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RESN-D-20-01322R2

Urinary cytology: a potential tool for differential diagnosis of acute kidney injury in patients with nephrotic syndrome

BMC Research Notes

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RESN-D-20-01322R1

Urinary cytology as a tool for differential diagnosis between acute tubular necrosis and proliferative glomerulonephritis in patients with nephrotic syndrome and acute kidney injury

BMC Research Notes

Dear Dr Akrom,

Thank you very much for your review of manuscript RESN-D-20-01322R1, 'Urinary cytology as a tool for differential diagnosis between acute tubular necrosis and proliferative glomerulonephritis in patients with nephrotic syndrome and acute kidney injury'.

We greatly appreciate your assistance.

Best wishes,

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BMC Research Notes

Urinary cytology as a tool for differential diagnosis between acute tubular necrosis and proliferative glomerulonephritis in patients with nephrotic syndrome and acute kidney injury

--Manuscript Draft--

Manuscript Number:	RESN-D-20-01322
Full Title:	Urinary cytology as a tool for differential diagnosis between acute tubular necrosis and proliferative glomerulonephritis in patients with nephrotic syndrome and acute kidney injury
Article Type:	Research note
Abstract:	Objective: Acute tubular necrosis (ATN) is a frequent cause of acute kidney injury (AKI). In patients with nephrotic syndrome (NS), AKI demands the differential diagnosis between ATN and rapidly progressive glomerulonephritis. In some cases, conclusive diagnosis is possible only by kidney biopsy. We aimed to study the potential use of urine cytology in the differential diagnosis between ATN and proliferative glomerular lesion in patients with NS. Results: Cell size analysis showed a higher proportion of small cells and a lower proportion of large cells in the urine of patients with AKI. Cells phenotypes were easily defined using cytological preparations. Leukocytes were found to be a primary classifier of NS groups, with higher number in patients with AKI and patients with proliferative glomerular lesions. Our data suggests that urinary cytology can be readily performed and support the differential diagnosis between proliferative glomerular lesion and ATN in patients with NS and AKI. Keywords: Acute tubular necrosis; glomerulonephritis; cytodiagnosis; acute kidney injury

Title page

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Urinary cytology as a tool for differential diagnosis between acute tubular necrosis and proliferative glomerulonephritis in patients with nephrotic syndrome and acute kidney injury

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Abstract

Objective: Acute tubular necrosis (ATN) is a frequent cause of acute kidney injury (AKI). In patients with nephrotic syndrome (NS), AKI demands the differential diagnosis between ATN and rapidly progressive glomerulonephritis. In some cases, conclusive diagnosis is possible only by kidney biopsy. We aimed to study the potential use of urine cytology in the differential diagnosis between ATN and proliferative glomerular lesion in patients with NS. **Results:** Cell size analysis showed a higher proportion of small cells and a lower proportion of large cells in the urine of patients with AKI. Cells phenotypes were easily defined using cytological preparations. Leukocytes were found to be a primary classifier of NS groups, with higher number in patients with AKI and patients with proliferative glomerular lesions. Our data suggests that urinary cytology can be readily performed and support the differential diagnosis between proliferative glomerular lesion and ATN in patients with NS and AKI.

Introduction

Acute tubular necrosis (ATN) is a leading cause of acute kidney injury (AKI) in hospitalized patients [1]. The prevalence of AKI also correlates with ATN severity in patients with nephrotic syndrome (NS) [2]. Emergence of AKI in patients with NS requires the differential diagnosis between ATN alone and glomerular proliferative lesions such as in crescentic glomerulonephritis [3] since the therapeutic approach differs between these conditions. Whereas proliferative glomerulopathies require immediate immunosuppression to avoid progression to end-stage renal disease, ATN requires support treatment without immunosuppression avoiding potential side effects [4]. Urinary sediment analysis has been used in the diagnosis of ATN and in the differential diagnosis between isolated ATN and proliferative glomerular lesion in patients with AKI [5,6]. The presence of renal tubular epithelial cells in the urinary sediment has been considered indicative of ATN, and high number of leukocytes has been considered indicative of glomerular lesions [5,6,7]. However, cell identification in unstained urine sediment can be difficult, particularly in conditions of glomerular lesions associated with nephrotic syndrome, where the urinary sediment may be complex. Furthermore, studies associating urinary sediment cytological findings with kidney histology are lacking. In this work, we compare the urinary cytology with the histological presentation of the kidney in biopsy of patients with NS and AKI. To avoid inconsistent cell identification, we used conventional

3

77 staining techniques used in cytology. Our data suggest that

78 conventionally stained urine cytology can be a rapid, consistent and easy-

79 to-use tool for supporting the differential diagnosis between proliferative

80 glomerular lesion and ATN in patients with NS and AKI. We propose a

81 cross-validation of this model to support larger studies for validation of

82 the test in the bedside.

83 ## Main text

84 **Methods**

85 *Patients:* A prospective cross-sectional study including 27 patients with

86 NS subjected to renal biopsy for glomerular disease diagnosis in referral

87 hospitals of Salvador, Brazil, from July 2013 to April 2015. The biopsies

88 were examined at the Fundação Oswaldo Cruz, Instituto Gonçalo Moniz

89 in Salvador, Brazil. Cases were excluded if the renal biopsy contained less

90 than 7 glomeruli, if the estimated interstitial fibrosis encompassed 30%

91 or more of the cortical area, if the patient had diabetes mellitus or

92 infections. The patients were allocated into 3 groups: PRO - 8 patients

93 with proliferative glomerulopathy, ATN - 10 patients with ATN without

94 proliferative glomerulonephritis and Non-ATN - 9 patients without ATN

95 or proliferative glomerular lesions. AKI was defined using the KDIGO

96 criteria. Ten healthy volunteers were used as a reference group of normal

97 urine cytology.

98 *Clinical data:* The following data were obtained from biopsy request forms

99 and by anamneses: age, sex, serum creatinine, albumin, cholesterol, 24-

100 hour urine proteinuria and diagnosis of systemic arterial hypertension.

101 *Histological analysis:* The renal specimens were obtained by 102 percutaneous biopsies, fixed in Bouin's solution or acid formalin, paraffin 103 embedded, cut into 2-µm thick sections, and stained with hematoxylin 104 and eosin. All the slides were reviewed by a pathologist (WLCS). The 105 intensity of ATN was estimated as a percentage of the renal cortex by 106 visual assessment. The following tubular changes were considered as 107 evidence of either current or recent ATN: tubular dilatation, thinning of 108 the tubular epithelium, cellular casts, interstitial edema, and the 109 evidence of epithelial regeneration (hyperchromatic nucleus, mitosis, and 110 binucleation). The percentage of cortical tubulointerstitial fibrosis was 111 estimated by visual assessment.

