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Polymorphisms in Renal Ammonia Metabolism Genes Correlate With 24-Hour Urine pH

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Introduction: Urine pH is critical for net acid and solute excretion, but the genetic factors that contribute to its regulation are incompletely understood.

Methods: We tested the association of single nucleotide polymorphisms (SNPs) from 16 genes related to ammonia (NH_3) metabolism (15 biological candidates selected *a priori*, 1 selected from a previous genome-wide association study analysis) to that of 24-hour urine pH in 2493 individuals of European descent across 2 different cohorts using linear regression, adjusting for age, sex, and body mass index.

Results: Of 2871 total SNPs in these genes, 13 SNPs in *ATP6V0A4* (a4 subunit of hydrogen− adenosine triphosphatase), *SLC9A3* (sodium/hydrogen exchanger, isoform 3), and *RHCG* (Rhesus C glycoprotein), and 12 SNPs from insulin-like growth factor binding protein 7 (*IGFBP7*) had a meta-analysis *P* value <0.01 in the joint analysis plus a consistent direction of effect and at a least suggestive association ($P < 0.1$) in both cohorts. The maximal effect size (in pH units) for each additional minor allele of the identified SNPs was −0.13 for *IGFBP7*, −0.08 for *ATP6V0A4*, 0.06 for *RHCG*, and −0.06 for *SLC9A3*; SNP rs34447434 in *IGFBP7* had the lowest meta-analysis *P* value ($P = 7.1 \times 10^{-8}$). After adjusting for net alkali absorption, urine pH remained suggestively associated with multiple SNPs in *IGFBP*, 1 SNP in *ATP6V0A4*, and a new SNP in *GLS* (phosphate-dependent glutaminase).

Discussion: Overall, these findings suggest that variants in common genes involved in ammonia metabolism may substantively contribute to basal urine pH regulation. These variations might influence the likelihood of developing disease conditions associated with altered urine pH, such as uric acid or calcium phosphate kidney stones.

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Urine pH is the net result of a wide variety of renal physiological processes, such as ammonia (NH_3), titratable acid, and bicarbonate excretion.[1–6] Urinary pH also influences the solubility of several crystals commonly found in urinary stones. Thus, abnormalities associated with these processes can lead to electrolyte disturbances and urinary stone disease. Understanding the mechanisms that regulate basal urine pH is critical to furthering our understanding of abnormal states.

The final determination of urine pH is a balance between renal tubular hydrogen (H^+) secretion, renal tubular bicarbonate secretion, the availability of titratable acids, and renal production and transport of the ammonia species NH_3 and NH_4^+. Many physiologic factors regulate this balance, including systemic pH, partial pressure of carbon dioxide, bicarbonate and serum electrolyte concentrations, and a variety of hormones such as angiotensin II, aldosterone, atrial

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natriuretic peptide, and endothelin.[7] The importance of a number of specific renal acid-base transporters in this process is evidenced by the identification of human genetic disorders that alter either renal transport protein expression and/or function that cause abnormal urinary pH.[8,9] Single nucleotide polymorphisms (SNPs) in genes are frequent (1/300 nucleotides) and can affect protein expression, function, and regulation. To date, genetic variation of proteins involved in urine pH regulation has not been explored.

The purpose of this study was to determine whether SNP genetic variants are associated with changes in urinary pH in a general population of non-Hispanic whites. We developed a list of 15 candidate genes *a priori* and 1 gene after preliminary genome-wide associated study analysis that consisted of genes involved in NH_3 production and were likely to influence urinary pH (Table 1). We evaluated SNP data from these genes compared with 24-hour urine pH from 2 large cohorts with available 24-hour urine pH and other phenotypic data. Our results implicated several genetic variants in renal proton secretion and NH_3 metabolism that correlated with basal urine pH.

METHODS

This study was approved by the Mayo Clinic's and Brigham and Women's Hospital's Institutional Review Boards.

