Urinary exosomes in the diagnosis of Gitelman and Bartter syndromes

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ABSTRACT

Background. Gitelman syndrome (GS) and Bartter syndrome (BS) are hereditary salt-losing tubulopathies (SLTs) resulting from defects of renal proteins involved in electrolyte reabsorption, as for sodium-chloride cotransporter (NCC) and furosemide-sensitive sodium-potassium-chloride cotransporter (NKCC2) cotransporters, affected in GS and BS Type 1 patients, respectively. Currently, definitive diagnosis is obtained through expensive and time-consuming genetic testing. Urinary exosomes (UE), nanovesicles released by every epithelial cell facing the urinary space, represent an ideal source of markers for renal dysfunction and injury, because UE molecular composition stands for the cell of origin. On these assumptions, the aim of this work is to evaluate the relevance of UE for the diagnosis of SLTs.

Methods. UE were purified from second morning urines collected from 32 patients with genetically proven SLTs (GS, BS1, BS2 and BS3 patients), 4 with unclassified SLTs and 22 control subjects (age and sex matched). The levels of NCC and NKCC2 were evaluated in UE by SDS–PAGE/western blotting with specific antibodies.

Results. Due to their location on the luminal side of tubular cells, NCC and NKCC2 are well represented in UE proteome. The NCC signal is significantly decreased/absent in UE of Gitelman patients compared with control subjects (Mann–Whitney t-test, P < 0.001) and, similarly, the NKCC2 in those of Bartter type 1 (P < 0.001). The difference in the levels of the two proteins allows recognition of Gitelman and Bartter type 1 patients from controls and, combined with clinical data, from other Bartter patients. Moreover, the receiver operating characteristic curve analysis using UE NCC densitometric values...
showed a good discriminating power of the test comparing GS patients versus controls and BS patients (area under the curve value = 0.92; sensitivity 84.2% and specificity 88.6%).

**Conclusions.** UE phenotyping may be useful in the diagnosis of GS and BS, thus providing an alternative/complementary, urine-based diagnostic tool for SLT patient recognition and a diagnostic guidance in complex cases.

**Keywords:** diagnosis, NCC, NKCC2, salt-losing tubulopathies, urinary exosomes

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**INTRODUCTION**

Urinary exosomes (UE) are 30–100 nm vesicles which are normally secreted by cells from all nephron segments, and which have aroused increasing interest in the nephrology community [1]. In fact, unlike renal biopsy, an invasive and expensive procedure that provides only a small sample from one of two kidneys, UE provide a full molecular representation of the entire urinary system [2]. Because the UE molecular composition reflects their cellular origin, they are considered a promising source of biomarkers for renal dysfunction and structural injury [1, 3].

Gitelman syndrome (GS) (OMIM 263800) and Bartter syndrome (BS) types 1–4 (BS1–4) (OMIM 601678, 241200, 607364, 602522 and 613090) [4] are rare salt-losing tubulopathies (SLTs) with autosomal recessive inheritance, with an incidence of 25/1,000,000 [5] and 1/1,000,000, respectively, caused by mutations of genes involved in Na/liquid reabsorption. SLTs are characterized by chronic hypokalaemic metabolic alkalosis and normotensive, hyperreninaemic hyperaldosteronism. The signs and symptoms may include growth retardation, nephrocalcinosis, cramps, tetanic crises, fatigue and cardiac arrhythmias [6]. In some cases, chronic kidney disease occurs [7]. These syndromes can be grouped into four types: (i) GS with mutations on the SCLC12A3 gene, (ii) antenatal BS (aBS or type 1 and 2), on the SLC12A1 and KCNJ1 genes, (iii) classic BS (cBS or type 3), on the CLCNKB gene and (iv) BS associated with sensorineural deafness (type 4a and 4b) caused by BSND gene mutations and a digenic defect on the CLCNKA and -KB genes, respectively [4].

