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Running Title: Urinary extracellular vesicles and lithium in TIH

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Abstract

Introduction: Thiazide diuretics are amongst the most widely used anti-hypertensive medicines worldwide. Thiazide-Induced Hyponatremia (TIH) is one of their most clinically significant adverse effects. A priori TIH must result from excessive saliuresis and/or water reabsorption. We hypothesised that pathways regulating the thiazide-sensitive NCC and the water channel AQP$_2$ may be involved. Our aim was to assess whether patients with TIH show evidence of altered NCC and AQP$_2$ expression in urinary extracellular vesicles (UEVs), and also whether abnormalities of renal sodium reabsorption were evident using Endogenous Lithium Clearance (ELC).

Methods: Blood and urine samples were donated by patients admitted to hospital with acute symptomatic TIH, after recovery to normonatremia, and also from normonatremic controls on and off thiazides. UEVs were isolated and target proteins evaluated by Western blotting and by Nanoparticle Tracking Analysis (NTA). ELC was assessed by Inductively Coupled Plasma Mass Spectrometry.

Results: UEVs analysis by Western blotting showed that patients with acute TIH display reduced total NCC and increased phospho-NCC and AQP$_2$ relative to appropriate control groups; smaller differences in NCC and AQP$_2$ expression persisted after recovery from TIH. These findings were confirmed by NTA. Renal ELC was lower in acute TIH compared to controls and convalescent cases.

Conclusion: Reduced NCC expression and increased AQP$_2$ expression would be expected to result in saliuresis and water reabsorption in TIH patients. This study raises the possibility that UEV analysis may be of diagnostic utility in less clear cut cases of thiazide associated hyponatremia and may help identify those at risk of TIH before thiazide initiation.

Keywords
1. Thiazide
2. Diuretic
3. Sodium
4. Hyponatremia
5. Hypertension
6. Urinary extracellular vesicles
Introduction

Thiazide diuretics have been used in the management of hypertension for more than half a century\(^1\). They lower blood pressure by inhibition of the sodium-chloride cotransporter, NCC, in the Distal Convoluted Tubule (DCT) and have all-cause mortality benefits equivalent to ACE inhibitors and calcium channel antagonists\(^1,2\). Although thiazides are generally well tolerated, their use is limited in a minority of patients due to hyponatremia\(^3\). Owing to the very large number of patients prescribed thiazides, Thiazide Induced Hyponatremia (TIH) is the most common cause of drug-induced hyponatremia requiring hospitalization\(^4,5\).

The mechanisms underlying TIH remain unclear. Risk factors for TIH include advanced age, reduced body mass and concurrent use of other medications which impair water excretion\(^6\). A priori TIH must result from excessive saliuresis and / or water reabsorption (or ingestion). It therefore seems likely that excessive saliuresis and / or water reabsorption (or ingestion) occurs via altered regulation of NCC and/or AQP\(_2\). As thiazide diuretics have not, to the best of our knowledge, been reported to produce saliuresis with a urinary sodium concentration greater than plasma, this suggests that excessive water reabsorption (or ingestion) is a fundamental part of the pathogenesis of TIH. Our aim in this study was to probe the molecular mechanisms underlying TIH by investigating whether patients with TIH exhibit evidence of altered regulation of NCC and AQP\(_2\).

NCC is located in the renal cortex, therefore thiazides reduce maximal urinary diluting ability by increasing sodium excretion without affecting the renal medullary sodium gradient required for water reabsorption from the collecting duct\(^7\). NCC exists in three isoforms\(^8\). NCC\(_{1\&2}\) vary by only 1 amino acid\(^8\). NCC\(_3\) however is 9 amino acids longer; it constitutes approximately 40% of NCC is the human nephron. NCC regulation occurs principally by two mechanisms: regulation of apical membrane abundance (Type 1 regulation) and regulation of transporter kinetics (Type 2 regulation), the latter being controlled by phosphorylation of key N-terminal serine and threonine residues (Thr 60, Thr 55 and Ser 91)\(^9,10\). Urinary concentration principally occurs through the vasopressin-AQP\(_2\) pathway in the collecting duct\(^11-13\). Moreover, microperfusion studies suggest thiazides may induce upregulation of aquaporin-2 (AQP\(_2\)), increasing the water permeability of the collecting duct\(^14,15\). Locally derived Prostaglandin E\(_2\) (PGE\(_2\)) is a key
modulator of this pathway\textsuperscript{16,17} and its reuptake into collecting duct cells is determined by a PGE\textsubscript{2} specific ProstaGlandin Transporter (PGT). A recent study has suggested modest genetic associations with TIH including aberrant PGE\textsubscript{2} reuptake in the distal nephron which may increase AQP\textsubscript{2}-mediated water reabsorption in a subset of patients who carry a variant within \textit{SLCO2A1}, the gene encoding PGT\textsuperscript{18}.