Study Cohort 1: Genetic Epidemiology Network of Arteriopathy

The Genetic Epidemiology Network of Arteriopathy (GENOA), a member of the Family Blood Pressure Program, recruited non-Hispanic white hypertensive sibships from Rochester, Minnesota for linkage and association studies to investigate the genetic underpinnings of hypertension in Phase I (1996–2001).[10] The Genetic Determinants of Urinary Lithogenicity (GDUL) study (2006–2012) is an ancillary study of the Phase III GENOA Genetics of Chronic Kidney Disease (CKD) Study. After informed consent, CKD and GDUL study participants were invited to collect 24-hour urine samples (once during the CKD study and twice during the GDUL study), complete a food frequency questionaire (Viocare Technologies, Princeton, New Jersey, USA; GDUL study participants only), and complete a detailed questionnaire about kidney stones (CKD study participants only).[11,12] Recruitment for the original GENOA study and all ancillary studies was not based on CKD status or the presence (or absence) of kidney stones, except that participants were excluded if they were in end-stage renal failure (stage 5 CKD). Most of the participants included in this analysis participated in both the CKD and the GDUL studies (87.4%). A total of 333, 295, and 183 participants had a total of 1, 2, or 3 urine collections, respectively. For individuals with 1 or 3 urine collections, values were averaged for analysis. The mean time between the earliest (CKD) and latest (GDUL) urine collections was 1.73 years (range: 0.9–3.6 years). The average time between the 2 GDUL collections was 22 days. Intraclass correlation coefficients for urine factors across collections revealed that most urine measures were relatively stable across time, and the intraclass correlation coefficients for urine pH was 0.52. Urine was collected with toluene as a preservative, and urine pH was measured with an ion electrode.

Table 1. Candidate genes, associated protein and function, and known number of single-nucleotide polymorphisms evaluated

Gene symbol, chromosome		Protein	RefSeq ID	Function	No. of SNPs
ATP1A1	1	Na^+-K^+-ATPase, α1 subunit	476	Maintenance of low intracellular Na^+	65
ATP1A2	1	Na^+-K^+-ATPase, α2 subunit	477	Maintenance of low intracellular Na^+	112
ATP4B	13	H^+-K^+-ATPase, β subunit	496	H^+ secretion, K^+ reabsorption (CD)	42
ATP6V0A4	7	H^+-ATPase, A4 subunit	50617	H^+ secretion (CD)	401
ATP6V1B1	2	H^+-ATPase, B1 subunit	525	H^+ secretion (CD)	145
GLS	2	Phosphate-dependent glutaminase	2744	Glutamine metabolism, NH_4^+ generation	118
GLUD1	10	Glutamine dehydrogenase	2746	Glutamate metabolism in ammoniagenesis	111
IGFBP7[a]	4	Insulin-like growth factor binding protein 7	3490	Ammoniagenesis through NHE3 activation (PT)	445
ODGH	7	Oxoglutarate (α-ketoglutarate) dehydrogenase	4967	Ammoniagenesis, oxoglutarate metabolism	204
PCK1	20	Phosphoenolpyruvate carboxykinase I	5105	Ammoniagenesis	67
RHBG	1	Rhesus B glycoprotein	57127	NH_3/NH_4^+ transport (distal nephron, CD)	59
RHCG	15	Rhesus C glycoprotein	51548	NH_3 transport (distal nephron, CD)	62
SLC4A4	4	Sodium bicarbonate cotransporter, isoform 1 (NBCe1)	8671	Bicarbonate transport (PT) necessary for regulation ammonia metabolism	599
SLC9A3	5	Na^+/H^+ exchanger, isoform 3 (NHE3)	6550	Proximal tubule H^+, NH_4^+ secretion	269
SLC12A1	15	Na^+-K^+-$2Cl^-$ cotransporter (NKCC2)	6557	NH_4^+ reabsorption (TAL)	141
SLC38A3	3	Na^+-coupled neutral amino acid transporter 3 (SNAT3)	10991	Glutamine transport (PT)	31

ATP, adenosine triphosphotase; CD, collecting duct; Cl, chloride; H, hydrogen; K, potassium; Na, sodium; NH_3/NH_4, ammonia/ammonium; NHE3, sodium/hydrogen exchanger; PT, proximal tubule; SNP, single nucleotide polymorphism; TAL, thick ascending limb.
[a]Identified in preliminary genome-wide association study conducted in cohort 2.