All these genes code for membrane proteins localized along the thick ascending limb (TAL) of Henle and the distal convoluted tubule (DCT), which are involved in the transport/re-absorption of electrolytes (\(\text{Na}^+, \text{K}^+, \text{Cl}^-, \text{Mg}^{2+}\) and \(\text{Ca}^{2+}\)). In particular, GS is due to defective thiazide-sensitive sodium-chloride cotransporter (NCC) that moves chloride ions against an electrochemical gradient across the luminal membrane into the epithelial cells in the DCT. Similarly, the furosemide-sensitive sodium-potassium-chloride cotransporter (NKCC2) also performs the transmembrane transport of ions from lumen into the epithelial cells of the TAL and is defective in BS1 [8].

Clinical history and biochemical work-up may not allow a definitive diagnosis of tubular disorders, due to their genetic heterogeneity and the overlapping phenotypes between BS3 and GS or between BS1 and BS2. Currently, genetic testing is the main diagnostic tool and it is by now feasible for all known genes responsible for GS and BS, although expensive and time-consuming. Some patients, however, remain without a genetic explanation for their phenotype (the mutation detection rate is ~80%), due to the very large number of private mutations [9] and the possibility of a mutation located in intronic and regulatory regions not analyzed or in new candidate genes. Diagnostic tests with diuretics have been proposed, but they are cumbersome and have no established cut-off values [10]. Moreover, the relationships between mutations and the molecular mechanism of the diseases have not been fully clarified. For all these reasons, we propose a new diagnostic phenotypic approach based on the UE molecular study.

Thanks to the availability of a cohort of patients with genetically well-characterized SLTs, we focussed on the most common form of SLTs, the GS, with its dysfunctional NCC protein and on BS1, with the NCC homologue protein NKCC2, because they are both luminal transporters in tubular cells.

**MATERIALS AND METHODS**

Milli-Q water was used for all solutions. Bicinchoninic acid (BCA) protein assay, methanol, bovine serum albumin (BSA) and N-cyclohexyl-3-aminopropanesulfonic acid were from SIGMA Chemical Co. (St. Louis, MO, USA); glycerol was from Merck (Darmstadt, Germany). Hybond-enhanced chemiluminescence (ECL) nitrocellulose membrane was from GE (Little Chalfont, Buckinghamshire, UK). NuPAGE® SDS-PAGE Gel Electrophoresis System components (mini-gels, running and loading buffer, molecular weight markers and Coomassie Blue staining) were supplied by Life Technologies (Paisley, Renfrewshire, UK). Anti-protease inhibitor cocktail (Complete) was from Roche (Monza, Italy). Anti-Flotillin-1 (FLOT1) monoclonal antibody (mAb) was purchased from Transduction Laboratories (Lexington, KY, USA); anti-aquaporin-2 (AQP2) mAb from Cell Signaling Technology (Beverly, MA, USA); anti-Tumour Susceptibility Gene 101 (TSG101), anti-Motility-related protein 1 (CD9) mAbs and anti-NCC polyclonal antibody (pAb) from Abcam (Cambridge, UK); anti-NKCC2 pAb from SIGMA Chemical Co. (St. Louis, MO, USA). Species-specific secondary peroxidase conjugated antibodies and ECL reagents were from Pierce (Rockford, IL, USA).

**Patients**

A total of 36 patients were included in the study (belonging to 33 unrelated families); all had normotensive and chronic ionic disturbances of tubular/uncertain origin, and their final diagnosis was GS in 19 patients, BS in 13 (5 of type 1, 3 of type 2 and 5 of type 3), unclassified in 4. The patients were studied and followed up at San Leopoldo Mandic Hospital, Merate (Italy). The diagnosis was supported by genetic testing, disclosing significant mutations of pertinent genes in homozygous or compound heterozygous state (Table 1). One patient with a single heterozygous mutation was also included in the GS group, because GS mutations may sometimes be missed [11]. Moreover, 22 healthy sex- and age-matched controls (Ctrl) were studied (Table 2 and Supplementary data, Table S1).
Molecular diagnostics

Genetic testing was carried out on genomic DNA from peripheral blood cells according to published methods for the SLC12A3 [12, 13], SLC12A1 [14], CLCNKB [15, 16] and KCNJ1 [17] genes by means of direct sequencing of the coding regions and the intron–exon boundaries. For the CLCNKB and SLC12A3 genes, a Multiplex Ligation Probe Assay analysis was performed to detect large rearrangements. The gene most likely to account for the phenotype in individual patients was analyzed.

