in the study group and 32.2 ± 11.7 years in the control group. In the study group, 19 (51.4%) were male. Twenty-two (59.5%) of the patients tested positive on the Montenegro skin test. All 37 patients had isolated skin lesions: 27 had only one lesion; 7 had two to four lesions; and 3 had more than four lesions. The mean duration of disease was 28.5 ± 20.6 days (range, 7–90 days). Demographic and clinical data are shown in Table 1.

In comparison with the controls, none of the patients had significant glomerular dysfunction (109.6 ± 32 vs 116.3 ± 23 ml/min per 1.73 $m^2$, $P = 0.694$). None of the patients presented a GFR of <60 ml/min.

**Table 1 | Demographic and clinical characteristics, by group**

| Characteristic | ACL (*n*=37) | Control (*n*=10) | *P* [a] |
|---|---|---|---|
| Age (years), mean ± s.d. | 35.6 ± 12 | 32.3 ± 11.7 | 0.442 |
| *Gender, n (%)* | | | |
| Male | 19 (51.4) | 6 (60) | 0.73 |
| Female | 18 (48.6) | 4 (40) | |
| Duration of disease (days), mean ± s.d. | 28.5 ± 20.6 | — | — |
| Montenegro skin test (±), *n* | 22/37 | — | — |
| *Number of skin lesions, n (%)* | | | |
| 1 | 27 (72.9) | — | — |
| 2–4 | 7 (18.9) | — | — |
| >4 | 3 (8.1) | — | — |
| Systolic blood pressure (mm Hg), mean ± s.d. | 122 ± 10 | 117 ± 9.5 | 0.221 |
| Diastolic blood pressure (mm Hg), mean ± s.d. | 80 ± 4.7 | 75 ± 8.5 | 0.079 |

Abbreviation: ACL, American cutaneous leishmaniasis.
[a]Student's *t*-test.

### Tubular defects

Post-desmopressin test urinary concentrating ability was lower in ACL patients than in controls. Urinary concentrating defect, defined as a urine osmolality ($U_{osm}$)/plasma osmolality ($P_{osm}$) ratio <2.8, was observed in 27 patients (77%). Although pre- and post-desmopressin test values of $U_{osm}$ were comparable in the study group, $U_{osm}$ was significantly lower in the patients than in the controls (618 ± 202 vs 985 ± 81, $P<0.05$).

Urinary acidification defect, defined as the inability to reduce urinary pH ($U_{pH}$) to <5.50 after $CaCl_2$ administration, was observed in 15 patients (40.5%). Although there was no significant difference between patients and controls in terms of the mean plasma bicarbonate concentration, the pre-$CaCl_2$ plasma bicarbonate concentration was <22 mEq/l in 12 of the 15 patients with urinary acidification defect.

Combined urinary concentration and acidification defect was observed in 12 patients. Only five patients had no defect in urinary concentration or acidification. Figure 1a and b illustrate $U_{osm}$ and $U_{pH}$ before and after the test with desmopressin and $CaCl_2$ in the ACL patients.

Comparing the patients who presented tubular (concentration or acidification) defects with the remaining patients, we found no differences in relation to age, gender, duration of disease, number of skin lesions, Montenegro skin test positivity, or body mass index. We detected no abnormalities in relation to plasma concentrations or fractional excretions of sodium, potassium, calcium, or phosphorus. Comparing the ACL patients with the controls, we found that