Study Cohort 2: Nurses Health Study I and II and Health Professionsals Follow-up Study

The Nurses Health Study (NHS) I was established in 1976 with >120,000 female registered nurses aged 30 to 55 years. The NHS II was established in 1989 with >116,000 female nurses aged 25 to 42 years. The Health Professionals Follow-Up Study (HPFS) was established in 1986 with >51,000 male health care professionals aged 40 to 75 years. All 3 groups have been followed by biennially mailed questionnaires, including questions on lifestyle practices, a food frequency questionaire, and newly diagnosed diseases such as nephrolithiasis.[13] Additional information was obtained from self-reported cases, including symptoms and kidney stone type. In validation studies, permission to obtain medical records was requested from newly diagnosed cases in all 3 groups. The diagnosis of stone disease was confirmed in >90% of these cases. Twenty-four−hour urine collections were obtained from participants with a history of confirmed nephrolithiasis and from randomly selected control subjects. Only data from the first urine collection was used in this analysis. Those with a history of kidney stones performed the collections after the diagnosis. All 24-hour urine collections were performed using the Mission Pharmacal system (San Antonio, Texas, USA). Urinary pH was measured with a pH electrode, and urinary calcium was measured by an atomic absorption spectrophotometer.[13] Data from NHS I, NHS II, and HPFS were analyzed as a combined data set (cohort 2), and only non-Hispanic white participants were included in the analysis (Table 2).

Table 2. Demographic and 24-hour urine data from both cohorts

	Cohort 1	Cohort 2		
Characteristic	GENOA (n = 811)	HPFS (n = 553)	NHS I (n = 494)	NHS II (n = 635)
Age (yr)	66 ± 9	64 ± 8	66 ± 8	50 ± 6
Female (%)	467 (58)	0 (0)	494 (100)	635 (100)
Height (cm)	168 ± 10	179 ± 7	164 ± 6	165 ± 7
Weight (kg)	88 ± 19	84 ± 12	71 ± 15	74 ± 19
Body mass index (kg/m^2)	31 ± 6	25 ± 8	26 ± 6	27 ± 7
Diabetes history (%)	17.3%[a]	2.4%	3.2%	3.9%
Percent with kidney stone history	13.1%[b]	57.0%	66.3%	53.7%
24-h urine measures				
24-h urine pH	6.21 ± 0.5	5.88 ± 0.45	6.06 ± 0.51	6.03 ± 0.47
Net alkali absorption[c] (mEq/24 h)	45 ± 21	25 ± 23	27 ± 21	18 ± 20
Calcium (mg/24 h)	156 ± 88	194 ± 97	201 ± 102	207 ± 95
Magnesium (mg/24 h)	108 ± 41	124 ± 42	102 ± 41	102 ± 38
Uric acid (mg/24 h)	443 ± 172	625 ± 220	443 ± 157	504 ± 157
Volume (ml/24 h)	1958 ± 700	1715 ± 643	1846 ± 680	1749 ± 725
Sodium (mEq/24 h)	139 ± 58	182 ± 71	139 ± 57	150 ± 64
Creatinine (mg/24 h)	1133 ± 425	1661 ± 364	1043 ± 218	1212 ± 261

GENOA, Genetic Epidemiology Network of Arteriopathy; HPFS, Health Professionals Follow-Up Study; NHS, Nurses Health Study.
[a]Diabetes status was not known for 104 cohort 1 participants.
[b]Kidney stone history was not known for 140 cohort 1 participants.
[c]Net alkali absorption was calculated according to the method of Oh[21]: (sodium + potassium + calcium/20.04) + (magnesium/12.15)) − (chloride + phosphorus/31)). In cohort 2, sodium, chloride, and magnesium were not measured, and were therefore omitted from the equation.
Values are mean ± SD, unless stated otherwise.

Candidate Genes

Candidate genes were selected by expert opinion after searching public databases PubMed and Online Mendelian Inheritance in Man as well as by review of *in vivo* and *in vitro* evidence regarding renal or intestinal acid base homeostasis regulation. The 16 genes investigated in this study are listed in Table 1, including gene symbol, chromosome, protein name and function, RefSeq ID, and number of SNPs that met quality control criteria. Based on an unpublished genome-wide association study analysis in study cohort 2, we also included *IGFBP7* (insulin-like growth factor [IGF] binding protein 7) as an additional gene. *IGFBP7* has been associated with insulin resistance,[14] and *in vitro* studies have suggested insulin stimulates renal ammoniagenesis from the substrate L-glutamine and through activation of the sodium $(Na^+)/H^+$ exchanger in the proximal tubule (NHE3).[15]