### Table 1. Genetic data of GS, BS and unclassified patients

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age</th>
<th>Mutations*</th>
<th>Effect on protein</th>
<th>Mutation type</th>
<th>Gene affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gitelman</td>
<td></td>
<td></td>
<td>c.[1196_1202dup7 bp];[1424C&gt;G]</td>
<td>p.[Ser402*];[Ser475Cys]</td>
<td>N/M</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5GE01^</td>
<td>M</td>
<td>10</td>
<td>c.[1196_1202dup7 bp];[1424C&gt;G]</td>
<td>p.[Ser402*];[Ser475Cys]</td>
<td>N/M</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5SA05^</td>
<td>F</td>
<td>17</td>
<td>c.[1175C&gt;T];[1844C&gt;T]</td>
<td>p.[Thr392Ile];[Ser615Leu]</td>
<td>M/F</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5SB05^</td>
<td>F</td>
<td>17</td>
<td>c.[1924C&gt;G];[2981G&gt;A]</td>
<td>p.[Arg642Gly];[Cys994Tyr]</td>
<td>M/M</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5PG97</td>
<td>F</td>
<td>13</td>
<td>c.[283delC];[2612G&gt;C]</td>
<td>p.[Ala877fs];[Arg871Pro]</td>
<td>F/M</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5SA72</td>
<td>F</td>
<td>38</td>
<td>c.[1175C&gt;T];[1844C&gt;T]</td>
<td>p.[Thr392Ile];[Ser615Leu]</td>
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<td>SLC12A3</td>
</tr>
<tr>
<td>5PG97</td>
<td>F</td>
<td>13</td>
<td>c.[283delC];[2612G&gt;C]</td>
<td>p.[Ala877fs];[Arg871Pro]</td>
<td>F/M</td>
<td>SLC12A3</td>
</tr>
<tr>
<td>5AS72</td>
<td>F</td>
<td>38</td>
<td>c.[1175C&gt;T];[1844C&gt;T]</td>
<td>p.[Thr392Ile];[Ser615Leu]</td>
<td>M/M</td>
<td>SLC12A3</td>
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</table>

Bartter type 1

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age</th>
<th>Mutations*</th>
<th>Effect on protein</th>
<th>Mutation type</th>
<th>Gene affected</th>
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<tbody>
<tr>
<td>3GG07</td>
<td>M</td>
<td>4</td>
<td>c.[730dupG];[1432G&gt;A]</td>
<td>p.[Arg244fs];[Gly478Arg]</td>
<td>F/M</td>
<td>SLC12A1</td>
</tr>
<tr>
<td>1TR02</td>
<td>M</td>
<td>9</td>
<td>c.[551T&gt;A];[611T&gt;C]</td>
<td>p.[Leu184Gln];[Val204Ala]</td>
<td>M/M</td>
<td>SLC12A1</td>
</tr>
<tr>
<td>6BA09</td>
<td>F</td>
<td>5</td>
<td>c.[572T&gt;A];[1493C&gt;T]</td>
<td>p.[Ile191Asn];[Ala498Val]</td>
<td>M/M</td>
<td>SLC12A1</td>
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Bartter type 2

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<th>Mutations*</th>
<th>Effect on protein</th>
<th>Mutation type</th>
<th>Gene affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2GA04^</td>
<td>M</td>
<td>6</td>
<td>c.[572C&gt;T];[572C&gt;T]</td>
<td>p.[Thr191Ile];[Thr191Ile]</td>
<td>M/M</td>
<td>KCNJ1</td>
</tr>
<tr>
<td>2GN03^</td>
<td>M</td>
<td>7</td>
<td>c.[572C&gt;T];[572C&gt;T]</td>
<td>p.[Thr191Ile];[Thr191Ile]</td>
<td>M/M</td>
<td>KCNJ1</td>
</tr>
<tr>
<td>2ED04</td>
<td>M</td>
<td>3</td>
<td>c.[572C&gt;T];[572C&gt;T]</td>
<td>p.[Thr191Ile];[Thr191Ile]</td>
<td>M/M</td>
<td>KCNJ1</td>
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</table>