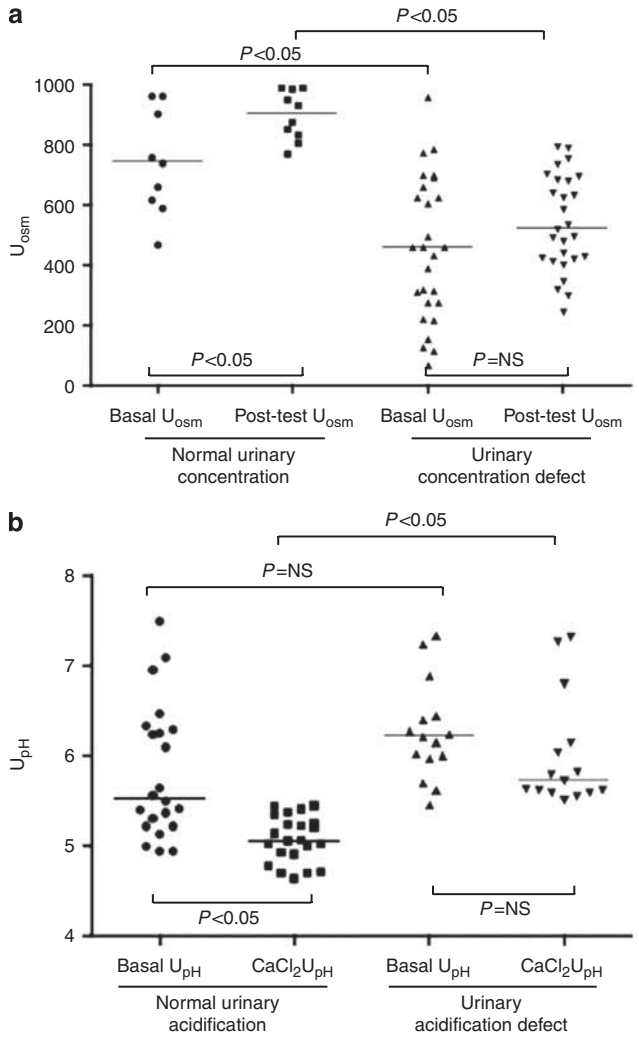


**Figure 1 | Urine concentration and acidification capacity in ACL patients pre- and post-desmopressin test and pre- and post-$CaCl_2$ test.** (**a**) $U_{osm}$ pre- and post-desmopression test in ACL patients with normal or abnormal urinary concentration. (**b**) $U_{pH}$ pre- and post-$CaCl_2$ test in ACL patients with normal or abnormal urinary acidification. ACL, American cutaneous leishmaniasis; NS, nonsignificant; $U_{osm}$, urine osmolality; $U_{pH}$, urinary pH.

the former showed higher urinary fractional excretion of calcium, potassium, and magnesium (Table 2). However, we found no difference between the groups in terms of serum electrolyte levels. It is noteworthy that plasma magnesium levels differed only between the patients with urinary acidification defect and those without ($2.15 \pm 0.06$ vs $2.33 \pm 0.04$, $P = 0.02$).

Microalbuminuria, defined as an albumin/creatinine ratio $>30$ mg/g, was detected in 12 (35.3%) of 34 patients tested (Table 2). Two patients had microalbuminuria alone, nine had microalbuminuria and urinary concentration defect, and another had urinary acidification defect alone. None of the patients with urinary acidification defect and urinary concentration defect had an albumin/creatinine ratio $>30$ mg/g.

**Table 2 | Renal function, by group**

| Variable | ACL ($n$=37), mean ± s.d. | Control ($n$=10), mean ± s.d. |
|---|---|---|
| $P_{creat}$ (mg/dl) | 0.81 ± 0.16 | 0.85 ± 0.18 |
| CrCl (ml/min per 1.73 $m^2$) | 109.6 ± 31.5 | 116.4 ± 22.7 |
| Post-DT $U/P_{osm}$ | 2.19 ± 0.73 | 3.47 ± 0.33* |
| Post-$CaCl_2$ $U_{pH}$ | 5.45 ± 0.64 | 4.82 ± 0.20* |
| Post-DT $U_{osm}$ (mOsm/kg $H_2O$) | 618 ± 202 | 965 ± 81* |
| FENa (%) | 1.15 ± 0.74 | 0.73 ± 0.39 |
| FEK (%) | 10 ± 6.6 | 7.50 ± 2.8* |
| FECa (%) | 1.07 ± 0.72 | 0.62 ± 0.34* |
| FEP (%) | 10.9 ± 9.98 | 9.10 ± 6.4 |
| FEMg (%) | 1.81 ± 1.70 | 0.90 ± 0.40* |
| Albumin/creatinine ratio (mg/g) | 23.6 ± 26 | 6.12 ± 4.06* |

Abbreviations: ACL, American cutaneous leishmaniasis; $CaCl_2$, calcium chloride test; CrCl, creatinine clearance; DT, desmopressin test; FE, fractional excretion; $P_{creat}$, plasma creatinine; $U/P_{osm}$, urinary/plasma osmolality ratio; $U_{osm}$, urinary osmolality; $U_{pH}$, urinary pH.
*$P < 0.05$ (Student's $t$-test; Mann–Whitney test).