112 *Cytology analysis:* Fresh urine was obtained from the patients by 113 spontaneous voiding before renal biopsy. Ten milliliters of urine were 114 centrifuged at 2,000 *g* per 10 min in a standard centrifuge. The 115 supernatant was removed by suction. The sediment was resuspended in 116 100 µl of Hank's balanced salt solution (HBSS) and cytocentrifuged onto 117 histological slides at 500 rpm per 5 min, fixed with a methanol-based 118 buffered preservative solution and stained with hematoxylin and eosin. 119 Ten low-power (x100), non-overlapping images were collected from each 120 patient's cytological smears using a camera attached to a light 121 microscope (CX41, Olympus, Tokyo, Japan) and Image-Pro Plus software 122 version 7.0 (MediaCybernetics, Inc., Bethesda, MD, USA). Cells were 123 classified as small, medium or large. Morphometric estimates of cell 124 diameter revealed that the cells classified as small measured up to 30

125 µm, those classified as medium measured 30-48 µm, and those classified

126 as large measured over 48 µm. The cells were further classified as

127 squamous cells (large cells with irregular cytoplasm and round and

128 central nuclei), urothelial cells (large cells with regular rounded

129 cytoplasm), renal tubular epithelial cells (small cells with small rounded

130 nuclei and basophilic cytoplasm) or leukocytes (small cells with lobulated

131 or oval hyperchromatic nuclei and basophilic cytoplasm).

132 *Cell immunophenotyping*: Cytological preparations were fixed in cold

133 acetone and labeled for tubular cells and leukocytes identification. KIM-

134 1/TIM-1 antibody (Abcam 47635) was used at 5 µg/mL diluted in

135 phosphate buffered saline (PBS) containing 1% bovine serum albumin

136 (BSA), 10% normal goat serum, 0.3 M glycine and 0.1% Tween 20,

137 followed by a secondary antibody conjugated to Alexa Fluor 490 (green)

138 at 1:200. For leukocyte identification, the slides were incubated with

139 CD45 (Abcam 27287) FITC (green) at 1:100 in 1% PBS-BSA. DAPI was

140 used to stain the cell nuclei.

141 *Prediction of model and cross-validation:* Models of prediction and cross-

142 validation were built using Orange Data Mining software (University of

143 Ljubljana). The classification based on cell morphology and the diagnosis

144 of AKI were tested for prediction of the patient groups. The accuracy of

145 the model, represented by area under the ROC curve (AUC) was assessed

146 using cross-validation between three learning models: logistic regression,

147 random forest and tree.

6

148 *Statistical analysis:* Continuous variables are summarized as the means

149 ± standard deviations or median and first and third quartiles and were

150 compared using the Kruskal-Wallis or ANOVA followed by Bonferroni's

151 multiple comparison tests when required. Comparisons of proportions

152 were performed using chi-square test or Fisher's exact probability test.

153 Principal component analysis (PCA) graph was used to illustrate

154 multivariate analysis of the data on cell morphology. The results were

155 considered statistically significant at $P<0.05$. Data were analyzed using

156 Prism 5.01 (GraphPad, San Diego, CA, USA) and Stata/IC 11 data

157 analysis and statistical software (StataCorp LLC, College Station, TX,

158 USA).

159 *Ethical statement:* All patients were informed about the research and

160 agreed to participate. This work was approved by Research Ethical

161 Committee of Fundação Oswaldo Cruz, Instituto Gonçalo Moniz,

162 Salvador, BA, Brazil, Protocol No. 184.419.

163 **Results**
164 **General patient characteristics**

165 The main characteristics of the enrolled patients are shown in table 1.

166 Hypoalbuminemia was more severe in the ATN (1.7 [1.6-1.8] g/dL) than

167 in the PRO (2.3 [1.7-2.8] g/dL) group (P=0.04). Serum cholesterol level

168 was significantly higher in the ATN (372 [278.5-581.8] mg/dL) than in

169 the PRO (235 [139-291] mg/dL) group (P=0.02) (Table 1). Although the

170 serum concentrations of creatinine were above the reference values for

171 the ATN (1.4 [0.7-2.0] mg/dL) and PRO (1.7 [0.7-2.0] mg/dL) groups no

172   statistically significant differences were observed among the groups. The

173   main diagnosis of patients of the ATN or Non-ATN group was minimal

174   change disease (MCD) (40% and 44%, respectively), followed by focal and

175   segmental glomerulosclerosis (FSGS) (30% and 33%, respectively). Lupus

176   nephritis (LN) was the most frequent histological diagnosis in patients of

177   the PRO group (50%) (Table 1). Evidence of recent ATN was found in 63%

178   of biopsies. Seven patients of the PRO group also presented ATN.

179   **Cytological analysis**

180   The urinary sediment of one patient without ATN and of three patients

181   with ATN did not provide enough cells for cytology analysis. Therefore,

182   cell characteristics were studied for 23 cases.

183   Cell size: Patients with AKI showed a higher proportion of small cells

184   (75.3±18.8%) and a lower proportion of large cells (10.4±11.9%) than did

185   patients without AKI (30.7±25.8%, P=0.0007; 57±27.4%, P=0.0003,

186   respectively) (Table 2). However, although a trend towards an increased

187   proportion of small cells was observed in patients with proliferative

188   glomerular lesion, this trend was not statistically significant.

189   Morphological identification of the cell populations: Patients with AKI

190   showed a higher proportion of leukocytes (41.4±35%) and a lower

191   proportion of squamous cells (4.2±3.2%) than did patients without AKI

192   (12.2±11.9%, P=0.03; 39.7±23.5%, P=0.0004, respectively) (Table 2).

193   Furthermore, patients of the PRO group showed a higher proportion of

8

194 leukocytes (50.2±32.4%) than did the other NS patients (Non-ATN group,

195 12.88±20%; ATN group, 12.86±9.9%; P=0.005) (Figure 1).

196 The reliability of the morphological identification was confirmed by

197 positive immunolabeling of leukocytes with anti-CD45 antibody and of

198 renal tubular epithelial cells with anti-KIM-1 antibody (supplementary

199 data, Figure S1).

Model of urine cytology for differential diagnosis of AKI

201 The tree learner model had the best performance analysis (AUC 0.864

202 and precision of 0.909, supplementary data, Table S1) for prediction of

203 AKI. Using this model for diagnosis of AKI and morphology of cells, a

204 cluster of patients with NS was observed in the three groups as shown in

205 the PCA analysis (supplementary data, Figure S2, A). In the binary tree

206 model, the counts and morphology of cells were able to classify the NS

207 groups the data set (supplementary data, Figure S2, B).