Bartter type 3

<table>
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<th>Sex</th>
<th>Age</th>
<th>Mutations*</th>
<th>Effect on protein</th>
<th>Mutation type</th>
<th>Gene affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>3BS07</td>
<td>F</td>
<td>4</td>
<td>c.[725C&gt;A];[1-?_2064+?del]</td>
<td>p.[Arg242Glu];[0]</td>
<td>LD/LD</td>
<td>CLCNKB</td>
</tr>
<tr>
<td>3SR82</td>
<td>F</td>
<td>29</td>
<td>c.[2-?_2064+?del];[1-?_2064+?del]</td>
<td>p.[0];[0]</td>
<td>LD/LD</td>
<td>CLCNKB</td>
</tr>
<tr>
<td>3BD82</td>
<td>M</td>
<td>30</td>
<td>c.[160T&gt;C];[?]</td>
<td>p.[Leu542Pro];[?]</td>
<td>M/-</td>
<td>SLC12A3</td>
</tr>
</tbody>
</table>

Unclassified

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sex</th>
<th>Age</th>
<th>Mutations*</th>
<th>Effect on protein</th>
<th>Mutation type</th>
<th>Gene affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>6CM96</td>
<td>F</td>
<td>15</td>
<td>c.[572C&gt;T];[572C&gt;T]</td>
<td>p.[Thr191Ile];[Thr191Ile]</td>
<td>M/M</td>
<td>KCNJ1</td>
</tr>
<tr>
<td>6CG84</td>
<td>M</td>
<td>27</td>
<td>c.[572C&gt;T];[572C&gt;T]</td>
<td>p.[Thr191Ile];[Thr191Ile]</td>
<td>M/M</td>
<td>KCNJ1</td>
</tr>
</tbody>
</table>

[*], undetected mutations; del, deletion; dup, duplication; M, missense mutation; N, nonsense mutation (stop); F, frameshift mutation; LD, large deletion; [0], putative null protein production because of complete deletion of the gene; c.[?], cDNA data not available because analyzed previously in a different laboratory. ^Sibling.

Genetic variants are shown for the two alleles.

§The mutation c.1925G>A yielded two transcripts in the expression study of Riveira-Munoz et al. [9].

### Table 2. Demographic description of subject by diagnosis

<table>
<thead>
<tr>
<th>N</th>
<th>Age</th>
<th>Male gender, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>22</td>
<td>11.9</td>
</tr>
<tr>
<td>GS</td>
<td>19</td>
<td>15.9</td>
</tr>
<tr>
<td>BS1</td>
<td>5</td>
<td>11.4</td>
</tr>
<tr>
<td>BS2</td>
<td>3</td>
<td>5.3</td>
</tr>
<tr>
<td>BS3</td>
<td>5</td>
<td>18.8</td>
</tr>
</tbody>
</table>
first; however, in the case of negative results or of heterozygous mutations for a single gene, and in the presence of strong clinical suspicion, also other pertinent genes were evaluated.

**Study design of UE**

All of the subjects were studied either as outpatients (mostly adults) or inpatients during a routine day hospital evaluation. Informed consent, approved by the Local Research Ethics Committee, was signed by the patients and healthy control subjects, or their parents. Second morning urine samples (~50 mL) were collected, according to the guidelines provided by the Human Kidney and Urine Proteome Project (HKUP, http://www.hkupp.org) and European Kidney and Urine Proteomics organization (EuroKUP, http://www.eurokup.org). All samples were centrifuged for sediment removal (10 min at 1000 g, 4°C) within 4 h from the collection and stored at ~80°C after the addition of protease inhibitors (Complete, Roche). An aliquot of the collected urines was submitted to routine chemical–physical examination. Moreover, a creatinine assay (Jaffé method, Roche) was performed on individual urine samples to normalize for differences in urine concentration [18].