### Urinary exosomes

As can be seen in Figure 2a, urinary protein expression of the collecting duct water channel aquaporin 2 (AQP2) was significantly lower in the patients than in the controls ($38.5 \pm 12$ vs $99.5 \pm 0.5\%$, $P = 0.006$). Figure 2b shows that the expression of Na-K-2Cl cotransporter (NKCC2) was significantly higher in the patients than in the controls ($147 \pm 12$ vs $102 \pm 2.5\%$, $P = 0.02$). In addition, Figures 3, 4a and b show that urinary protein expression of the Na/H exchanger (NHE3), $H^+$-ATPase, and pendrin, respectively, was significantly higher in the patients than in the controls ($176 \pm 15$ vs $100 \pm 0.6\%$, $P = 0.015$; $190 \pm 8$ vs $98 \pm 0.2\%$, $P = 0.04$; and $176 \pm 15$ vs $100 \pm 0.6\%$, $P = 0.015$).

## DISCUSSION

Here, we have demonstrated that ACL can cause tubular dysfunction without a drop in GFR. Our findings also indicate that, in patients with ACL, reduced expression of AQP2 leads to impaired urine concentrating ability. In addition, ACL patients showed a marked increase in the urinary protein expression of NKCC2.

In this study, ACL was associated with dysregulation of major acid–base transporters in the proximal tubule (NHE3) and the distal nephron ($H^+$-ATPase and pendrin). Therefore, ACL can impair the ability of the kidneys to respond appropriately to an acute acid load.

Although it has been shown that patients with VL develop renal dysfunction,[5–8] there are few data regarding such dysfunction in patients with ACL. Studies of ACL have attributed renal dysfunction to the use of pentavalent antimonials.[10] However, in this study, we showed that, before antimonial treatment, patients with ACL had asymptomatic renal tubular dysfunction, as evidenced by their inability to concentrate and acidify urine, albeit without glomerular dysfunction.