Urinary Extracellular Vesicles (UEVs) are spherical, structured membrane vesicles which are formed by cells throughout the nephron\textsuperscript{19}. Because UEVs contain cell derived membrane transporters and channels, their analysis is an attractive and non-invasive method to study renal tubular pathophysiology in patients\textsuperscript{20-22}.

Since lithium is absorbed along with sodium throughout the nephron it is possible to use measurement of lithium clearance to assess tubular sodium reabsorption. Renal lithium clearance likely reflects mostly proximal tubular sodium reabsorption because this is where the majority of sodium is reabsorbed\textsuperscript{23}. Measuring Endogenous Lithium Clearance (ELC) is preferable to exogenous lithium clearance following ingestion of lithium tablets, since it negates any potential effect which therapeutic doses of lithium may have on distal nephron physiology including water trafficking\textsuperscript{24} and is also more convenient for research volunteers.

To fulfil our aim of investigating whether patients with TIH exhibit evidence of altered regulation of NCC and AQP\textsubscript{2} we report analysis of urinary extracellular vesicle NCC and AQP\textsubscript{2} in TIH patients and controls. We also report assessment of renal tubular sodium reabsorption in TIH patients by measurement of endogenous lithium clearance.

**Methods**

**Clinical recruitment**

This study was conducted in line with the standards of ICH/Good Clinical Practise sections 8.2.8 in adherence to the Declaration of Helsinki and was approved by the UK national research ethics committee (reference 11/EM/0233). Informed consent was given by all participants. Between April 2012 and August 2015 a daily search of the biochemistry data base of all patients admitted to the department of internal medicine at Nottingham University Hospitals NHS Trust, UK was undertaken to identify those patients with serum sodium <130mM (cases). Patients were reviewed by the
investigators to establish those whose hyponatremia was due to a thiazide diuretic (TIH). Normonatremic thiazide and non-thiazide controls (serum sodium 135-145 mM) were identified by primary care surgeries in Nottinghamshire and matched as closely as possible to the cases for age, sex, comorbidities and polypharmacy. TIH cases were also assessed in the outpatient clinic two months after thiazide cessation (termed normonatremic TIH cases off thiazides). The cases here were a subset of those previously reported\(^\text{18}\) and are composed of the small number of acutely hyponatremic TIH patients who were able to produce a mid-morning spot urine sample suitable for the current study.

**Urine collection and processing**

Second morning spot urine samples (30ml) were collected in sterile containers from TIH patients and control groups (Supplementary Table 1). Protease inhibitor cocktail tablets (Roche Diagnostics) were added to urine samples immediately following collection and samples stored at -80\(^\text{°}\)C. Fresh urine samples were processed the same day.

**UEV isolation and total protein estimation**

Urinary nanovesicles, including UEVs were isolated from both fresh and frozen urine by Ultrafiltration (Vivaspin 20 MWCO 100 kDa - Sartorius Stedim, UK). Before processing of urine samples, nanomembrane concentrators were washed with PBS buffer to remove glycerol and other preservatives and then centrifuged at 2500 g for 5 min at RT. Frozen urine samples were thawed on ice, 1ml DTT (100 mg/ml final concentration) was added, extensively vortexed and incubated for 30 mins at 37\(^\text{°}\)C before UEVs isolation. All samples were centrifuged (2500 g for 15 min at 4\(^\text{°}\)C) for urinary cells removal. Cell pellet (CP) was suspended in lysis buffer (1.5% SDS and 50 mM Tris-HCl with protease inhibitor, pH 6.8) and stored at -80\(^\text{°}\)C. Further filtration of supernatant using 0.22 µm filters (Millipore, UK) was undertaken to remove debris. 25 mL of filtered supernatant was centrifuged at 15000 g for 30 min at 4\(^\text{°}\)C. The urine supernatants were then added to Vivaspin nanomembrane concentrators and centrifuged at 4800 g for 1 h. at 4\(^\text{°}\) C. Concentrated UEVs were treated with 50 µl DTT, added directly in to the filters and incubated for 10 min at 37\(^\text{°}\) C. Isolated urinary extracellular vesicles were directly suspended in lysis buffer and stored at -80\(^\text{°}\)C. Total urine (TU) and flow through (FT) fractions were also stored at -80\(^\text{°}\)C and -20\(^\text{°}\)C. Total soluble protein concentration was quantified with BCA assay (Pierce®, Thermo Scientific, UK).
Electrophoresis and Western immunoblotting