Genotyping

The majority of GENOA participants were genotyped on the Affymetrix Genome-Wide Human SNP Array 6.0 (Santa Clara, CA), and a small number of participants were genotyped on the Illumina Human 1M-Duo, 660-Quad, or 610-Quad BeadChips (San Diego, CA). Before imputation, SNPs and samples with a call rate of <95% were excluded. Haplotypes were prephased using SHAPEIT version 2,[16] and imputation was performed with IMPUTE version 2[17] using the cosmopolitan reference panel of the 1000 Genomes Project Phase I Integrated Release Version 3 (March 2012). Prephasing and imputation was performed separately for participants genotyped on the Affymetrix and Illumina platforms. Following imputation, allelic dosage for each SNP was calculated by combining the probabilities of the 3 possible genotypes reported in the IMPUTE output files. For example, if the probability of each genotype for a given SNP was represented as P(AA), P(AB), and P(BB), then the dosage of the B allele is calculated as 0*P(AA)+1*P(AB)+2*P(BB). The resulting dosage ranges from 0 to 2 and represents the expected number of coded (B) alleles.

NHS I and II and HFPS were genotyped on the Illumina Infinium Human610-Quad BeadChip. Imputation of all three groups was performed simultaneously with the Markov Chain Haplotyping algorithm (MaCH), using the CEU reference panel (composed of Utah residents with Northern and Western European ancestry from the Centre d''Etude du Polymorphisme

Humain [CEPH] collection) of the 1000 Genomes Project Phase I reference panel (November 2010).[18,19] MACH directly outputs the allelic dosage, which is comparable to the calculated dosages used in GENOA.

Although genotypes from cohorts 1 and 2 were imputed using separate imputation programs and reference panels, they were meta-analyzed together. It was consistently shown that using different platforms for imputation, including MACH and IMPUTE, produce similar and highly accurate results.[20] Because the cohorts consisted of non-Hispanic whites, we expected little difference in the imputation results obtained when using the CEU (European ancestry) reference panel versus the updated reference panel, which consists of multiple ethnicities. Genetic principal components were calculated for both cohorts. We did not include principal components in the final models because our initial modeling of urine pH in cohort 1 showed no association between urine pH and each of the first 4 principal components ($P > 0.05$).

Statistical Analysis

The candidate gene regions of interest were defined as all SNPs within the candidate gene, plus all SNPs 5kb upstream and downstream of the gene. For each candidate gene, imputed SNP dosages were tested for association with 24-hour urine pH using simple linear regression models (cohort 2) or linear mixed effects modeling with sibship as a random intercept (cohort 1). All regression models included age, sex, and body mass index (BMI) as adjustment covariates. In a second meta-analysis, models also included net alkali absorption, which was calculated in cohort 1 using the method of Oh[21]: net alkali absorption = (sodium + potassium + [calcium/20.04] + [magnesium/12.15]) − (chloride + [phosphorus/31]). In cohort 2, Na, chloride, and magnesium were not measured, and were therefore omitted from the equation. These 2 methods for calculating net alkali were highly correlated in cohort 1 ($r = 0.66$). We did not have complete data in both cohorts for other variables that might have influenced urine pH (diabetes status, kidney stone history, medications) and thus could not include them in our primary analysis. However, we did evaluate their effects on urine pH and/or perform sensitivity analysis when appropriate.

Fixed-effects meta-analysis was conducted with inverse variance weighting by the SE of the β coefficient using the METAL package in R.[22] Only SNPs with a combined minor allele frequency >0.01 and imputation quality score (R^2 or INFO) >0.5 for both cohorts were included in the meta-analysis. SNPs were considered to be suggestively associated with urine pH if the following criteria were met: $P < 0.01$ in the joint meta-analysis; association $P < 0.1$ in both cohort 1 (GENOA) and cohort 2 (the combined sample of HPFS/NHS I/NHS II); and consistent direction of effect in both cohorts. SNPs were considered to be significantly associated with urine pH if the following, more stringent, criteria were met: $P < 1.7 \times 10^{-5}$ in the joint meta-analysis (Bonferroni-corrected α level for 2871 tests); association $P < 0.05$; and consistent direction of effect in both cohorts. LocusZoom plots were created to visualize the suggestive and significant SNP associations within the gene regions, using reference data from the 1000 Genomes 2012 EUR reference panel (hg19).[23]

Sensitivity Analysis

Because the presence of diabetes was previously shown to influence urine pH,[15] we conducted a sensitivity analysis to investigate whether suggestively associated SNPs still met criteria for association after excluding participants with diabetes from cohort 1. SNPs that met all criteria for suggestive association in the sensitivity analysis are indicated by an asterisk in Tables 3 and 4.