Discussion

209 In this study, we analyzed the potential use of urine cytology in the

210 differential diagnosis of AKI in patients with NS. Similar studies have

211 been conducted with patients without NS. In NS, the high protein

212 concentration and the presence of different proteins in the urine may

213 interfere with cell representation in urine sediment [8]. Furthermore,

214 urine sediment may be enriched in some patients with NS. Nevertheless,

215 we found that small cells predominated in the urine sediment of patients

216 with AKI. Although a trend towards an increased population of small cells

217 was observed in patients with proliferative glomerulopathy, cell size alone

9

218 could not distinguish the potential cause of AKI. This small cell

219 population included tubular epithelial cells and leukocytes, as identified

220 by cell morphology and confirmed morphologically and by

221 immunofluorescence assay. Of these two cell populations, the leukocytes

222 proportion was higher in the urine sediment of patients with proliferative

223 glomerulopathy than in that of the remaining patient groups. Similar

224 change has been reported in urine of patients with glomerular

225 proliferative disease without NS [5]. The number of leukocytes was found

226 to be a primary classifier of NS groups of patients in the learning model

227 applied to this image dataset. Perazella et al. [6], using phase contrast

228 microscopy, found a significant increase in the proportion of epithelial

229 tubular cells in patients with ATN compared with patients with pre-renal

230 AKI. Although we found a trend toward an increase in tubular epithelial

231 cells in patients with nephrotic syndrome and ATN alone relative to the

232 other patient groups, this difference was not statistically significant. A

233 possible reason for the difference between studies is that patients with

234 glomerular diseases were excluded from the study by Perazella and

235 coworkers [6]. Patients with glomerular disease might present a more

236 complex urinary sediment, which might affect the proportions of the

237 different cell populations. Furthermore, histological confirmation of ATN

238 was lacking in the study by Perazella and coworkers [6].

239 The staining of urinary sediment is rapid, easily performed, and

240 inexpensive, and staining reagents are widely available. We show that

241 this procedure allows even professionals with little experience in cytology

10

242 to confirm the diagnosis of AKI and to distinguish proliferative
243 glomerulonephritis as the potential cause of this condition in patients
244 with the emergence of kidney dysfunction in the course of NS. The
245 clustering of patients with the three different causes of AKI based in the
246 number and cell morphological types and using the decision tree reported
247 herein support further studies with a larger number pf patients.

Conclusions

249 1) Using urine cytology with conventional staining might constitute a
250 helpful tool for the differential diagnosis between proliferative
251 glomerular lesion and ATN in patients with NS and AKI in the
252 absence of kidney biopsy.

253 2) The classification method based in cell number and types has
254 potential use in the distinction of AKI etiology in patients with NS.

Limitations

256 1) Although the use of urine cytology provided some direction in the
257 differential diagnosis of AKI in patients with NS, renal biopsy is still
258 needed for confirmation.

259 2) Larger sample size and different hospital settings are needed to
260 validate urine cytology as an alternative tool for diagnosis of AKI.

List of abbreviations

262 **AKI** – Acute kidney injury

263 **ATN** – Acute tubular necrosis

11

264   **AUC** – area under the ROC curve

265   **BSA** - bovine serum albumin

266   **DPGN** - diffuse-proliferative glomerulonephritis

267   **FITC** - fluorescein isothiocyanate

268   **FSGS** - focal and segmental glomerulosclerosis

269   **HBSS** - Hank's balanced salt solution

270   **KIM-1/TIM-1** - Kidney Injury Molecule -1/ T-cell immunoglobulin and
271   mucin-containing molecule

272   **LN** - lupus nephritis

273   **MCD** - minimal change disease

274   **MN** – membranous nephropaty

275   **MPGN** - membranoproliferative glomerulonephritis

276   **Non-ATN** – group og patients without ATN or proliferative glomerular
277   lesions

278   **NS** – Nephrotic syndrome

279   **PBS** - phosphate buffered saline

280   **PCA** – principal component analysis

281   **PRO** – group of patients with proliferative glomerulopathy

282   **SAH** - systemic arterial hypertension

Declarations

Ethics approval and consent to participate:

All patients were informed about the research and consented to participate. This work was approved by Research Ethical Committee of Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, BA, Brazil, Protocol No. 184.419.

Consent for publication:

Not applicable.

Availability of data and materials:

All data obtained during this study is included in this article.

Competing interests:

The authors have declared that no competing interests exist.

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Author's contributions:

CVBM, MBT and PNF conducted the experimental procedures. CVBM and CASS performed the cytological analysis. WLCS, MBT and CVBM analyzed the renal biopsies. MBO and RDC provided the laboratory and clinical data of the patients. WLCS, MBO and MBT supervised the project.

13

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335   sedimentation rate of human blood. Biochemical Journal, 1943;37(3):

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337

Tables

339 **Table 1.** General characteristics of the patients with nephrotic syndrome

340 and the healthy individuals enrolled in the study.

Parameter	Control	Non-ATN	ATN	PRO	P value
Total (*n*)	10	9	10	8	
Gender					
Female	5 (50%)	4 (44%)	5 (50%)	5 (63%)	-
Age (years) †	30 [25.5-36.75]	25 [21.5-50.5]	50.5 [20-56]	25.5 [22-46.7]	ns
Serum urea (mg/dL) †	25 [22.75-28.9]	33 [20-47]	64 [37.5-107]	36.5 [24.2-89.5]	ns
Serum creatinine (mg/dL) †	0.9 [0.82-0.97]	1.1 [0.8-1.7]	1.4 [0.7-2.0]	1.7 [0.7-2.0]	ns
Serum albumin (g/dL) †	-	1.9 [1.6-2.2]	1.7 [1.6-1.8]	2.3 [1.7-2.8]	0.04*
Total cholesterol (mg/dL) †	-	360.5 [256-470]	372 [278.5-581.8]	235 [139-291]	0.02*
24 hour protein (mg) †	-	6965 [3913–13437]	7540 [3482–13844]	8488 [2860–16081]	ns
SAH	-	8 (89%)	9 (90%)	5 (63%)	ns

Histological diagnostic					
MCD	-	4 (44%)	4 (40%)	0	-
FSGS	-	3 (33%)	3 (30%)		
MN	-	2 (22%)	2 (20%)	0	-
NL	-	0	0	4 (50%)	-
DPGN	-	0	0	2 (25%)	-
MPGN	-	0	1 (10%)	2 (25%)	-
Tubulointerstitial fibrosis ‡		7.7±7.9%	9.4±7.6%	8.1±7%	-
Acute tubular necrosis (ATN)	-	0	10 (100%)	7 (88%)	
Intensity ATN ‡	-	3.3±2.5%	46±26.1%	29.3±26.7%	-

341 Notes: †Data expressed as medians and interquartile intervals. ‡Data expressed as
342 medians ± standard deviations. SAH= systemic arterial hypertension, MCD= minimal
343 change disease, FSGS= focal and segmental glomerulosclerosis, MN= membranous
344 nephropathy, LN= lupus nephritis, DPGN= diffuse-proliferative glomerulonephritis,
345 MPGN= membranoproliferative glomerulonephritis. *=acute tubular necrosis group
346 (ATN) vs. inflammatory-proliferative glomerular lesion group (PRO). ns= not significant.
347

348 **Table 2.** Estimates of cell populations in the urine of patients with
349 nephrotic syndrome and healthy volunteers included in the study.

