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Phenotypic and pharmacogenetic evaluation of patients with thiazide-induced hyponatremia

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Introduction
Cardiovascular disease is the leading cause of mortality worldwide (1, 2) and hypertension one of its most important modifiable causes. Thiazide diuretics inhibit the thiazide-sensitive sodium chloride cotransporter NCC in the distal convoluted tubule (DCT) of the kidney (3) and are among the most widely used class of medicines in the management of hypertension (4).

However, some patients given thiazides develop thiazide-induced hyponatremia (TIH) (5). Severe TIH (serum sodium <125 mM) causes debilitating symptoms (5) and is the most common form of drug-induced hyponatremia requiring hospital admission (6). The mechanism of TIH is poorly understood. Serum sodium concentration in the thiazide-treated general population is virtually unchanged by thiazide therapy (7), implying that TIH occurs in a susceptible subgroup, but that this subgroup cannot be prospectively identified, and so TIH is largely unpredictable at the point of thiazide initiation. Pharmacogenetic predisposition to a range of adverse drug effects (8–14) raises the possibility that TIH might also have genetic causation. This hypothesis is supported by the highly reproducible nature of TIH even on single-dose rechallenge in individuals for whom environmental factors are controlled (15–17). We therefore set out to study the phenotypic and genetic characteristics of 2 cohorts of patients admitted to the hospital with severe symptomatic TIH in the United Kingdom.

Results
Characteristics of cohort 1 and cohort 2 TIH cases and controls
Two cohorts of patients of mixed European descent who were hospitalized with symptomatic TIH were recruited (Figure 1, Methods, and Supplemental Methods; supplemental material available online with this article; https://doi.org/10.1172/JCI89812DS1). The characteristics of the TIH cases and controls from cohorts 1 and 2 are presented in Table 1. In both cohorts, hyponatremic TIH cases on thiazides were typically aged over 70 years, with a predominance of females (Table 1).

Phenotypic differences between hyponatremic TIH cases on thiazides and non-hyponatremic thiazide controls
Cohort 1 hyponatremic TIH cases on thiazides had lower serum potassium compared with cohort 1 normonatremic thiazide controls (Supplemental Table 2). In cohort 2, hyponatremic TIH cases...
on thiazides also demonstrated significantly ($P < 10^{-5}$) lower serum potassium on treatment compared with off treatment. Cohort 2 hyponatremic TIH cases on thiazides also had lower blood pressure, lower blood glucose, and a lower serum concentration of chloride, magnesium, calcium, zinc, and vitamin D relative to cohort 2 normonatremic thiazide controls.

Twenty-four hour urine volume and osmolarity were significantly lower in hyponatremic TIH cases on thiazides compared with normonatremic thiazide controls (Supplemental Table 3). Fractional urinary excretion of potassium and phosphate was reduced and that of zinc was increased (Supplemental Table 4). Twenty-four hour urinary urea excretion, an important determinant of free water clearance, was also significantly lower in TIH cases both on and off thiazide, compared with relevant controls (Supplemental Table 3).

**Evaluating the baseline physiology of TIH cases after thiazide withdrawal**

Serum abnormalities resolved following thiazide cessation with the exception of levels of chloride and zinc (Supplemental Table 2). Although hypochloridemia and hypozincemia improved following thiazide cessation, cohort 2 normonatremic case remained hypochloridemic and hypozincemic 2 months after stopping thiazide therapy (Supplemental Table 2).

**TIH cases display an exaggerated increase in free water reabsorption**

Cohort 2 TIH cases reabsorbed 48% more free water when on thiazides compared with when off thiazides, which is in marked contrast with cohort 2 controls, who showed only a 9% increase in free water reabsorption while on thiazide (Supplemental Table 3). This suggests that TIH cases display an exaggerated increase in free water reabsorption in response to thiazide exposure. All groups in cohort 2 were in a state of net free water reabsorption (Supplemental Table 3). Although solute-free water reabsorption was lower in cohort 2 hyponatremic TIH cases on thiazides than in cohort 2 normonatremic thiazide controls, continued water reabsorption and production of a concentrated urine by hyponatremic TIH cases on thiazides is clearly inappropriate in the context of profound hyponatremia and intravascular volume expansion (as assessed by increased fractional urate clearance).