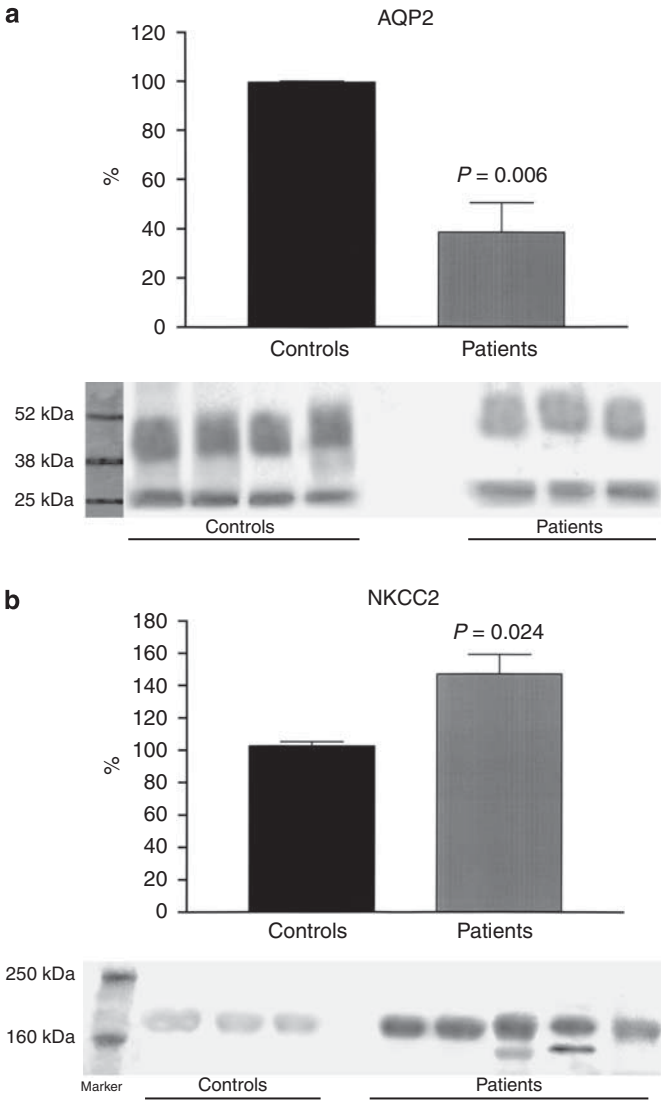


**Figure 2 | Western blotting analysis in the urinary exosome fraction, normalized to urinary creatinine, in controls and in patients with ACL.** Abundance of (**a**) AQP2 and (**b**) NKCC2. ACL, American cutaneous leishmaniasis; AQP2, aquaporin 2; NKCC2, Na-K-2Cl cotransporter.

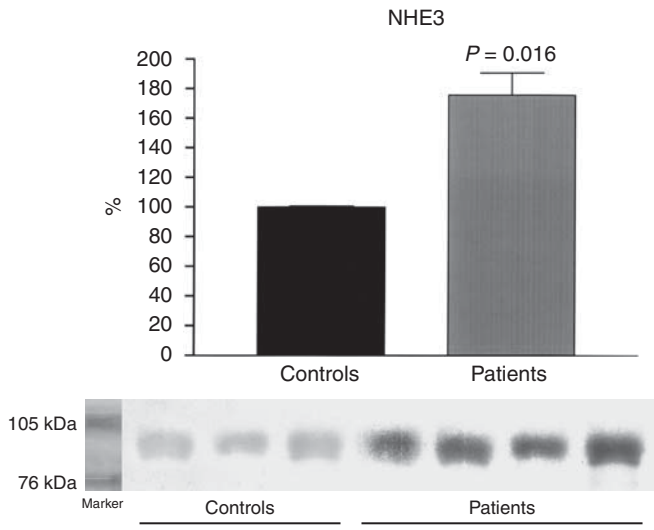


**Figure 3 | Abundance of NHE3 by western blotting analysis in the urinary exosome fraction, normalized to urinary creatinine, in controls and in patients with ACL.** ACL, American cutaneous leishmaniasis; NHE3, Na/H exchanger.

Because the development of ACL depends solely on the level of exposure to the vector, it can occur at any age.[1] The mean age of the patients studied by Lima Verde *et al.*[14] was 29 ± 11 years, compared with 36 ± 15 years for those studied by Oliveira *et al.*[13] The mean age of our study sample was 35.6 ± 12 years. Therefore, patient age cannot be considered a source of bias in this study.

Among our patients, the mean duration of disease was 28.5 ± 20.6 days (range, 7–90 days), which is not characteristic of chronic exposure to highly antigenic material. In a previous retrospective study of 151 patients with ACL, 97.2% tested positive on the Montenegro skin test, higher than the 59.0% observed in our study.[20] In our study sample, 72.9% of the patients had only one skin lesion, compared with 69.5% in a study conducted by Schubach *et al.*[20] In studies involving only patients with localized disease, Montenegro skin test positivity has been shown to be 82–89%.[21,22] The test does not distinguish recent infection from past infection, and its importance in endemic areas is questionable.

Various studies of VL have reported glomerular involvement. In a prospective study of 50 patients with VL, Lima Verde *et al.*[14] observed that 14 (28%) had a reduction in GFR of $<80$ ml/min per 1.73 $m^2$. In another study of VL patients, AKI was observed in 76 (33.9%) of the 224 patients evaluated.[13] Reports of glomerular dysfunction in ACL have been associated with the use of pentavalent antimonials.[10] Other infectious diseases that primarily affect the skin and nerves, such as leprosy, can also lead to glomerular dysfunction. In a prospective study of 59 patients with leprosy, Oliveira *et al.*[23] observed a reduction in GFR in 50% of the cases. However, in our study sample, no significant GFR abnormality was observed, suggesting that ACL, *per se*, does not cause glomerular dysfunction.