Category	Control	NS patients		P value
		With AKI	Without AKI	
Total (*n*)	10 (%)	9 (%)	9 (%)	
Small cells	31±29.9	75.3±18.8	30.7±25.8	0.0007*

Category	Control	NS patients		P value
		With AKI	Without AKI	
Medium cells	4.8±5	14.2±9.8	12.2±13	ns
Large cells	64.1±30.3	10.4±11.9	57±27.4	0.0003*
Squamous cells	64±35.1	4.2±3.2	39.7±23.5	0.0004*
Urothelial cells	11.3±14.9	27.3±21.8	33.8±19.9	ns
Renal epithelial tubular cells	4±5.5	26.7±23.1	14.1±19.6	ns
Leukocytes	21.1±22.9	41.4±35	12.2±11.9	0.03*

350 Notes: Data expressed as medians of proportion ± standard deviation. NS= nephrotic

351 syndrome, AKI= acute kidney injury. *difference between groups with or without AKI.

352

353 **Figures**

354 **Figure 1**: Representative photomicrograph of the urinary sediment,

355 stained with H/E, of a patient with nephrotic syndrome (A) (x200). Dotted

356 arrows identify small cells; white indicates renal tubular epithelial cell,

357 black indicates leukocyte. Head arrows identify large cells: white

358 indicates urothelial cell, black indicates squamous cell. Continuous

359 arrows identify medium cells: urothelial cells. (B) Proportions of small

360 cells, i.e., renal epithelial tubular cells (RTEC) and leukocytes, in groups

361 without ATN (Non-ATN), with ATN (ATN) and with glomerular proliferative

362 lesion (PRO).

363 **Supplementary material**

364 **Figure S1:** Representative photomicrograph of the urinary sediment

365 stained with H/E of a patient with nephrotic syndrome (x400) (A).

366 Immunofluorescence of the urinary sediment of an acute tubular necrosis

367 patient showing positive marking of KIM-1 (green) and nucleus (blue) (B),

368 and immunofluorescence of the urinary sediment of an inflammatory-

369 proliferative glomerular lesion patient showing positive marking of CD45

370 (green) and nucleus (blue) (C).

371

372 **Figure S2:** Algorithmic models of patients without ATN or proliferative

373 glomerular disease (Non-ATN – Group 1/blue), with ATN (ATN – Group

374 2/red) and with glomerular proliferative lesion (PRO – Group 3/green).

375 **(A)** Principal component analysis of groups of patients based in AKI

376 diagnosis and cell numbers and types. **(B)** Binary tree model of groups of

377 patients based in cell numbers and types. Transition of colors means the

378 classification of groups accordingly as follows: blue - Non-ATN/Group 1;

379 red – ATN/Group 2 and green – PRO/Group 3.

380

Table 1

Table 1. General characteristics of the patients with nephrotic syndrome and the healthy individuals enrolled in the study.

Parameter	Control	Non-ATN	ATN	PRO	P value
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Gender					
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Acute tubular necrosis (ATN)	-	0	10 (100%)	7 (88%)	
Intensity ATN ‡	-	3.3±2.5%	46±26.1%	29.3±26.7%	-

Notes: †Data expressed as medians and interquartile intervals. ‡Data expressed as medians ± standard deviations. SAH= systemic arterial hypertension, MCD= minimal change disease, FSGS= focal and segmental glomerulosclerosis, MN= membranous nephropathy, LN= lupus nephritis, DPGN= diffuse-proliferative glomerulonephritis, MPGN= membranoproliferative glomerulonephritis. *=acute tubular necrosis group (ATN) vs. inflammatory-proliferative glomerular lesion group (PRO). ns= not significant.

Table 2. Estimates of cell populations in the urine of patients with nephrotic syndrome and healthy volunteers included in the study.

Category	Control	NS patients		P value
		With AKI	**Without AKI**	
Total (_n_)	10 (%)	9 (%)	9 (%)	
Small cells	31±29.9	75.3±18.8	30.7±25.8	0.0007*
Medium cells	4.8±5	14.2±9.8	12.2±13	ns
Large cells	64.1±30.3	10.4±11.9	57±27.4	0.0003*
Squamous cells	64±35.1	4.2±3.2	39.7±23.5	0.0004*
Urothelial cells	11.3±14.9	27.3±21.8	33.8±19.9	ns
Renal epithelial tubular cells	4±5.5	26.7±23.1	14.1±19.6	ns
Leukocytes	21.1±22.9	41.4±35	12.2±11.9	0.03*