**Increased fractional uric acid clearance in TIH suggests volume expansion**

Increased fractional renal excretion of uric acid is observed in the syndrome of inappropriate antidiuretic hormone secretion (SIADH) and is caused by arterial blood volume expansion (18, 19). Mean serum and urinary uric acid concentration in cohort 2 hyponatremic TIH cases on thiazides was significantly lower than in cohort 2 normonatremic thiazide controls (Supplemental Figure 1). Fractional uric acid clearance in cohort 2 hyponatremic TIH cases on thiazides was significantly increased compared with both normonatremic thiazide and nonthiazide control groups and recovered to normal following thiazide cessation (Figure 2A).

**Plasma antidiuretic hormone concentration is suppressed during TIH**

Mean plasma antidiuretic hormone (ADH) concentration in cohort 2 hyponatremic TIH cases on thiazides was significantly lower than in cohort 2 normonatremic thiazide controls and normonatremic nonthiazide controls.
In our GWAS data set, rs4854769, the intronic sentinel SNP within SLCO2A1 showed association with TIH at $P = 3.92 \times 10^{-6}$ (odds ratio [OR] = 2.58). Targeted resequencing of SLCO2A1 in cohort 1 TIH cases and cohort 1 controls confirmed the presence of the nonsynonymous variant encoding p.A396T (rs34550074) in complete linkage disequilibrium ($r^2 = 1$) with the sentinel GWAS SNP rs4854769. Association with rs34550074 between cohort 1 cases and the carefully phenotyped normonatremic cohort 1 controls on thiazide was observed at $P = 0.0005$ (OR = 3.3; Bonferroni’s corrected threshold with $\alpha = 0.05 = 0.0017$; Supplemental Table 7).

The minor allele frequency (MAF) of rs34550074 in cohort 1 TIH cases was 0.35 (54% of TIH cases in cohort 1 carry at least 1 copy of the variant allele) compared with 0.14 in cohort 1 controls (25% carry at least 1 copy of the variant allele) and 0.18 in HAPMAP_CEU.

The total burden of rare protein-altering variants in other genes prioritized by the GWAS did not differ significantly between cohort 1 cases and controls after correcting for multiple testing (Supplemental Table 8). SLCO2A1 was nominally associated by the c-\alpha test ($P = 0.0019$), driven by the association with p.A396T. Sanger sequencing also confirmed the presence of rs34550074 in cohort 1 hyponatremic TIH cases on thiazides (Supplemental Figure 5).

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### Table 1. Demographic and medical details of TIH patients and controls in cohorts 1 and 2

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Cohort 1</th>
<th>Cohort 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyponatremic TIH cases on thiazides</td>
<td>Normonatremic thiazide controls</td>
</tr>
<tr>
<td>Number</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>Age, yr (±SD)</td>
<td>76 ± 9</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>Female (%)</td>
<td>54</td>
<td>68</td>
</tr>
<tr>
<td>ACEi (%)</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>ARB (%)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Beta blocker (%)</td>
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<td>19</td>
</tr>
<tr>
<td>CCB (%)</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>Loop diuretic (%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>K+-sparing diuretic (%)</td>
<td>6</td>
<td>2.5</td>
</tr>
<tr>
<td>SSRI (%)</td>
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<td>4</td>
</tr>
<tr>
<td>Low-dose aspirin (%)</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>NSAID (%)</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comorbidities</th>
<th>Cohort 2 hyponatremic TIH cases on thiazides versus cohort 2 normonatremic thiazide controls, $P &lt; 0.05$.</th>
<th>Cochort 1 hyponatremic TIH cases on thiazides versus cohort 1 normonatremic thiazide controls, $P &lt; 0.05$.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated hypothyroidism (%)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Diabetes mellitus, glucose &lt; 13.9 mM (%)</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Mild LV impairment (%)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>eGFR 30–60 ml/min (%)</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>

| Thiazide type           | BFZ (%)                        | 73                                             | 94\(^a\)                                      | 72                                             | 81                                             |
|                        | Chlortalidone (%)              | 15                                             | 1                                               | 6                                              | 4                                              |
|                        | Indapamide (%)                 | 4                                              | –                                               | 20                                             | 6\(^a\)                                       |
|                        | HCTZ (%)                       | 6                                              | 4                                               | 2                                              | 10                                             |
|                        | Cyclopenthiazide(%)            | 2                                              | 1                                               | –                                              | –                                              |