There is considerable evidence that antimonial treatment causes impaired urine concentrating ability in ACL. In a study involving 11 ACL patients treated with meglumine antimoniate at a daily dose of 40 mg/kg BW (double dose) for 30 days, Sampaio *et al.*[16] found that 8 of the patients had impaired urine concentrating ability after 16 h of water deprivation. Veiga *et al.*[24] reported that five ACL patients treated with a conventional dose, but for a longer period, developed an inability to concentrate urine after treatment.[24] Lima Verde *et al.*[14] reported impaired post-desmopressin test urine concentrating ability in 68% of the 50 VL patients studied.[14] In an experimental study with toad bladder, which has a phylogenetic structure similar to the renal collecting

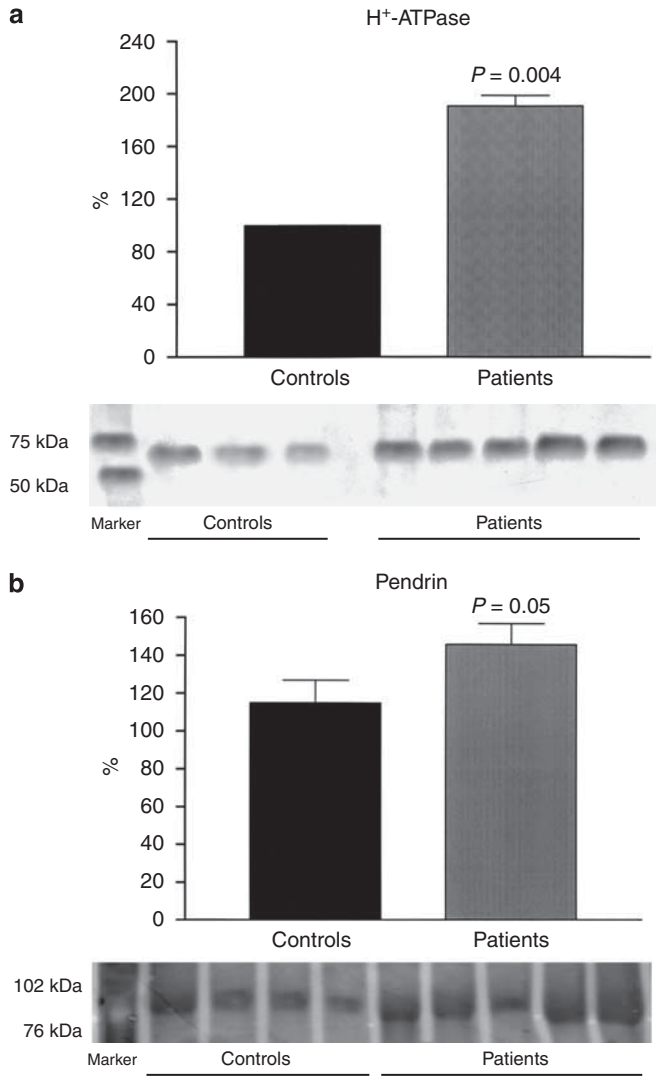


**Figure 4 | Western blotting analysis in the urinary exosome fraction, normalized to urinary creatinine, in controls and in patients with ACL.** Abundance of (**a**) $H^+$-ATPase and (**b**) pendrin. ACL, American cutaneous leishmaniasis.

tubule, it was demonstrated that antimonial administration inhibits osmotic stimulated water flow.[25] In an experimental study with rats receiving the antimonial sodium stibogluconate at a daily dose of 30 mg/100 g for 30 days, there was post-treatment tubular abnormality, without glomerular dysfunction. However, when a dose of 200 mg/100 g was administered, there was significant glomerular dysfunction.[19] Nevertheless, in our sample of ACL patients evaluated before treatment, we demonstrated that $U_{osm}$ was significantly reduced, indicating that ACL, in and of itself, can impair urine concentrating ability.

According to the literature on leishmaniasis, urinary acidification defect is less common than is urinary concentrating defect. In a study of patients with VL treated with the standard dose of meglumine antimoniate, 64% showed an inability to reduce $U_{pH}$ to $<5.50$ after the $CaCl_2$ test.[19] Although the patients evaluated in this study had ACL rather than VL, we identified an inability to acidify urine in 46%, calling into question the role that antimonial treatment has in the development of tubular dysfunction in leishmaniasis.