Notes: Data expressed as medians of proportion ± standard deviation. NS= nephrotic syndrome, AKI= acute kidney injury. *difference between groups with or without AKI.
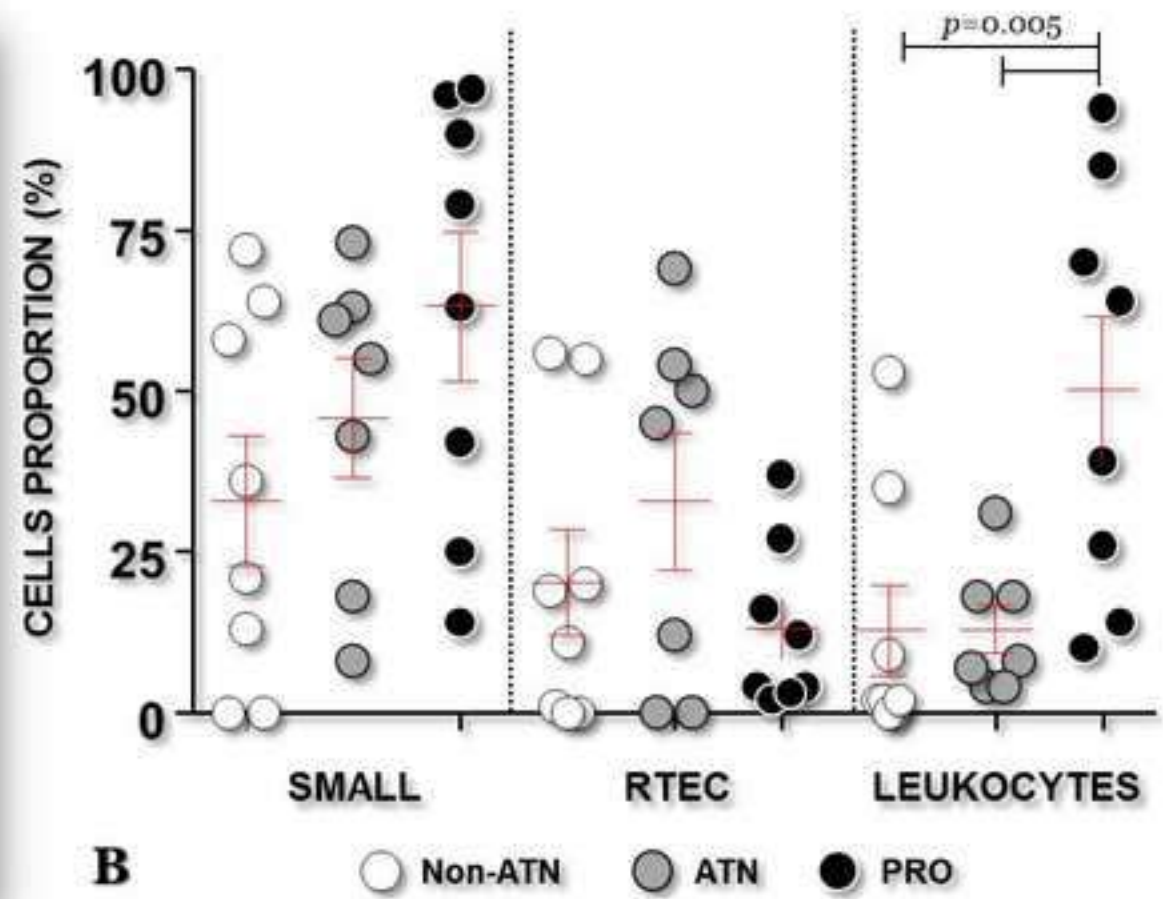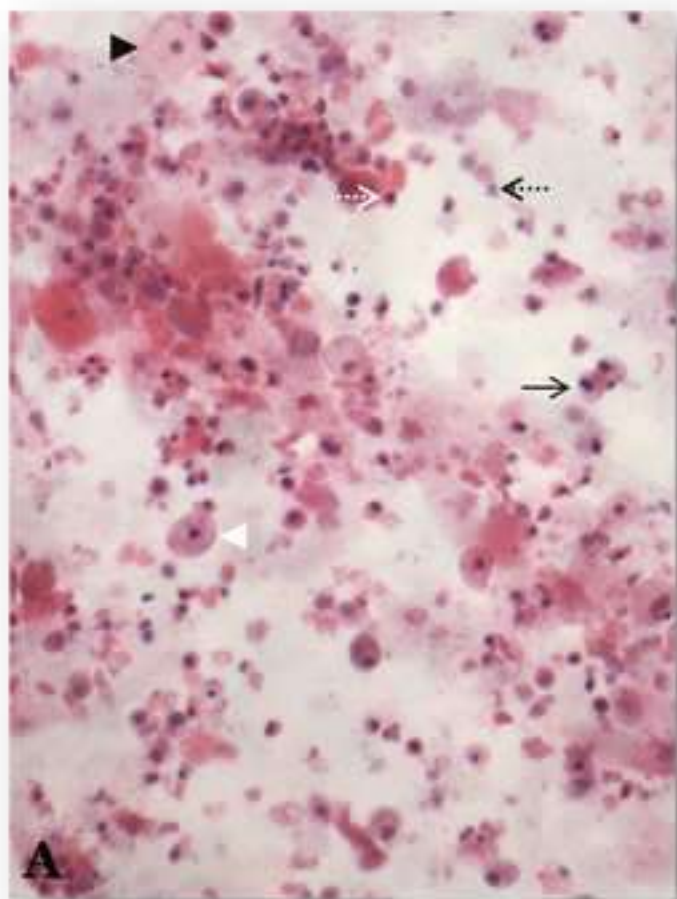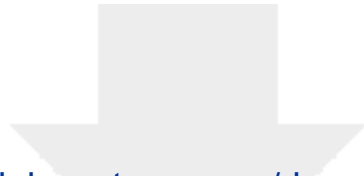
Figure 1

Table S1

Figure S1

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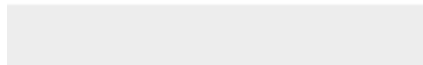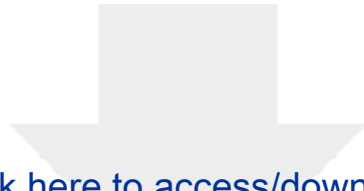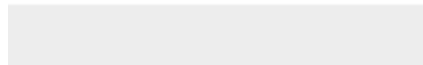Supplementary Material
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Figure S2

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Urinary cytology: a potential tool for differential diagnosis of acute kidney injury in patients with nephrotic syndrome

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Abstract

Objective: Acute tubular necrosis (ATN) is a frequent cause of acute kidney injury (AKI). In patients with nephrotic syndrome (NS), AKI demands the differential diagnosis between ATN and rapidly progressive glomerulonephritis. In some cases, conclusive diagnosis is possible only by kidney biopsy. We aimed to study the potential use of urine cytology in the differential diagnosis between ATN and proliferative glomerular lesion in patients with NS.

Results: Cell size analysis showed a higher proportion of small cells and a lower proportion of large cells in the urine of patients with AKI. Cells phenotypes were easily defined using cytological preparations. Leukocytes were found to be a primary classifier of NS groups, with higher number in patients with AKI and patients with proliferative glomerular lesions. Although renal biopsy is still required for confirmative diagnosis, our data suggests that urinary cytology can be readily performed and support the differential diagnosis between proliferative glomerular lesion and ATN in patients with NS and AKI.

Keywords: Acute tubular necrosis, Glomerulonephritis, Cytodiagnosis, Acute kidney injury

Introduction

Acute tubular necrosis (ATN) is a leading cause of acute kidney injury (AKI) in hospitalized patients [1]. The prevalence of AKI also correlates with ATN severity in patients with nephrotic syndrome (NS) [2]. Emergence of AKI in patients with NS requires the differential diagnosis between ATN alone and glomerular proliferative lesions such as in crescentic glomerulonephritis [3], since the therapeutic approach differs between these conditions. Whereas proliferative glomerulopathies require immediate immunosuppression to avoid progression to end-stage renal disease, ATN requires support treatment without immunosuppression avoiding potential

side effects [4]. Urinary sediment analysis has been used in the diagnosis of ATN and in the differential diagnosis between isolated ATN and proliferative glomerular lesion in patients with AKI [5, 6]. The presence of renal tubular epithelial cells in the urinary sediment has been considered indicative of ATN, and high number of leukocytes has been considered indicative of glomerular lesions [5–7]. However, cell identification in unstained urine sediment can be difficult, particularly in conditions of glomerular lesions associated with nephrotic syndrome, where the urinary sediment may be complex. Furthermore, studies associating urinary sediment cytological findings with kidney histology are lacking. In this work, we compare the urinary cytology with the histological presentation of the kidney in biopsy of patients with NS and AKI. To avoid inconsistent cell identification, we used conventional staining techniques used in cytology. Our data suggest that conventionally stained urine

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cytology can be a rapid, consistent and easy-to-use tool for supporting the differential diagnosis between proliferative glomerular lesion and ATN in patients with NS and AKI. We propose a cross-validation of this model to support larger studies for validation of the test in the bedside.