All patients were of mixed European descent. ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; SSRI, selective serotonin reuptake inhibitor; BFZ, bendroflumethiazide; HCTZ, hydrochlorothiazide. Comparisons are by 1-way ANOVA/\chi^2 with Bonferroni’s correction. \(^a\)Cohort 2 hyponatremic TIH cases on thiazides versus cohort 2 normonatremic thiazide controls, $P < 0.05$. \(^b\)Cohort 1 hyponatremic TIH cases on thiazides versus cohort 1 normonatremic thiazide controls, $P < 0.05$. Treated hypothyroidism (no clinical features of hypothyroidism and TSH within normal range), diabetes mellitus (plasma glucose < 13.9 mM), mild left ventricular (LV) impairment (no clinical features of heart failure and ECHO mild left ventricular impairment). eGFR, estimated glomerular filtration rate.
Positive in the medulla, colocalizing with AQP1 and AQP2. Strong staining was detected in the proximal straight tubule, with comparatively weaker labeling of outer medullary collecting ducts and no PGT expression in the thick ascending limb loop of Henle (Figure 3 and Supplemental Figure 6, E–I). Expression of PGT increased as it transitioned into the inner medullary collecting ducts, with faint staining of the thin limb loop of Henle detectable only at higher laser/detector settings (Figure 3 and Supplemental Figure 6, J–M).

Species conservation of rs34550074 is shown in Supplemental Table 10. The tissue expression of genes near GWAS loci is shown in Supplemental Table 11.

Urinary prostaglandin E2 and PGE2 metabolite concentrations are increased in TIH
Mean prostaglandin E2 (PGE2) and PGE2 metabolite (PGE2M) concentrations from 24-hour urine samples were significantly higher in cohort 2 hyponatremic TIH cases on thiazide compared with controls. The MAF for rs34550074 in cohort 2 TIH cases on thiazide was 0.26 (45% carried at least 1 copy of the variant allele) and 0.18 in normonatremic thiazide controls (test of association: \( P = 0.0304 \), OR = 1.70) (Supplemental Table 9). When data were combined across both cohorts, the pooled effect estimate for the association between rs34550074 and severe TIH was OR = 2.13 (\( P = 1.70 \times 10^{-4} \), Supplemental Table 9).

Prostaglandin transporter is expressed in the collecting duct of human cadaveric kidneys and colocalizes with AQP2
SLCO2A1 is principally expressed in the kidneys, adrenal glands, and lungs. Similarly to the renal cortex of the rat, human glomeruli and renal capillaries stained positive for prostaglandin transporter (PGT) (the protein product of \( \text{SLCO2A1} \)) (Figure 3 and Supplemental Figure 6, A and B) (20). Cortical tubules were primarily negative for PGT (Figure 3 and Supplemental Figure 6, C–E). PGT stained positive in the medulla, colocalizing with AQP1 and AQP2. Strong staining was detected in the proximal straight tubule, with comparatively weaker labeling of outer medullary collecting ducts and no PGT expression in the thick ascending limb loop of Henle (Figure 3 and Supplemental Figure 6, E–I). Expression of PGT increased as it transitioned into the inner medullary collecting ducts, with faint staining of the thin limb loop of Henle detectable only at higher laser/detector settings (Figure 3 and Supplemental Figure 6, J–M).

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Urinary prostaglandin E, and PGE, metabolite concentrations are increased in TIH
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The Journal of Clinical Investigation

RESEARCH ARTICLE

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jci.org   Volume 127   Number 9   September 2017

The charge and size will mimic phospho-Thr and recapitulate physiological effects of phosphorylation at a regulatory site. Figure 2E shows that the 396Glu moiety had a transport rate of 65% compared with 396Ala, suggesting that the 396Thr variant may result in reduced PGT function relative to 396Ala.

Discussion

TIH remains a substantial clinical problem: population-based studies suggest that as many as 9% of subjects taking a thiazide may develop hyponatremia. Here, we report the largest and most detailed phenotypic description of TIH patients to date.

In vitro assessment of SLC02A1 396 Ala/Thr/Glu variants

Figure 2E shows functional assays of PGE$_2$ transport rate by 396Thr compared with that of 396Ala. The ratio of transport was not significantly different from 1. Because urinary PGE$_2$ levels were higher in 396Thr compared with 396Ala subjects (Figure 2, C and D), we hypothesized that 396Thr may be subject to regulation by phosphorylation. Accordingly, we substituted Glu for Thr, since the charge and size will mimic phospho-Thr and recapitulate physiological effects of phosphorylation at a regulatory site. Figure 2E shows functional assays of PGE$_2$ transport rate by 396Thr compared with that of 396Ala. The ratio of transport was not significantly different from 1. Because urinary PGE$_2$ levels were higher in 396Thr compared with 396Ala subjects (Figure 2, C and D), we hypothesized that 396Thr may be subject to regulation by phosphorylation. 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Systematic review of existing data suggests that TIH affects an older and predominantly female demographic with low serum osmolality, and limited spot urine testing suggests inappropriately concentrated urine and saliuresis (21). Our study supported this and also demonstrated a phenotype resembling SIADH, with low plasma osmolarity, inappropriately concentrated urine, more than minimal urinary sodium excretion, and normal thyroid function, but with low or normal ADH levels. Moreover, the phenotype of TIH also involved severe hypochloridemia, mild hyperglycemia, and intravascular volume expansion. Hypochloridemia and hypozincemia improved following thiazide cessation, but remained present 2 months after thiazide medication was stopped. Acutely hyponatremic TIH cases on thiazides demonstrated inappropriate net free water reabsorption and an exaggerated increase in free water reabsorption in response to thiazide exposure. This study therefore suggests there is much more to the phenotype of acute TIH than has been previously described. There are also phenotypic abnormalities that far outlast any kinetic or dynamic effect of thiazide medications, raising the possibility that such features might be present at baseline before thiazide commencement.