25µg total soluble protein per sample was electrophoresed on 7.5% (NCC and PNCC), 10% (ALIX and PGT) and 12.5% (AQP₂) acrylamide gels. SDS-PAGE was performed on PVDF membrane (Bio-Rad, UK). UEVs AQP₂ was detected by probing the blots with 1:500 diluted anti-AQP₂ antibody (Sigma, UK) followed by anti-rabbit IgG HRP-conjugated antibody (Sigma, UK). UEVs NCC₃ was probed with 1:500 diluted anti-NCC primary antibody (Millipore, UK) followed by anti-rabbit IgG HRP-conjugated antibody (Sigma, UK). UEVs NCC₃₁&₂ was probed with 1:500 diluted anti-NCC primary antibody (21st Century Biochemicals, USA) followed by anti-rabbit IgG HRP-conjugated antibody (Sigma, UK). UEVs PNCC (phosphorylated at T55, T60 and S91) was detected by probing blots with anti-PNCC antibodies (University of Dundee, UK), each at 1:500 (0.2–1.2 µg/ml) followed by anti-sheep IgG HRP-conjugated antibody (Sigma, UK). To detect specific phosphorylated forms of NCC, the corresponding nonphosphorylated peptide was incubated with primary antibody (10 µg/ml). UEVs PGT was probed with 1:200 diluted anti-PGT primary antibody (Cayman, UK) followed by anti-rabbit IgG HRP-conjugated antibody (Sigma, UK). The anti-AQP2 and NCC antibodies used target intracellular domains of AQP2 and NCC. Vesicles did not undergo prior permeabilisation. Urinary cell pellet was used as a negative control for Western blots. UEVs ALIX was detected by probing the blots with 1:1000 diluted anti-ALIX antibody (Abcam, UK) followed by anti-rabbit IgG HRP-conjugated antibody (Sigma, UK). Western blot data were corrected for ALIX as indicated the relevant figure legends. This method of correction by ALIX is as previously reported27. The immunocomplex was detected by ECL chemiluminescence (GE Healthcare, UK) on X-Omat AR films (Kodak, UK). Normalization was carried using UEVs ALIX and bands were quantified using Image J® software. Density of each band was divided by the band from the same sample stained with amido black. The differential expression of UEVs proteins densitometry values among all different groups was evaluated by using one-way ANOVA followed by Tukeys multiple comparison tests (Graph Pad Prism V6.05). P < 0.05 was considered significant.

Nanoparticle Tracking Analysis

NTA is a light-scatter microscopy method of tracking microparticles and nanoparticles on the basis of direct and real-time tracking of the particles Brownian movement, which results in a description of the particle size and concentration distribution in a given solution. NTA can be used to count and measure specific subgroups of nanoparticles using fluorescent antibodies
against nanoparticle proteins, including extracellular vesicles derived from kidney cells in culture and in urine\textsuperscript{25}.

AQP\textsubscript{2} and NCC-expressing UEVs were analyzed using the NanoSight LM 10 instrument (NanoSight Ltd, Amesbury, United Kingdom). The analysis settings were optimized and kept constant between samples, and each video was analyzed to give the mean, mode, median, and estimated concentration for each particle size. All experiments were carried out at a 1:1000 dilution of unprocessed urine, yielding particle concentrations in the region of $1.3 \times 10^8$ particles/ml in accordance with the manufacturer’s recommendations. For fluorescent NTA analysis, a 532-nm (green) laser diode excited the fluorescent-loaded ECVs with a long-pass filter (430 nm). For quantification of AQP\textsubscript{2} (Millipore, Billerica, MA, USA and NCC (Chemicon USA), primary antibodies were fluorescent-labeled by conjugation to Quantum dots. All samples were analyzed in triplicate to give one value per patient. NCC and AQP\textsubscript{2} data were normalized by urinary creatinine.