Main text
Methods
Patients
A prospective cross-sectional study including 27 patients with NS subjected to renal biopsy for glomerular disease diagnosis in referral hospitals of Salvador, Brazil, from July 2013 to April 2015. The biopsies were examined at the Fundação Oswaldo Cruz, Instituto Gonçalo Moniz in Salvador, Brazil. Cases were excluded if the renal biopsy contained less than 7 glomeruli, if the estimated interstitial fibrosis encompassed 30% or more of the cortical area, if the patient had diabetes mellitus or infections. The patients were allocated into 3 groups: PRO—8 patients with proliferative glomerulopathy, ATN—10 patients with ATN without proliferative glomerulonephritis and Non-ATN—9 patients without ATN or proliferative glomerular lesions. AKI was defined using the KDIGO criteria. Ten healthy volunteers were used as a reference group of normal urine cytology.

Clinical data
The following data were obtained from biopsy request forms and by anamneses: age, sex, serum creatinine, albumin, cholesterol, 24-h urine proteinuria and diagnosis of systemic arterial hypertension.

Histological analysis
The renal specimens were obtained by percutaneous biopsies, fixed in Bouin's solution or acid formalin, paraffin embedded, cut into 2-μm thick sections, and stained with hematoxylin and eosin. All the slides were reviewed by a pathologist (WLCS). The intensity of ATN was estimated as a percentage of the renal cortex by visual assessment. The following tubular changes were considered as evidence of either current or recent ATN: tubular dilatation, thinning of the tubular epithelium, cellular casts, interstitial edema, and the evidence of epithelial regeneration (hyperchromatic nucleus, mitosis, and binucleation). The percentage of cortical tubulointerstitial fibrosis was estimated by visual assessment.

Cytology analysis
Fresh urine was obtained from the patients by spontaneous voiding before renal biopsy. Ten milliliters of urine were centrifuged at 2000*g* per 10 min in a standard centrifuge. The supernatant was removed by suction. The sediment was resuspended in 100 μl of Hank's balanced salt solution (HBSS) and cytocentrifuged onto histological slides at 500 rpm per 5 min, fixed with a methanol-based buffered preservative solution and stained with hematoxylin and eosin. Ten low-power ($\times 100$), non-overlapping images were collected from each patient's cytological smears using a camera attached to a light microscope (CX41, Olympus, Tokyo, Japan) and Image-Pro Plus software version 7.0 (MediaCybernetics, Inc., Bethesda, MD, USA). Cells were classified as small, medium or large. Morphometric estimates of cell diameter revealed that the cells classified as small measured up to 30 μm, those classified as medium measured 30–48 μm, and those classified as large measured over 48 μm. The cells were further classified as squamous cells (large cells with irregular cytoplasm and round and central nuclei), urothelial cells (large cells with regular rounded cytoplasm), renal tubular epithelial cells (small cells with small rounded nuclei and basophilic cytoplasm) or leukocytes (small cells with lobulated or oval hyperchromatic nuclei and basophilic cytoplasm).

Cell immunophenotyping
Cytological preparations were fixed in cold acetone and labeled for tubular cells and leukocytes identification. KIM-1/TIM-1 antibody (Abcam 47635) was used at 5 μg/ml diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), 10% normal goat serum, 0.3 M glycine and 0.1% Tween 20, followed by a secondary antibody conjugated to Alexa Fluor 490 (green) at 1:200. For leukocyte identification, the slides were incubated with CD45 (Abcam 27287) FITC (green) at 1:100 in 1% PBS-BSA. DAPI was used to stain the cell nuclei.

Prediction of model and cross-validation
Models of prediction and cross-validation were built using Orange Data Mining software (University of Ljubljana). The classification based on cell morphology and the diagnosis of AKI were tested for prediction of the patient groups. The accuracy of the model, represented by area under the ROC curve (AUC) was assessed using cross-validation between three learning models: logistic regression, random forest and tree.

Statistical analysis
Continuous variables are summarized as the means ± standard deviations or median and first and third quartiles and were compared using the Kruskal–Wallis or ANOVA followed by Bonferroni's multiple comparison tests when required. Comparisons of proportions were performed using chi-square test or Fisher's exact probability test. Principal component analysis

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(PCA) graph was used to illustrate multivariate analysis of the data on cell morphology. The results were considered statistically significant at P < 0.05. Data were analyzed using Prism 5.01 (GraphPad, San Diego, CA, USA) and Stata/IC 11 data analysis and statistical software (StataCorp LLC, College Station, TX, USA).

Ethical statement

All patients were informed about the research and agreed to participate. This work was approved by Research Ethical Committee of Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, BA, Brazil, Protocol No. 184.419.

Results

General patient characteristics

The main characteristics of the enrolled patients are shown in Table 1. Hypoalbuminemia was more severe in the ATN (1.7 [1.6–1.8] g/dl) than in the PRO (2.3 [1.7–2.8] g/dl) group (P = 0.04). Serum cholesterol level was significantly higher in the ATN (372 [278.5-581.8] mg/dl) than in the PRO (235 [139-291] mg/dl) group (P = 0.02) (Table 1). Although the serum concentrations of creatinine were above the reference values for the ATN (1.4

[0.7–2.0] mg/dl) and PRO (1.7 [0.7–2.0] mg/dl) groups no statistically significant differences were observed among the groups. The main diagnosis of patients of the ATN or Non-ATN group was minimal change disease (MCD) (40% and 44%, respectively), followed by focal and segmental glomerulosclerosis (FSGS) (30% and 33%, respectively). Lupus nephritis (LN) was the most frequent histological diagnosis in patients of the PRO group (50%) (Table 1). Evidence of recent ATN was found in 63% of biopsies. Seven patients of the PRO group also presented ATN.

Cytological analysis

The urinary sediment of one patient without ATN and of three patients with ATN did not provide enough cells for cytology analysis. Therefore, cell characteristics were studied for 23 cases.