RESULTS

A total of 2493 individuals of European descent from 2 cohorts participated in this study (Table 2). Cohort 1 (GENOA) had a prevalence of stone formers similar to that in the general population (13.1%),[24] but they also had relatively high prevalence of diabetes (17.3%) and a high BMI. Cohort 2 (combined sample of HPFS, NHS I, and NHS II) was purposefully enriched to contain approximately 50% with a kidney stone history, with female NHS II participants being younger in age. Overall mean 24-hour urine pH was 6.21 ± 0.5 in cohort 1 and 5.88 ± 0.45, 6.06 ± 0.51, and 6.03 ± 0.47 for the 3 studies included in cohort 2.

Within the 16 genes, 2871 SNPs with combined minor allele frequency >0.01 and imputation quality >0.5 in both cohorts were evaluated. Adjustment variables in the regression models included age, sex, BMI (models 1 and 2) and net alkali absorption (model 2). We assessed the possibility of also including other potential influencers of urine pH (diabetes status, kidney stone history, medications), but we did not have complete information on these variables for both cohorts. Both kidney stone history and diabetes status were unknown for 104 cohort 1 (GENOA) participants (12.8%). However, of cohort 1 participants with diabetes data, the presence of diabetes was associated with decreased pH ($P < 0.0001$). Thus, we performed a sensitivity analysis for diabetes (described in the following). For individuals with kidney stone data, urine pH did not differ between stone formers and

Table 3. Meta-analysis results from model 1

Gene	SNP	Allele (C/N)	CAF	β, cohort 1	*P* value, cohort 1	β, cohort 2	*P* value, cohort 2	β, joint	*P* value, joint
ATP6V0A4	rs6955765[a]	T/C	0.13	−0.147	0.0030	−0.056	0.0285	−0.075	0.0009
	rs6943456[a]	C/T	0.12	−0.132	0.0060	−0.056	0.0281	−0.073	0.0012
	rs76046773[a]	C/A	0.12	−0.131	0.0096	−0.061	0.0306	−0.078	0.0016
	rs35735948	T/C	0.12	−0.082	0.0802	−0.070	0.0126	−0.073	0.0024
	rs6973199[a]	C/T	0.11	−0.112	0.0093	−0.049	0.0617	−0.067	0.0031
	rs13223647	C/T	0.12	−0.100	0.0233	−0.054	0.0385	−0.066	0.0033
	rs34587733	C/T	0.13	−0.081	0.0574	−0.057	0.0256	−0.063	0.0038
	rs7808193	G/A	0.13	−0.078	0.0691	−0.057	0.0246	−0.062	0.0042
	rs35448898	T/G	0.13	−0.082	0.0607	−0.056	0.0293	−0.063	0.0047
IGFBP7	rs34447434	A/C	0.18	−0.062	0.0726	−0.113	1.8×10^{-7}	−0.098	7.1×10^{-8}
	rs11726223	T/C	0.44	−0.062	0.0179	−0.059	0.0003	−0.060	1.3×10^{-5}
	rs11734140	A/G	0.44	−0.060	0.0211	−0.059	0.0003	−0.059	1.5×10^{-5}
	rs2412775	T/C	0.44	−0.057	0.0288	−0.059	0.0003	−0.059	2.1×10^{-5}
	rs17761305	T/C	0.13	−0.073	0.0465	−0.076	0.002	−0.075	0.0002
	rs17183073	C/G	0.13	−0.062	0.0948	−0.076	0.0018	−0.072	0.0004
	rs116189043	A/G	0.03	−0.147	0.0449	−0.119	0.0076	−0.127	0.0009
	rs28370990	T/C	0.08	0.088	0.0639	0.069	0.0218	0.075	0.0034
	rs28623722	G/A	0.08	0.085	0.0663	0.069	0.0219	0.074	0.0034
	rs2271807	G/A	0.08	0.084	0.0739	0.069	0.0219	0.074	0.0038
	rs73242632	A/C	0.08	0.082	0.0816	0.069	0.0219	0.073	0.0041
	rs1713980	C/A	0.30	−0.049	0.0776	−0.036	0.0401	−0.040	0.0073
RHCG	rs74032414[a]	G/C	0.17	0.067	0.0989	0.060	0.0238	0.062	0.0052
SLC9A3	rs890976	T/C	0.40	0.050	0.0781	0.043	0.0091	0.044	0.0017
	rs890979	G/A	0.40	0.049	0.0787	0.043	0.0092	0.044	0.0017
	rs10475280[a]	C/T	0.42	−0.060	0.0760	−0.054	0.0188	−0.056	0.0032