Exosomes were prepared from the stored urine samples by ultracentrifugation [18] according to HKUPP (http://www.hkupp.org) and EuroKUP (http://www.eurokup.org). Briefly, thawed urines were centrifuged for 15 min at 17 000 g, 4°C, to eliminate large membrane fragments and debris. Supernatants were subjected to ultracentrifugation for 1 h at 200 000 g, 4°C: crude exosome pellets were washed in phosphate buffered saline solution and then resuspended in bidistilled water, in the presence of protease inhibitors. The exosome samples were stored at ~80°C until use. Moreover, urine samples after sediment removal (U), and supernatants after 200 000 g ultracentrifugation (Sn), were analyzed as well. To concentrate proteins, U and Sn samples were subjected to ultrafiltration using VivaSpin 500 devices (Sartorius), pre-treated with 5% Triton X-100 for improved recovery of low-concentrated samples, according to the manufacturer’s instructions [18]. Protein concentration was assessed by BCA assay (SIGMA Chemical Co), using BSA as standard.

**Electrophoresis and western blotting**

Equal amounts (10 µg) of exosomal U and Sn proteins were separated by 4–12% NuPAGE (Life Technologies), stained by Coomassie Blue or transferred to nitrocellulose membranes, and the blots were developed as described [18]. Densitometric analysis was performed by ImageQuant TL software (GE Healthcare): the volumes of band proteins were expressed as protein bands was similar in U and in Sn; instead, UE showed the different distribution of NCC and NKCC2 UE densitometric values, among all different groups (controls, GS, BS1, BS2 and BS3) was evaluated by the Kruskal–Wallis test, while the Mann–Whitney test was used for two by two comparison. To evaluate the diagnostic performance of NCC UE densitometric values to discriminate GS patients, we draw the receiver operating characteristic (ROC) curve and estimated its area under the curve (AUC) (GraphPad Prism 5, GraphPad Software, Inc.). As an exploratory analysis, we identified an optimal cut-off as the one maximizing the Youden index (sensitivity + specificity – 1).

We classified the mutations of GS patients (missense, M; nonsense (stop), N; frameshift, F; large deletion, LD) in three groups of severity: severe (F/F; F/LD), intermediate (F/M; LD/M; N/M) and mild (M/M; M/L). The results were plotted as a box plot of NCC levels by severity, considering also normal levels (healthy controls).

**RESULTS**

**Case studies**

GS and BS patients displayed alterations of laboratory tests suggestive for SLTs: hypokalaemia, hypo-/hypercalciuria, normo-/hypomagnesaemia and metabolic alkalosis (Figure 1). All patient and control subject urines were negative for proteins (except for two GS patients, who had mild proteinuria, <37 mg/dl), glucose, ketone, bilirubin, urobilinogen and blood (data not shown). They were not subject to any diagnostic test with diuretics, because its extensive use has several practical limitations [10].

In our cohort of patients, the majority of mutations in SLC12A1, SLC12A3 and KCNJ1 genes were point mutations (missense, nonsense, splice site and small deletions/insertions), whereas in CLCNKB gene the most frequent mutation was the deletion of the entire gene (Table 1). No hot spot could be identified. All of the variants were considered loss-of-function mutations. Twenty-two (on a total of 57 allele mutations found in 29 families) were nonsense, frameshift, splice site mutations and gross deletions, leading to a stop codon. The remaining 35 missense allele mutations were either previously published as inactivating mutations or are novel variants targeting on amino acids conserved among the species, evaluated as pathogenetic by the application of in silico prediction models [9, 11–13, 20–32]. None of the novel mutations were found in 100 control chromosomes.

**UE isolation and characterization**

After urine collection and urinary vesicle isolation, the pattern of isolated vesicles was analysed by NuPAGE, followed by Coomassie Blue staining (Figure 2). The distribution of protein bands was similar in U and in Sn; instead, UE showed a different and typical protein pattern: in fact albumin, the main band in the non-exosomal fractions, was depleted, while the Tamm–Horsfall protein (THP), a glycoprotein released by renal cells, was predominant in the UE profile. We did not apply any THP depletion method, because in preliminary experiments it caused lower UE recovery (data not shown). Moreover, apart from THP, the exosomal protein composition was similar along the different control samples and was not substantially affected by the age of children at the time of collection (Supplementary data, Figure S1).