It has been demonstrated that, in patients with VL, hypomagnesemia is accompanied by an increase in urinary excretion of magnesium.[26] In ACL patients, we demonstrated an increase in the fractional excretion of magnesium. However, we found that serum levels of magnesium were depressed only in patients with urinary acidification defect. We speculate that the tubular defects seen in VL are also present in ACL, although they are probably less common in the latter.

There have been few studies evaluating microalbuminuria in leishmaniasis. In one study of patients with VL, 81.8% presented microalbuminuria.[6] In a study of patients with multibacillary leprosy, Oliveira *et al.*[23] identified microalbuminuria in 8.5%. In this study, microalbuminuria was identified in 35% of the ACL patients evaluated. Microalbuminuria is a widely accepted marker of incipient diabetic nephropathy. However, further studies are needed in order to determine the importance of microalbuminuria for the early detection of glomerular dysfunction in infectious diseases. Of the 15 patients with urinary acidification defect, only 1 had microalbuminuria, suggesting a separate process.

Exosomes containing vesicular membranes and intracellular fluid are normally secreted into the urine from all nephron segments. Those exosomes contain proteins that can be altered, in terms of their abundance or physical properties, as a consequence of various renal diseases. A number of studies have demonstrated that AQP2 is excreted in urine through small membranous vesicles and that its abundance in the urine is correlated with its expression in renal tissue.[27–29] After the discovery of AQP2, other urinary transporters were identified and came to be used in the study of tubular dysfunction.[30] It has been demonstrated that most membrane transporters are naturally expressed as high-molecular-weight complexes and can be identified in urine.[31] The collection and analysis of urine samples is an ideal non-invasive means of evaluating biomarkers for the diagnosis of renal diseases, because urine contains exosomes with membrane and intracellular proteins from all segments of the nephron.[32,33] Among the relevant transporters are AQP2, NHE3, NKCC2, $H^+$-ATPase, and pendrin.[34] In this study, a high percentage of the patients had urinary concentrating defect, as evidenced by reduced urinary expression of AQP2. This was accompanied by increased expression of NKCC2, which could represent a compensatory mechanism. Located in the proximal tubule, the NHE3 isoform is the principal agent of bicarbonate generation and reabsorption. One-third of the renal cortical collecting duct cell population comprises intercalated cells, which are ultimately responsible for regulating the acid–base balance.[35] In response to acid or alkali loading, cortical collecting duct cells reabsorb or secrete more bicarbonate, respectively. This is accomplished through the coordinated activity of two types of intercalated cells:

α-intercalated cells, which are able to secrete acid through an apical $H^+$-ATPase and basolateral $Cl^-/HCO_3^-$ exchanger; and β-intercalated cells, which are able to secrete bicarbonate through an apical $Cl^-/HCO_3^-$ exchanger and a basolateral $H^+$-ATPase. Pendrin is an apical $Cl^-/HCO_3^-$ exchanger in β-intercalated cortical collecting duct cells. It has been shown that metabolic acidosis decreases the activity of the apical pendrin in β-intercalated cells of the rat cortical collecting duct by reducing the overall expression of pendrin.[36,37] Mohebbi *et al.*[38] showed that protein expression of pendrin increases during NH4CL loading in FK506 rats, altering the expected renal response to acidosis. In this study, we demonstrated that urinary expression of pendrin is increased in the urinary exosomes of patients with ACL. The increase in pendrin expression might be the key mechanism of urinary acidification defect. However, we cannot explain why the expression of this protein is increased in ACL. Further studies, in humans and in animal models, are warranted in order to confirm this hypothesis. We also found that there was an increase in NHE3 and $H^+$-ATPase expression in urinary exosomes. It is possible that the marked increase in NHE3 and $H^+$-ATPase expression presented by the ACL patients represents a compensatory response to the increase in pendrin expression and consequently to the urinary acidification defect. However, we cannot rule out the possibility that other transporters are involved in ACL-related metabolic acidosis.