**Endogenous Lithium measurement**

Serum and urine samples were prepared for analysis with a 1:25 dilution in 0.5% nitric acid (Romil, Cambridge, UK) containing 0.05% Triton-X 100 (Romil, Cambridge, UK) and 40ng/mL beryllium (Alfa Aesar, Massachusetts, USA) as an internal standard. Measurements were performed on an Agilent 7900 ICP-MS (Agilent Technologies, USA) operated in no-gas mode with the RF generator power set to 950W giving a “cold” plasma. Lithium was measured at m/z ratio of 7 and was normalised to the beryllium internal standard at m/z 9. Calibration solutions were prepared by diluting a 1000 ug/mL lithium standard (Alfa Aesar, Massachusetts, USA) in 2% nitric acid (Romil, Cambridge, UK) to give concentrations of 0.0352, 0.141, 0.563, 2.25, 9 and 36 umol/L. Seronorm trace elements urine quality control materials (Sero, Billingstad, Norway) and an in-house serum control were analysed in each batch.

**Results**

**UEV characterization and validation**

Western blot analysis of AQP\textsubscript{2}, NCC\textsubscript{3} and PGT was undertaken in healthy volunteers to establish (a) the principal urinary fraction(s) these transporters are excreted in (urinary extracellular vesicles (UEVs), Cell Pellet (CP) or Total Urine (TU)) and (b) the optimal
method of storage of urine samples for UEVs analysis (fresh vs. frozen urine stored at either -20°C or -80°C).

The abundance of AQP₂, NCC₃ and PGT was much greater in UEVs than either CP or TU, confirming that UEVs are the most appropriate fraction of urine to study (Figure 1). The abundance of AQP₂, NCC₃, and PGT in UEVs from freshly collected urine was not significantly different to urine which had been stored at -80°C with protease inhibitor (Supplementary Figure 1); however AQP₂ and NCC₃ abundance was significantly less in the UEVs of urine stored at -20°C compared to that from urine stored at -80°C. (Supplementary Figure 2).

Figure 1: Western blot analysis of AQP₂, NCC₃ and PGT in different urinary fractions demonstrates expression predominantly in urinary extracellular vesicles. Abundance of AQP₂ (A), NCC₃ (B) and PGT (C) are shown in Urinary Extracellular vesicles (UEVs), Cell Pellet (CP) and Total Urine (TU) fractions. Blots shown are representative of individual experiments. N=8 in each group. Data are corrected for exosomal expression of ALIX and are shown as mean ± SEM. ****p < 0.0001.

Expression of UEVs AQP₂, NCC and PGT in TIH

The clinical characteristics of TIH patients and controls are shown in Supplementary Table 1. UEV AQP₂ was significantly more abundant in acutely hyponatremic TIH cases on thiazides compared to normonatremic control patients on or off thiazides (Figure 2A).
Even when TIH cases had recovered to normonatremia, AQP\textsubscript{2} expression was still greater than controls (Figure 2A).

UEV NCC\textsubscript{3} and NCC\textsubscript{1&2} were significantly less abundant in acutely hyponatremic TIH cases on thiazides compared to convalescent normonatremic TIH cases off thiazides and normonatremic controls on or off thiazides (Figure 2B and 2C). Although NCC abundance increased in TIH cases on recovery to normonatremia off thiazides, NCC expression was still 30% less than normonatremic controls off thiazide (Figure 2B and 2C).

Extracellular vesicle ProstaGlandin Transporter (PGT) was significantly more abundant in acutely hyponatremic TIH cases on thiazides compared to normonatremic controls on thiazides. Despite reduction in PGT in TIH cases following cessation of thiazide and recovery to normonatremia, PGT expression was still significantly greater than normonatremic controls off thiazides (Figure 2D).

We also used a second method of UEVs analysis (Nanoparticle Tracking Analysis, NTA) to analyse differences in NCC\textsubscript{3} and AQP\textsubscript{2} excretion between these patient groups. NTA has been used previously to quantify the abundance of UEVs containing AQP\textsubscript{2} and NCC\textsubscript{3} in unprocessed urine samples. NTA analysis in our study showed that TIH patients had increased numbers of AQP\textsubscript{2} positive UEVs in both acutely hyponatremic and convalescent normonatremic states compared to controls (Supplementary Figure 3). The numbers of NCC\textsubscript{3} positive UEVs were not significantly different between the groups. NTA analysis confirmed the changes in AQP\textsubscript{2} expression in TIH patients seen by Western blot analysis.
Figure 2: Western blot analysis of AQP2, NCC and PGT in patients with TIH and controls. Abundance of UEV (A) AQP2, (B) NCC3, (C) NCC1&2 and (D) PGT are shown. Blots shown are representative of individual experiments. Data are corrected for exosomal expression of ALIX and are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Hyponatremic TIH cases on thiazides (HC) n=8, normonatremic TIH cases off thiazides (NC) n=16, normonatremic thiazide controls (TC) n=16 and normonatremic non-thiazide controls (NTC) n=16.