To further validate the exosome purification protocol, we evaluated four commonly used UE markers, FLOT1, TSG101, CD9 (ubiquitary exosomal markers) and AQP2 (specific for urine) [1]. The results showed that the markers were highly
enriched in the vesicle fraction of patients and controls, compared with U or Sn, where their signals were nearly undetectable (Figure 3A). The assessment of UE protein markers was extended to UE isolated from all cases and demonstrated that their purity was comparable in all the preparations (Figure 3B).

**NCC and NKCC2 in UE**

The NCC and NKCC2 transporters were easily detectable in control UE but not in urine from which they derive (Figure 4A). The NKCC2 signal was detected as a doublet, due to glycosylation [33]. The NCC signal was almost undetectable in the GS patient UE, while present and clearly visible in those of the control subjects; the same pattern was observed for NKCC2, absent in BS1 UE, while easily detectable in those of the control subjects (Figure 4B). UE NCC and NKCC2 levels differed among the groups considered (Kruskal–Wallis test, P < 0.0001), after band densitometric analysis (Table 3 and Supplementary data, Table S1). In particular, NCC levels were very low in GS patients compared with the other groups and NKCC2 were very low in BS1 patients, suggesting that it is possible to discriminate among the different syndromes (Figure 4C and Table 3). We also verified that NCC and NKCC2 levels did not differ by age and sex, neither in cases nor in controls (data not shown).

A ROC curve was constructed using UE NCC densitometric values and comparing GS patients versus Not-GS subjects (controls and BS patients). The AUC value was 0.92 (95% CI 0.84–1.00). Considering as cut-off 21.3 (optimal by Youden index), sensitivity was 84.2% and specificity was 88.6% (Figure 5 and Supplementary data, Table S2). To mimic a situation in which only ‘suspected’ patients are submitted to the test, we compared GS patients with the BS ones only (BS1 + BS2 + BS3), the AUC value was calculated as 0.88, (95% CI 0.76–1.00). Using the same cut-off, sensitivity resulted 84.2% and specificity 76.9%, still pointing out a good discriminating power of the test (Supplementary data, Table S2).

We took into consideration the level of the impairment of NCC protein that could be expected, based on the severity of the mutations. The results showed that, when both alleles carry a severe mutation (nonsense, frameshift and large deletions) leading to a stop codon, no signal was distinguished.
from background in UE, and the densitometric values were equal to zero, while the signal increased in the presence of one or two missense mutations (Figure 6 and Supplementary data, Table S1). The same interesting remark can be made for the NKCC2 protein, although the population studied is too small to draw any conclusions: among the five genetically determined BS1 patients, the two patients showing a frameshift mutation display an OD equal to zero (Supplementary data, Table S1).

Of note, we tested also some patients whose diagnosis was suggested by the clinical symptoms and biochemistry, but were negative to genetic tests: in particular, 6CG84 appears clinically as a GS patient, and despite negative genetic outcome on SLC12A3 gene, the NCC protein is almost undetectable in UE (OD/uCr = 17). Conversely, 6GI09 is an SLT patient with atypical presentation and early onset at 2 months: no mutation could be identified in CLCNKB, SLC12A1 and KCNJ1 genes. As both NCC and NKCC2 were detected in UE (131 and 303 OD/uCr, respectively), we can rule out GS and BS (Supplementary data, Table S1). Another puzzling case is 6CM96: the clinician excluded BS3/GS based on clinical and biochemical data and was undecided between BS1 and BS2. However, the visible NKCC2 signal in UE (OD/uCr = 105) suggests that BS1 may definitely be excluded.