C, coded; CAF, coded allele frequency; N, non-coded.
[a]Indicates that the SNP remained at least suggestively associated after excluding participants with diabetes (n = 122) and participants with missing data for diabetes (n = 104) from cohort 1.
For all single nucleotide polymorphisms (SNPs), the coded allele is the minor allele.
The results are adjusted for age, sex, and body mass index with meta-analysis $P < 0.01$ in the joint analysis, consistent direction of effect, and $P < 0.1$ in both cohorts.

non-formers ($P = 0.57$), and the only significant difference ($P < 0.05$) in other key variables was that stone formers had higher BMI than non-formers (mean: 32.0 and 30.7 kg/m^2, respectively; $P = 0.01$). In cohort 2 (HPFS/NHS I/NHS II), there was a significant difference in BMI between stone formers and non-formers in NHS II, but not in the other 2 studies. Urine pH was significantly different between formers and non-formers in HPFS, but not the other 2 studies, with $0.01 < P < 0.05$.[13] Because neither cohort showed

Table 4. Meta-analysis results from model 2

Gene	SNP	Allele (C/N)	CAF	β, cohort 1	*P* value, cohort 1	*P* value, cohort 2	*P* value, cohort 2	β, joint	*P* value, joint
ATP6V0A4	rs6955765	T/C	0.13	−0.087	0.0334	−0.042	0.0733	−0.053	0.0090
GLS	rs145048940[a]	C/A	0.02	−0.235	0.0025	−0.126	0.0687	−0.175	7.9×10^{-4}
IGFBP7	rs34447434	A/C	0.18	−0.051	0.0750	−0.087	0.0000	−0.075	4.24×10^{-6}
	rs17761305	T/C	0.13	−0.058	0.0555	−0.059	0.0086	−0.059	0.0011
	rs28623722	G/A	0.08	0.065	0.0900	0.076	0.0055	0.073	0.0012
	rs28370990	T/C	0.08	0.066	0.0952	0.077	0.0054	0.073	0.0012
	rs17183073	C/G	0.13	−0.051	0.0967	−0.059	0.0082	−0.057	0.0018
	rs7688831	A/G	0.09	0.064	0.0925	0.060	0.0254	0.062	0.0052
	rs1713968	A/G	0.15	0.061	0.0494	0.044	0.0420	0.050	0.0052
	rs116189043[a]	A/G	0.03	−0.112	0.0648	−0.083	0.0413	−0.092	0.0064
	rs1611781	G/A	0.14	0.061	0.0506	0.043	0.0515	0.049	0.0065
	rs6554409	C/T	0.13	0.059	0.0656	0.044	0.0467	0.049	0.0073
	rs1713961	C/T	0.13	0.059	0.0666	0.043	0.0497	0.049	0.0078
	rs1718883	T/A	0.13	0.059	0.0666	0.043	0.0500	0.048	0.0079
	rs7670536	T/C	0.13	0.059	0.0670	0.043	0.0500	0.048	0.0079
	rs1713962	G/A	0.13	0.059	0.0669	0.043	0.0500	0.048	0.0079
	rs1718874	C/T	0.13	0.059	0.0678	0.043	0.0504	0.048	0.0080
	rs1713967	T/A	0.13	0.059	0.0679	0.043	0.0504	0.048	0.0081
	rs1718873	C/T	0.13	0.059	0.0680	0.043	0.0505	0.048	0.0081
	rs13107451	A/G	0.13	0.058	0.0689	0.043	0.0511	0.048	0.0083
	rs11726321	C/T	0.13	0.058	0.0690	0.043	0.0512	0.048	0.0083
	rs77053948	G/A	0.16	0.056	0.0696	0.042	0.0537	0.047	0.0087
	rs1718834	T/C	0.13	0.057	0.0733	0.043	0.0529	0.048	0.0090
	rs1718872	C/G	0.15	0.052	0.0838	0.043	0.0513	0.046	0.0094
	rs6817232	G/C	0.16	0.055	0.0654	0.040	0.0650	0.045	0.0099