We can conclude that ACL can cause asymptomatic renal tubular dysfunction, manifesting as the inability to concentrate and acidify urine, without impairing glomerular filtration. The analysis of urinary transporters was useful in the detection of dysfunction at the molecular level. Further studies, involving larger patient samples and including other infectious diseases, are needed in order to confirm our findings.

## MATERIALS AND METHODS

### Participants

This was a prospective study of 37 patients with a confirmed diagnosis of ACL (based on epidemiologic, clinical, and biochemical findings), recruited from among those treated between July 2008 and July 2009 at a public health facility in the city of Barbalha, Brazil. The following exclusion criteria were applied: being under 15 years of age or over 60 years of age; having used pentavalent antimonials within the last 30 days; presenting with hypertension (systolic blood pressure $\geqslant$140 mm Hg or diastolic blood pressure $\geqslant$90 mm Hg); having diabetes mellitus; having a urinary tract infection; having systemic lupus erythematosus or another collagenosis; and having a history of renal disease. All patients were scheduled to undergo standard treatment with the antimonial meglumine antimoniate (Glucantime) at a daily dose of 20 mg/kg of BW for 20 days. All tests were conducted before the initiation of the treatment. We also recruited a group of 10 healthy volunteers to be evaluated as controls.

The study protocol was approved by the Research Ethics Committee of the Federal University of Ceará School of Medicine Walter Cantídio University Hospital, located in the city of Fortaleza, Brazil. All participants gave written informed consent.

### Diagnosis

The diagnosis of ACL was confirmed on the basis of epidemiological and clinical criteria, as well as on the results of the Montenegro skin test and the identification of parasites in a tissue biopsy.

### Clinical and biochemical characteristics

At the time of the medical consultation, all symptoms and signs were evaluated. We also collected data related to race, age, gender, history of chronic diseases, number of skin lesions, duration of disease, use of drugs, body mass index, blood pressure, Montenegro skin test results, and ACL classification. The following laboratory tests were conducted in blood or plasma: urea, creatinine, pH, bicarbonate, sodium, potassium, chloride, magnesium, calcium, phosphorus, albumin/globulin, amylase, fasting glucose, and erythrocyte sedimentation rate. In urine samples, we evaluated the following: creatinine, urea, sodium, potassium, chloride, calcium, phosphorus, magnesium, microalbuminuria, urinalysis results, $U_{osm}$, and $U_{pH}$.

### Renal function

We estimated GFR using the Cockroft and Gault formula, defining abnormal GFR as $\leqslant$90 ml/min per 1.73 $m^2$. In an isolated urine sample collected before the concentration and acidification tests, sodium, potassium, chloride, and magnesium were measured, and the albumin/creatinine ratio was determined.

All patients underwent food and water deprivation for 12 h. We calculated fractional excretions (sodium, potassium, calcium, phosphorus, and magnesium) using the standard formula. Microalbuminuria was normalized to urinary creatinine.

We measured $U_{osm}$ before and 4 h after intranasal administration of desmopressin acetate (20 μg/kg BW).[39,40] Urinary concentrating ability was evaluated by determining the urinary/plasma osmolality ratio ($U/P_{osm}$) after 12 h of water deprivation.

Urinary acidification was evaluated by measuring $U_{pH}$ before and after oral administration of $CaCl_2$ (2 mEq/kg BW).[41] Urinary acidification defect was defined as a post-test $U_{pH} > 5.50$.