**DISCUSSION**

This study highlights the role of UE in the diagnosis of GS and BS. Recently, UE-based approaches have emerged as tools for the detection of relevant biomarkers in kidney diseases [18, 34–39]. Indeed, UE sampling allows the detection of low abundance membrane proteins (potential markers) [3] that otherwise would be masked by soluble species. In fact, this study demonstrated a reproducibly high enrichment of the involved transporters, expressed on the luminal membranes [8], in UE in comparison with whole urine. Moreover, it was reported that the abundance of some proteins in UE correlates with their content in the kidney, as in the case of NCC and NKCC2 [40].

In fact, it has been suggested that UE analysis may be useful for the classification of disease processes involving the renal tubule, i.e. GS and BS [40–46]. Recently, some papers, focussing on hypertension, showed that the evaluation of urinary exosomal NCC could provide an estimation not only of its content in the tubular cells, but also of NCC biological activity *in vivo*, affected by its phosphorylation state [40, 45, 47]. However, we did not address the issue of phosphorylation or activity impairment in our study, because all GS patients showed very low or undetectable NCC signals in UE and did not carry any mutation on phosphorylation sites.

This pilot study shows that it is possible to recognize and discriminate GS and BS1 patients from other SLTs by using UE analysis, thus providing a basis for a simple and reliable test for those patients with SLT suggestive signs and symptoms. It has to be emphasized that the patients were carefully characterized from the clinical, biochemical and genetic point of view. Moreover, the urine collection from patients and controls was accomplished by following a rigorous protocol, specifically established by the HKUPP and EuroKUP, for exosome proteomic analysis.
Raj et al. [48] showed that some exosomal proteins have different abundance in relation to age. However, in our patient and control cohort, stratification of NCC and NKCC2 levels by age and sex did not show any significant variation.

Nevertheless, a partial overlap of some values among control and patient groups (e.g. controls with low UE NCC level) was shown, likely due to biological and analytical variability. However, it has to be underlined that overlapping distributions also occur using common diagnostic tests, such as prostate-specific antigen, where the presence of a number of outliers between the two populations under comparison does not affect the overall reliability of the test. The ROC curve showed a good diagnostic utility of UE NCC evaluation when comparing GS patients versus controls and Not-GS subjects. Nevertheless, we

**Figure 4:** Ion transporters in UEs. (A) Immunoblotting of NCC and NKCC2 in vesicle fractions (Exo), in comparison with total urine after sediment removal (U), and supernatant (Sn) obtained after 200,000 g ultracentrifugation from a representative control subject. NCC level in UEs. (B) Immunoblotting of NCC and NKCC2 in UEs of Ctrl, GS, BS1, BS2 and BS3 patients (two representative cases for each group). Equal amounts of proteins were loaded on all lanes of each gel. (C) Densitometric analysis of NCC and NKCC2 signals normalized by uCr values. Box-and-whiskers plot of all patient data. Not-GS = BS1 + BS2 + BS3 patients; Not-BS1 = GS + BS2 + BS3 patients. Statistical analysis (Mann–Whitney t-test): *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

Table 3. Descriptive statistics of NCC and NKCC2 level (OD/uCr) in UE isolated from control and patients

<table>
<thead>
<tr>
<th></th>
<th>NCCa</th>
<th>NKCC2a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Ctrl</td>
<td>22</td>
<td>122.7</td>
</tr>
<tr>
<td>GS</td>
<td>19</td>
<td>13.65</td>
</tr>
<tr>
<td>BS1</td>
<td>5</td>
<td>104.4</td>
</tr>
<tr>
<td>BS2</td>
<td>3</td>
<td>882.6</td>
</tr>
<tr>
<td>BS3</td>
<td>5</td>
<td>29.20</td>
</tr>
</tbody>
</table>

SE, standard error.

aSignificant differences observed using the Kruskal–Wallis test (P < 0.0001).
bP-value from the Mann–Whitney test comparing the level of NCC with the GS group as reference and of NKCC2 with BS1.

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need a larger cohort of patients to definitively identify and validate an appropriate cut-off value and consequently to establish sound predictive values.