Assessment of NCC phosphorylation at three principal N-terminal locations known to accelerate transporter kinetics (T55, T60 and S91) was undertaken. Patients acutely hyponatremic on thiazides with TIH displayed significantly greater phosphorylation at
T55, T60 and S91 than either convalescent normonatremic TIH cases off thiazides or normonatremic controls on thiazides (Figure 3).

**Figure 3: Western blot analysis of N-terminal NCC phosphorylation in TIH:** Abundance of UEV NCC phosphorylation at (A) T60, (B) T55 and (C) S91 is shown in acute TIH cases, convalescent TIH cases, and controls on and off thiazide respectively. Blots shown are representative of individual experiments. Data are corrected for exosomal expression of ALIX and are shown as mean ± SEM. * p < 0.05, **p < 0.01, ***p < 0.001. Hyponatremic TIH cases
Endogenous Lithium clearance in TIH

Renal Endogenous Lithium Clearance (ELC) was lower in acutely hyponatremic TIH cases on thiazides compared to convalescent TIH cases on recovery to normonatremia following thiazide cessation (Figure 4). ELC was also lower in acutely hyponatremic TIH cases compared to normonatremic controls on thiazide (Figure 4).

![Graph: Renal Endogenous Lithium Clearance (ELC) is reduced in acute TIH.](image)

**Figure 4: Renal Endogenous Lithium Clearance (ELC) is reduced in acute TIH.**

ELC was lower in hyponatremic TIH cases on thiazides compared to either the normonatremic TIH cases off thiazides or normonatremic thiazide controls. N=7 in each group. Data represented as mean ± SEM. * p < 0.05, **p < 0.01.

Discussion

Here we report a non-invasive method of obtaining, preserving and analysing urinary extracellular vesicles from volunteer patients suffering from Thiazide Induced Hyponatremia who were admitted to an acute internal medicine department. In addition, analysis of distal nephron sodium, water and prostaglandin transporters contained within the extracellular vesicles suggests a possible mechanism which may underlie the
pathophysiology of TIH. These data add to what has been previously been reported by demonstrating that patients with acute TIH display reduced total NCC and increased phospho-NCC and AQP$_2$ in their UEVs relative to appropriate control groups.

We have shown that the extracellular vesicles fraction is the most appropriate component of human urine in which to study the expression of NCC, AQP$_2$ and PGT. Further we demonstrate that it is not necessary to analyse only fresh urine samples and that samples frozen with protease inhibitor are equally suitable for use. This is important since if analysis was limited to fresh samples, the time consuming sample processing and immediate exosome isolation would make the study of acute medical conditions such as TIH very difficult. Since acquisition of a spot urine sample, addition of protease inhibitor tablet and storage in a freezer can be performed by the majority of clinical and research staff with very limited training required, such sample acquisition from acute medical patients becomes a much more feasible proposition.

A priori TIH must result from excessive saliuresis and/or water reabsorption. As thiazide diuretics have not been observed to produce saliuresis with urinary sodium concentration greater than plasma, excess water reabsorption (or ingestion) is likely to be a fundamental part of TIH pathophysiology. Reduced renal expression of NCC and increased expression of AQP$_2$ in acute TIH would support the hypothesis that the pathophysiology of TIH results from a combination of both of these processes. We have successfully identified both NCC$_{182}$, NCC$_3$ and determined the phosphorylation status of three key N terminal phosphorylation sites known to regulate NCC activity from the urinary extracellular vesicles. In addition reduced Endogenous Lithium Clearance (ELC) in acute TIH supports increased proximal sodium reabsorption reflecting augmented distal nephron sodium loss and so is complementary to the UEV data.