### Analytical methods

Urea concentration was determined by colorimetric-enzymatic assay (Labtest, Lagoa Santa, Brazil), and the results are expressed in mg/dl. Plasma and urinary levels of creatinine were determined by colorimetric (picric acid) assay (Labtest) and by the Bonsnes–Taussky method, respectively, and the results are expressed in mg/dl. Plasma and urinary sodium and potassium were determined by photometry with a flame photometer (B462; Micronal, São Paulo, Brazil), and the results are expressed in mEq/l. Albumin and globulin were determined by bromocresol reaction (Labtest), and the results are expressed in g/dl. Glucose was determined by the colorimetric glucose oxidase method (Labtest), and the results are expressed in mg/dl. Alkaline phosphatase was determined by the modified Bowers and McComb (phosphatase kinetics) method (Labtest), and the results are expressed in U/l. Amylase was determined by the modified Caraway colorimetric method, and the results are expressed in U/dl. We determined plasma pH and bicarbonate ($HCO_3^-$) using a blood gas analyzer (Rapidlab 238; Chiron Diagnostics, Bayer, Tarrytown, NJ), and the results for bicarbonate are expressed in mEq/l. We determined $U_{pH}$ using digital pH-metry (pG1000; Gehaka, São Paulo, Brazil). Urinary osmolality was determined in a vapor pressure osmometer (5100C; Wescor, Logan, UT), and the results are expressed in mOsm/kg $H_2O$. Urinalysis (to determine glucose, urobilin, bilirubin, ketone,

protein, nitrite, leukocytes, hemoglobin, pH, and density) was performed with reagent strips (Inlab URI-TEST; Mettler-Toledo, Giessen, Germany). Urothelial cells, leukocytes, erythrocytes, and bacteria were identified in urinary sediment under light microscopy (magnification, $\times 400$). Microalbuminuria was identified through immunoturbidimetry with a commercial kit (Tina-quant; Roche, Mannheim, Germany), and the results are expressed in mg of albumin/g creatinine.

### Exosome sample processing

Urine samples were collected from the subjects (patients, $n = 10$; controls, $n = 5$) after overnight (12 h) water deprivation. The samples were placed into sterile 50-ml plastic tubes and immediately stored in liquid nitrogen.

The samples were taken out of cold storage and placed on ice. As the samples thawed, protease inhibitors (protease inhibitor cocktail; Sigma, St Louis, MO) were added at a concentration of 10 µl/ml of urine and the samples were subjected to extensive vortexing. The mixed sample was then centrifuged at 17,000 *g* for 15 min at 4 °C to remove whole cells, large membrane fragments, and other debris. The supernatant was centrifuged at 200,000 *g* for 1 h at 4 °C to obtain low-density membrane pellets. The pellets were suspended in isolation solution (200 mmol/l Mannitol, 80 mmol/l HEPES, and 41 mmol/l KOH, pH 7.5) with protease inhibitors. All of the urinary protein samples were stored at −80 °C until use.[42,43]

### Electrophoresis and immunoblotting

Samples of membrane fractions were run either on 12.5% polyacrylamide minigels (for AQP2) or on 10% polyacrylamide minigels (for NHE3, NKCC2, H + -ATPase, and pendrin). Loading amounts for gel electrophoresis were adjusted to equalize urinary creatinine concentration. After transfer by electroelution to nitrocellulose membranes (PolyScreen, PVDF Transfer; Life Science Products, Boston, MA), blots were blocked with 5% milk and 0.1% Tween 20 in PBS (sodium chloride 8.7 g/l, dibasic phosphate 7.2 mmol/l, and monobasic phosphate 2.8 mmol/l) for 1 h. Blots were then incubated with one of the following: anti-AQP2 antibody (1:2000), NKCC2 antibody (0.12 µg/ml), NHE3 antibody (1:500), $H^+$-ATPase (1:500), or pendrin (1:1000). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody via the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Bands corresponding to protein expression of AQP2, NHE3, NKCC2, H + -ATPase, and pendrin were quantified by densitometric analysis using Image Master VDS (Pharmacia Biotech, Buckinghamshire, UK) and are expressed as percentages of control.

### Antibodies

We obtained the peptide-derived polyclonal antibodies specific to AQP2, NKCC2, NHE3, and pendrin from Santa Cruz Biotechnology (Santa Cruz, CA).

The peptide-derived polyclonal antibody specific to V1ATPase B1 subunit was kindly supplied by Dr Søren Nielsen (Institute of Anatomy, University of Aarhus, Denmark).

### Statistical analysis

All quantitative data are expressed as mean ± s.e.m. The normality of data distribution to quantitative variables was evaluated by Shapiro–Wilk test. Differences between two parameters were analyzed either by paired *t*-test or by nonparametric methods (Mann–Whitney test). Values of $P < 0.05$ were considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

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