We speculated about the application scenario of this potential test. First, it may be used as a preliminary test for the exclusion of GS and BS1 diagnosis especially in adult patients referred to nephrology units for hypokalaemia. Indeed, a negative result from genetic investigation of SLC12A3 gene is more frequent in adults (35%) versus paediatric patients (19%) [personal observation]. In such cases, if the NCC transporter signal is present in UE, the diagnostic flowchart may be changed and the genetic test may be spared.

Furthermore, this UE-based test could be useful in the differential diagnosis between GS and BS3, one of the critical points of SLTs diagnosis, given their phenotypic (i.e. clinical and biochemical) similarity. In fact, even if a partial overlapping of NCC levels between GS and BS3 UE can be observed, the mean values resulted statistically different (Mann-Whitney t-test, \( P = 0.0162 \)); actually, it may reflect the tightly coupled ion transport mechanisms where mutations affecting one element of transepithelial transport lead to the complete breakdown of absorption in the affected epithelial cells, involving both NCC and the chloride channel ClC-Kb \([8]\). In particular, after a negative genetic screening of SLT genes, two situations can be hypothesized. If NCC and NKCC2 are detectable in the UE, GS and BS1 can be excluded; therefore, the hypothesis of a possible involvement of further primary causative genes of BS3 is reinforced as well as that of mutations in secondary genes (modifying genes). On the other hand, in the case of absence of NCC at the UE level, as in patient 6CG84, we can first avoid the in-depth BS3 gene analysis and second hypothesize defects in SLC12A3 regulatory regions or the involvement of epigenetic mechanisms (miRNAs) \([49]\).

Moreover, we suggest the potential application of UE testing in the differential diagnosis between BS1 and BS2 as well. As shown for patient 6CM96, negative to all screened Bartter genes, the presence/absence of NKCC2 in UE may address the diagnosis.

Despite these promising results, a limiting point of this preliminary study remains the low number of BS patients, explained by the low incidence of these genetic diseases. This point, coupled to the not so practical exosome isolation and immunoblot analysis, impairs an easy transition to clinical laboratory, partially solvable by the developing of immunometric formats, such as enzyme-linked immunosorbent assay \([45]\) or protein microarray.

A final observation, worthy of further studies, concerns the correlation of the type of gene mutation with the exosomal level of NCC protein. Our data suggested an inverse trend between the content of NCC protein and the severity of the mutations, showing the potential of UE analysis as a new approach for SLT molecular characterization.

In conclusion, our results indicate that the strategy consisting of phenotyping the UE shows diagnostic potential, providing an alternative, urine-based, approach for SLT recognition and a diagnostic guidance in complex cases. Moreover, we believe that the in-depth characterization of the UE-derived proteins involved in SLTs will improve the understanding of their pathophysiological mechanisms and their potential future correction.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.
AKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

All the authors declared no competing interests.

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Impact of westernization on fibroblast growth factor 23 levels among individuals of African ancestry

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ABSTRACT

Introduction. The Western diet is associated with high consumption of processed foods preserved with phosphate. Higher dietary phosphate consumption stimulates production of fibroblast growth factor 23 (FGF23), which heightens risk for cardiovascular disease and mortality. We hypothesized that adults living in a more westernized society have higher levels of FGF23 due to increased phosphate consumption as measured by urinary phosphate excretion.

Methods. We measured plasma C-terminal FGF23 levels and urinary phosphate and creatinine levels in timed urine collections among 100 African adults living in the rural area of Igbo-Ora, Nigeria (52 women, 48 men), and 100 African Americans (32 women, 68 men) living in Maywood, IL, an urban suburb of Chicago, IL, USA. Among these 200 participants, urine collections were adequate in 76 and 68 of the Maywood and Igbo-Ora participants, respectively.

Results. In the total group, the mean age and body mass index, respectively, were 34.6 ± 8.2 years and 22.1 ± 3.9 kg/m² in Igbo-Ora, and 42.8 ± 7.2 years and 25.8 ± 6.5 kg/m² in Maywood. Demographic characteristics for each site were very similar after excluding participants without adequate urine collections. Among participants with adequate urine collections, the mean 24-h urinary phosphate excretion was 283–290.

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