The increased N-terminal phosphorylation state of NCC in acute TIH suggests some degree of physiological compensation to excessive distal nephron sodium loss, by attempting to maximally activate what NCC exists in the DCT. Why type 1 regulation of NCC (apical membrane expression) should be impaired in TIH but type 2 regulation (transporter activation by N terminal phosphorylation) remain intact is unclear. It is notable that thiazides inhibit NCC by binding to the co-transporter from the luminal membrane and one hypothesis may be that the physical binding of thiazides to NCC in
induces a conformational change which targets the cotransporter for endocytosis and degradation in patients susceptible to TIH. Alternatively saliuresis and resulting kaliuresis and trend to hypokalemia seen in acute TIH patients may drive phosphorylation and activation of remaining NCC\textsuperscript{27}.

Thiazides reduce the ability of the late diluting segment (DCT) to generate solute-free water directly and also act by reducing effective vascular volume and thus solute delivery from the end-proximal tubule\textsuperscript{28}. The increased proximal renal sodium reabsorption observed would support a reduction in the amount of fluid available to reach the diluting segments of the nephron, which is then further reduced by the role of thiazides and the observed reduction in NCC expression acting to reduce dilution. Study of NHE3 expression in the proximal tubule may therefore be informative for future studies.

Measuring differences in the UEV profile of acutely hyponatremic patients may also have clinical utility as an aid to TIH diagnosis where other potential contributors to hyponatremia exist. The use of UEV analysis as a diagnostic tool would require further studies to identify whether acute differences in UEV profiling exist in other causes of hyponatremia and whether profiling of a panel of UEV markers could reliably differentiate one cause of hyponatremia from another.

We chose also to study PGT since it is constitutively expressed in the CD where it mediates PGE\textsubscript{2} uptake, one of the most important physiological regulators of distal nephron water reabsorption\textsuperscript{28}. The protein-altering variant in \textit{SLCO2A1} (p.A396T), the gene which encodes PGT was also recently reported to show modest association with TIH\textsuperscript{18}. Here we show that PGT is significantly expressed in UEVs and that PGT expression is substantially elevated in acute TIH. This provides further support that PGT may be involved in the molecular pathways causing inappropriate water reabsorption in TIH.

That differences in the extracellular vesicle profile of convalescent normonatremic TIH cases exist two months after thiazides have been discontinued suggests that patients who are predisposed to TIH may exhibit these urinary extracellular vesicle characteristics before they were exposed to thiazides. Although a prospective study
would be required to confirm this observation, it raises the possibility that pre-
scription profiling of the relative abundance of NCC to AQP\textsubscript{2} could potentially identify
patients at higher risk of TIH. These individuals could then be offered alternative anti-
hypertensive therapy, for example medicines from the ACE inhibitor or calcium channel
antagonist groups or, if a diuretic was required, an alternative loop or potassium sparing
type could be selected instead of a thiazide.

In summary, we show here that TIH patients display reduced NCC expression and
increased AQP\textsubscript{2} expression in their UEVs which would be expected to produce
excessive saliuresis and water resorption. Reduced ELC also suggests compensatory
increased proximal sodium reabsorption in TIH. That differences in the renal
transporters responsible for these processes can be detected in urinary extracellular
vesicle both acutely and following recovery from TIH raises the possibility that such
techniques may have utility in prospectively identifying those at high risk of TIH and in
aiding the acute diagnosis of TIH in cases which are confounded by comorbidity and/ or
polypharmacy.

Disclosure
The authors have declared that no conflict of interest exists.

Supplementary material
1. Supplementary table 1. Clinical characteristics of TIH patients and controls.
2. Supplementary Figure 1: Western blotting of urinary extracellular vesicle demonstrates
that abundance of (A) AQP2, (B) NCC3 and (C) PGT is not significantly different in
fresh (F) vs. frozen (FR) urine.
3. Supplementary Figure 2: Western blotting of urinary extracellular vesicle demonstrates
that abundance of (A) AQP2 and (B) NCC is significantly different in urine stored at -
80^\circ\text{C} vs. -20^\circ\text{C}.
4. Supplementary Figure 3: Nanoparticle Tracking Analysis.
5. Supplementary Figure 4: Western blotting analysis of urinary extracellular vesicle (A)
AQP2 (B) NCC and (C) PGT in TIH patients and controls.
6. Supplementary Figure 5: Western blotting analysis of urinary extracellular vesicle
pNCC (A) T60 (B) T55 and (C) S91 in TIH patients and controls.

Supplementary information is available at KI Report’s website

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