Cell size

Patients with AKI showed a higher proportion of small cells (75.3 ± 18.8%) and a lower proportion of large cells (10.4 ± 11.9%) than did patients without AKI (30.7 ± 25.8%, P = 0.0007; 57 ± 27.4%, P = 0.0003, respectively) (Table 2). However, although a trend

Table 1 General characteristics of the patients with nephrotic syndrome and the healthy individuals enrolled in the study

Parameter	Control	Non-ATN	ATN	PRO	P value
Total (*n*)	10	9	10	8	
Gender					
Female	5 (50%)	4 (44%)	5 (50%)	5 (63%)	–
Age (years)[a]	30 [25.5–36.75]	25 [21.5–50.5]	50.5 [20–56]	25.5 [22–46.7]	ns
Serum urea (mg/dl)[a]	25 [22.75–28.9]	33 [20–47]	64 [37.5–107]	36.5 [24.2–89.5]	ns
Serum creatinine (mg/dl)[a]	0.9 [0.82–0.97]	1.1 [0.8–1.7]	1.4 [0.7–2.0]	1.7 [0.7–2.0]	ns
Serum albumin (g/dl)[a]	–	1.9 [1.6–2.2]	1.7 [1.6–1.8]	2.3 [1.7–2.8]	0.04*
Total cholesterol (mg/dl)[a]	–	360.5 [256–470]	372 [278.5–581.8]	235 [139–291]	0.02*
24-h protein (mg)[a]	–	6965 [3913–13,437]	7540 [3482–13,844]	8488 [2860–16,081]	ns
SAH	–	8 (89%)	9 (90%)	5 (63%)	ns
Histological diagnostic					
MCD	–	4 (44%)	4 (40%)	0	–
FSGS	–	3 (33%)	3 (30%)		
MN	–	2 (22%)	2 (20%)	0	–
NL	–	0	0	4 (50%)	–
DPGN	–	0	0	2 (25%)	–
MPGN	–	0	1 (10%)	2 (25%)	–
Tubulointerstitial fibrosis[b]		7.7 ± 7.9%	9.4 ± 7.6%	8.1 ± 7%	–
Acute tubular necrosis (ATN)	–	0	10 (100%)	7 (88%)	
Intensity ATN[b]	–	3.3 ± 2.5%	46 ± 26.1%	29.3 ± 26.7%	–

SAH systemic arterial hypertension, *MCD* minimal change disease, *FSGS* focal and segmental glomerulosclerosis, *MN* membranous nephropathy, *LN* lupus nephritis, *DPGN* diffuse-proliferative glomerulonephritis, *MPGN* membranoproliferative glomerulonephritis, *ns* not significant

* Acute tubular necrosis group (ATN) vs. inflammatory-proliferative glomerular lesion group (PRO)

[a] Data expressed as medians and interquartile intervals

[b] Data expressed as medians ± standard deviations

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Table 2 Estimates of cell populations in the urine of patients with nephrotic syndrome and healthy volunteers included in the study

Category	Control	NS patients		P value
		With AKI	Without AKI	
Total (n)	10 (%)	9 (%)	9 (%)	
Small cells	31 ± 29.9	75.3 ± 18.8	30.7 ± 25.8	0.0007*
Medium cells	4.8 ± 5	14.2 ± 9.8	12.2 ± 13	ns
Large cells	64.1 ± 30.3	10.4 ± 11.9	57 ± 27.4	0.0003*
Squamous cells	64 ± 35.1	4.2 ± 3.2	39.7 ± 23.5	0.0004*
Urothelial cells	11.3 ± 14.9	27.3 ± 21.8	33.8 ± 19.9	ns
Renal epithelial tubular cells	4 ± 5.5	26.7 ± 23.1	14.1 ± 19.6	ns
Leukocytes	21.1 ± 22.9	41.4 ± 35	12.2 ± 11.9	0.03*

Data expressed as medians of proportion ± standard deviation

NS nephrotic syndrome, AKI acute kidney injury

* Difference between groups with or without AKI

towards an increased proportion of small cells was observed in patients with proliferative glomerular lesion, this trend was not statistically significant.

Morphological identification of the cell populations: Patients with AKI showed a higher proportion of leukocytes (41.4 ± 35%) and a lower proportion of squamous cells (4.2 ± 3.2%) than did patients without AKI (12.2 ± 11.9%, P = 0.03; 39.7 ± 23.5%, P = 0.0004, respectively) (Table 2). Furthermore, patients of the PRO group showed a higher proportion of leukocytes (50.2 ± 32.4%) than did the other NS patients (Non-ATN group, 12.88 ± 20%; ATN group, 12.86 ± 9.9%; P = 0.005) (Fig. 1).
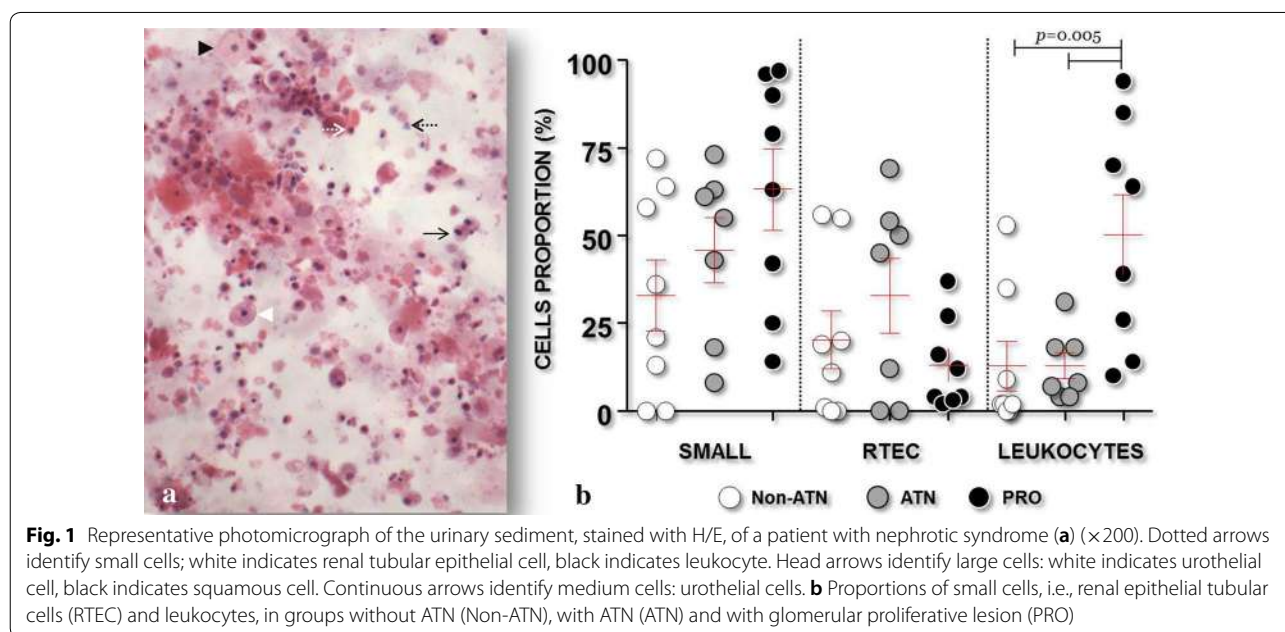
The reliability of the morphological identification was confirmed by positive immunolabeling of leukocytes with anti-CD45 antibody and of renal tubular epithelial cells with anti-KIM-1 antibody (Additional file 1: Figure S1).