Having defined the phenotype of TIH, we set out to identify possible genetic predisposition to this condition. Given the small number of cases available in our initial cohort, we chose to prioritize GWAS associations with suggestive evidence for follow-up using a less conservative \( P \) value cut off \( (P < 10^{-5}) \). We focused on \( \text{SLCO2A1} \) (encoding PGT) because augmented renal water reabsorption seemed most relevant to the principal abnormality of hyponatremia from our phenotype studies. PGT is known to regulate this process, and there was direct association between a protein-altering variant in \( \text{SLCO2A1} \) (\( \text{p.A396T} \)) and TIH. This association was replicated in a second independent cohort. \( \text{SLCO2A1} \ \text{p.A396T} \) was twice as frequent in TIH cases compared with control and general populations, with approximately half of all TIH patients carrying this variant. In vitro data suggest that this variant may reduce PGT activity and lead to the higher urinary \( \text{PGE}_2 \) concentrations observed in TIH patients who carry the variant allele. However, since half of TIH patients do not carry the \( \text{SLCO2A1} \) variant and at least a quarter of unaffected patients do, other genetic or environmental determinants must also contribute to the development of TIH.

One potential criticism of this study is the limited sample size in the discovery cohort. This was inevitable, since we were careful to include only those with severe thiazide-induced hyponatremia and excluded those who lacked mental capacity or who had relevant comorbidities.

So how does one reconcile suppressed ADH with elevated \( \text{PGE}_2 \) and intravascular volume expansion? And how might a reduction in \( \text{SLCO2A1} \) activity cause TIH? \( \text{SLCO2A1} \) is expressed at the apical membrane of the renal collecting duct, where it scavenges newly synthesized \( \text{PGE}_2 \), away from luminal EP4 receptors (22, 23). Activation of these apical EP4 receptors increases the water permeability of this nephron segment 10- to 15-fold, even in the absence of ADH (24, 25). Thus, reduced activity of collecting duct apical \( \text{SLCO2A1} \) would be predicted to increase hydraulic conductivity of the collecting duct, even in the absence of ADH (Figure 4). Although urinary osmolarity in the TIH subjects was 15% higher than in control subjects (Supplemental Table 3) (probably as a result of increased renal medullary \( \text{PGE}_2 \); ref. 26), which would mitigate water reabsorption, the proportionally larger increase in collecting duct water permeability observed would more than offset the slight reduction in osmotic driving force.

Although the putative effects of \( \text{SLCO2A1} \) inactivation appear to be compensated under normal conditions, they are made manifest when the patients are given a thiazide diuretic. 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 (27). We suggest therefore that, in individuals carrying the \( \text{SLCO2A1} \ \text{A396T} \) variant, the combination of thiazide-specific

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**Figure 4. Hypothesis for the role of \( \text{SLCO2A1} \) in contributing to TIH in individuals carrying the \( \text{SLCO2A1} \) \( \text{A396T} \) variant.**

(A) Under low ADH conditions, apical PGT in the renal collecting duct scavenges \( \text{PGE}_2 \), resulting in insertion of \( \text{AQP2} \), and minimal osmotic water reabsorption. (B) With reduced or absent apical PGT, \( \text{PGE}_2 \) reaching the lumen is able to stimulate apical EP4 receptors, resulting in insertion of \( \text{AQP2} \) and osmotic water reabsorption.
effects on free water generation and the increase in collecting duct water permeability from reduced SLCO2A1 activity combine to produce TIH (Figure 4).