Model of urine cytology for differential diagnosis of AKI

The tree learner model had the best performance analysis (AUC 0.864 and precision of 0.909, Additional file 2: Table S1) for prediction of AKI. Using this model for diagnosis of AKI and morphology of cells, a cluster of patients with NS was observed in the three groups as shown in the PCA analysis (Additional file 3: Figure S2A). In the binary tree model, the counts and morphology of cells were able to classify the NS groups the data set (Additional file 3: Figure S2B).

Discussion

In this study, we analyzed the potential use of urine cytology in the differential diagnosis of AKI in patients with NS. Similar studies have been conducted with patients without NS. In NS, the high protein concentration and the presence of different proteins in the urine may interfere with cell representation in urine sediment [8]. Furthermore, urine sediment may be enriched in some patients with NS. Nevertheless, we found that small cells predominated in the urine sediment of patients with AKI. Although a trend towards an increased population of small cells was observed

Fig. 1 Representative photomicrograph of the urinary sediment, stained with H/E, of a patient with nephrotic syndrome (**a**) (×200). Dotted arrows identify small cells; white indicates renal tubular epithelial cell, black indicates leukocyte. Head arrows identify large cells: white indicates urothelial cell, black indicates squamous cell. Continuous arrows identify medium cells: urothelial cells. **b** Proportions of small cells, i.e., renal epithelial tubular cells (RTEC) and leukocytes, in groups without ATN (Non-ATN), with ATN (ATN) and with glomerular proliferative lesion (PRO)

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in patients with proliferative glomerulopathy, cell size alone could not distinguish the potential cause of AKI. This small cell population included tubular epithelial cells and leukocytes, as identified by cell morphology and confirmed morphologically and by immunofluorescence assay. Of these two cell populations, the leukocytes proportion was higher in the urine sediment of patients with proliferative glomerulopathy than in that of the remaining patient groups. Similar change has been reported in urine of patients with glomerular proliferative disease without NS [5]. The number of leukocytes was found to be a primary classifier of NS groups of patients in the learning model applied to this image dataset. Perazella et al., using phase contrast microscopy, found a significant increase in the proportion of epithelial tubular cells in patients with ATN compared with patients with pre-renal AKI [6]. Although we found a trend toward an increase in tubular epithelial cells in patients with nephrotic syndrome and ATN alone relative to the other patient groups, this difference was not statistically significant. A possible reason for the difference between studies is that patients with glomerular diseases were excluded from the study by Perazella and coworkers [6]. Patients with glomerular disease might present a more complex urinary sediment, which might affect the proportions of the different cell populations. Furthermore, histological confirmation of ATN was lacking in the study by Perazella and coworkers [6].

The staining of urinary sediment is rapid, easily performed, and inexpensive, and staining reagents are widely available. We show that this procedure allows even professionals with little experience in cytology to confirm the diagnosis of AKI and to distinguish proliferative glomerulonephritis as the potential cause of this condition in patients with the emergence of kidney dysfunction in the course of NS. The clustering of patients with the three different causes of AKI based in the number and cell morphological types and using the decision tree reported herein support further studies with a larger number of patients.

Conclusions

1. Using urine cytology with conventional staining might constitute a helpful tool for the differential diagnosis between proliferative glomerular lesion and ATN in patients with NS and AKI in the absence of kidney biopsy.
2. The classification method based in cell number and types has potential use in the distinction of AKI etiology in patients with NS.

Limitations

1. Although the use of urine cytology provided some direction in the differential diagnosis of AKI in patients with NS, renal biopsy is still needed for confirmation.
2. Larger sample size and different hospital settings are needed to validate urine cytology as an alternative tool for diagnosis of AKI.

Supplementary information

Additional file 1: Figure S1. Representative photomicrograph of the urinary sediment stained with H/E of a patient with nephrotic syndrome (x400) (A). Immunofluorescence of the urinary sediment of an acute tubular necrosis patient showing positive marking of KIM-1 (green) and nucleus (blue) (B), and immunofluorescence of the urinary sediment of an inflammatory-proliferative glomerular lesion patient showing positive marking of CD45 (green) and nucleus (blue) (C).

Additional file 2: Table S1. Cross-validation of models in urine cytology for differential diagnosis of AKI.

Additional file 3: Figure S2. Algorithmic models of patients without ATN or proliferative glomerular disease (Non-ATN – Group 1/blue), with ATN (ATN – Group 2/red) and with glomerular proliferative lesion (PRO – Group 3/green). **(A)** Principal component analysis of groups of patients based in AKI diagnosis and cell numbers and types. **(B)** Binary tree model of groups of patients based in cell numbers and types. Transition of colors means the classification of groups accordingly as follows: blue - Non-ATN/Group 1; red – ATN/Group 2 and green – PRO/Group 3.

Abbreviations

AKI: Acute kidney injury; ATN: Acute tubular necrosis; AUC: Area under the ROC curve; BSA: Bovine serum albumin; DPGN: Diffuse-proliferative glomerulonephritis; FITC: Fluorescein isothiocyanate; FSGS: Focal and segmental glomerulosclerosis; HBSS: Hank's balanced salt solution; KIM-1/TIM-1: Kidney Injury Molecule-1/T cell immunoglobulin and mucin-containing molecule; LN: Lupus nephritis; MCD: Minimal change disease; MN: Membranous nephropaty; MPGN: Membranoproliferative glomerulonephritis; Non-ATN: Group og patients without ATN or proliferative glomerular lesions; NS: Nephrotic syndrome; PBS: Phosphate buffered saline; PCA: Principal component analysis; PRO: Group of patients with proliferative glomerulopathy; SAH: Systemic arterial hypertension.

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Availability of data and materials
All data obtained during this study is included in this article.

Ethics approval and consent to participate
All patients were informed about the research, read and signed the document consenting to participate. This work was approved by Research Ethical Committee of Fundação Oswaldo Cruz, Instituto Gonçalo Moniz, Salvador, BA, Brazil, Protocol No. 184.419.

Consent for publication
Not applicable.

Competing interests
The authors have declared that no competing interests exist.

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