Methods

Clinical recruitment. Two cohorts of patients of mixed European descent hospitalized with symptomatic TIH were recruited (Figure 1 and Supplementary Methods). Cohort 1 comprised 48 hyponatremic TIH cases on thiazides (serum sodium < 130 mM) recruited during their acute admission to Nottingham University Hospitals NHS Trust and matched healthy normonatremic thiazide controls from primary care. Cohort 2 comprised a further 109 hyponatremic TIH cases on thiazides recruited during their acute admission to the same hospital together with 2 matched control groups from primary care (cohort 2 normonatremic thiazide controls and cohort 2 normonatremic nonthiazide controls). Cohort 2 TIH cases were also assessed 2 months after thiazide cessation (termed cohort 2 normonatremic TIH cases off thiazides).

GWAS. A GWAS was performed using 48 cohort 1 TIH cases genotyped using the Illumina Omni1Quad array and 2,922 general population controls from the British 1958 birth cohort (28), genotyped using the Illumina 1.2M chip. Controls were all aged 44 to 45 years at the time of DNA collection (2002 to 2004) and 48% were female. Following quality control of the genotype data (Supplemental Methods), 502,663 SNPs that were genotyped in both cases and controls remained for association testing. A case-control association analysis was undertaken using a logistic regression model, with adjustment for 10 principal components and assuming an additive genetic model, using Plink v 1.07.

Resequencing and replication studies. 101 samples (48 cohort 1 hyponatremic TIH cases on thiazides and 53 cohort 1 normonatremic thiazide controls) underwent resequencing of the genes nearest to SNPs that were associated with TIH at \( P < 10^{-5} \), followed by association testing (Supplemental Table 7 and Supplemental Methods). Next Generation sequencing data were deposited at the European Nucleotide Archive (ENA accession number PRJEB21924; http://www.ebi.ac.uk/ena). Replication for one SNP of interest identified in the sequencing analysis of cohort 1 (rs34550074, p.A396T) was undertaken using Sanger sequencing in the second cohort of TIH cases and controls (Supplemental Methods). Rare variant association was carried out using Plink/Seq (29). Rare variants were assessed using the burden test, which is a collapsing allelic sum test using adaptive permutation to derive an empirical \( P \) value. Combined rare and common variants were also assessed using the c-u test (30), also implemented in Plink/Seq.

Kidney immunofluorescence. Formalin-fixed paraffin-embedded human tissue sections were obtained from the Cambridge Human Research Tissue Bank. After antibody labeling, confocal imaging was performed (Supplemental Methods).

In vitro functional studies of SLCO2A1 p.A396T. Human SLCO2A1 cDNA (encoding PGT) was modified by site-directed mutagenesis using standard methods so as to generate cDNAs encoding amino acids Ala, Thr, or Glu at position 396. DNA sequences were confirmed by direct sequencing. Human embryonic kidney (HEK) cells were transiently transfected with one of the 3 cDNAs, and the timed uptake of \(^3\)H-PGE2, was assayed as described previously (26).

Statistics. Significance between groups was assessed using a 2-tailed Student’s \( t \) test. Where comparisons between more than 2 groups were undertaken, 1-way ANOVA and Bonferroni’s post hoc test were used. The data were analyzed using Graph Pad Prism V6.05. \( P < 0.05 \) was considered significant. Statistical methods used for GWAS and resequencing experiments are contained within the relevant Methods and Supplemental Methods sections.

Study approval. This study was conducted in line with the standards of ICH/Good Clinical Practice sections 8.2.8 and was given approval by the Queen’s Medical Centre Ethics Committee (cohort 1; reference GM030208) and the UK National Research Ethics Committee (cohort 2; reference 11/EM/0233). Written informed consent was received from participants prior to inclusion in the study.

Author contributions

JSW, SC, LVW, SKC, VEJ, EE, RL, KS, WJ, NS, APH, and MG performed experiments and analyzed data. SK, MJ, JC, and MG recruited patients. KMO, VLS, MDT, IPH, and MG designed the study. All authors contributed to the drafting and revision of the manuscript, which was led by MG and IPH.

We are grateful to David Strachan for access to the 1958 British birth cohort resource. This work was supported by an Academy of Medical Sciences grant for clinical lecturers (to JSW and MG), British Heart Foundation grant PG/09/089 (to KMO), the National Institute for Health Research (NIHR) Royal Brompton Cardiovascular Biomedical Research Unit (to JSW and SC), the Fondation Leducq (to JSW and SC), and the British Heart Foundation (to JSW and SC). MDT holds a Medical Research Council Senior Clinical Fellowship (G0902313). This work was supported by the Medical Research Council (grant numbers G510364 and G1000861). This research used the ALICE and SPECTRE High Performance Computing Facilities at the University of Leicester.

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