[a]Indicates that the SNP remained at least suggestively associated after excluding participants with diabetes (n = 122) and participants with missing data for diabetes (n = 104) from cohort 1.
For all single nucleotide polymorphisms (SNPs), the coded allele is the minor allele.
The results are adjusted for age, sex, body mass index, and net alkali absorption, with meta-analysis $P < 0.01$ in the joint analysis, consistent direction of effect, and $P < 0.1$ in both cohorts.

dramatic differences in urine pH or other key variables between stone formers and non-formers, and a stratified analysis of only stone formers and/or non-formers would have had a prohibitively small sample size, we did not perform a formal sensitivity analysis for kidney stones. Finally, we assessed the effects of medications known to influence urine pH (bicarbonate/citrate salts or allopurinol). In cohort 1, medication data were available for 707 participants; of these, 50 (7.1%) were taking at least 1 medication known to influence urine pH (K bicarbonate, allopurinol, or both). The effects of these medications were not significant (K bicarbonate, $P = 0.78$) or only moderately significant (allopurinol, $P = 0.01$) after adjusting for age, sex, BMI, and net alkali. In cohort 2, most of the participants were not taking allopurinol, and information about bicarbonate/citrate salts was not collected, and thus it could not be assessed (although it was likely quite small).[13]

Model 1 Analysis (Adjusting for Age, Sex, and BMI)

Urine pH was tested for associations with the 2871 SNPs in additive genetic models that adjusted for age, sex, and BMI (Table 3). Altogether, 25 SNPs across 4 genes (12 *IGFBP7*, 9 *ATP6V0A4*, 3 *SLC9A3*, 1 *RHCG*) met criteria for suggestive association with urine pH ($P < 0.01$ in the joint meta-analysis, a consistent direction of effect, and association $P < 0.1$ in both cohorts). Only 2 SNPs in *IGFBP7* met the stringent criteria for significance ($P < 1.7 \times 10^{-5}$ in the joint meta-analysis, $P < 0.05$ and consistent direction of effect in both cohorts). The lowest meta-analysis value ($P = 7.1 \times 10^{-8}$) was observed for rs34447434 in the *IGFBP7* gene, with an average effect size (in pH units) of −0.13 for each additional minor allele. In cohort 1, this SNP was only marginally associated ($P = 0.07$), but it still explained approximately 0.5% of the variance in urine pH beyond adjustment variables, and the mean urine pH for those with zero, 1, or 2 minor alleles was 6.23, 6.17, and 6.18, respectively. The maximal effect sizes for each additional minor allele of the most strongly associated SNP in the other genes were −0.08 for *ATP6V0A4*, 0.06 for *RHCG*, and −0.06 for *SLC9A3*. In cohort 1, the most strongly associated SNP in *ATP6V0A4*, rs6955765, explained an additional 1.5% of the variance in urine pH, and the mean urine pH for those with zero, 1, or 2 minor alleles was 6.23, 6.16, and 6.01, respectively.

All of the associated SNPs were located in gene intron regions. LocusZoom plots of the meta-analysis results were made from each gene that had at least 2 suggestively associated SNPs (*ATP6V0A4*, *IGFBP7*, and *SLC9A3*). The plots (Figure 1) show that the most strongly associated SNPs within each of the genes represent a single association signal for that gene. That is, most of the associated SNPs exhibit at least moderate linkage disequilibrium ($r^2 > 0.4$) with the lead SNP in the gene (the most strongly associated SNP that met criteria for suggestive association), shown in purple. In *SLC9A3* and *IGFBP7*, there were a few strongly associated SNPs that were not in linkage disequilibrium with the lead SNP. However, these SNPs did not meet all criteria for suggestive association ($P > 0.1$ in at least 1 of the cohorts), and thus do not constitute an independent signal.

Model 2 Analysis (Adjusting for Age, Sex, BMI, and Net Alkali Absorption)

A second model was run that additionally adjusted for net alkali absorption (Table 4), a potential indicator of total body net acid/alkali load, and therefore, driver for urinary acidification.[25]

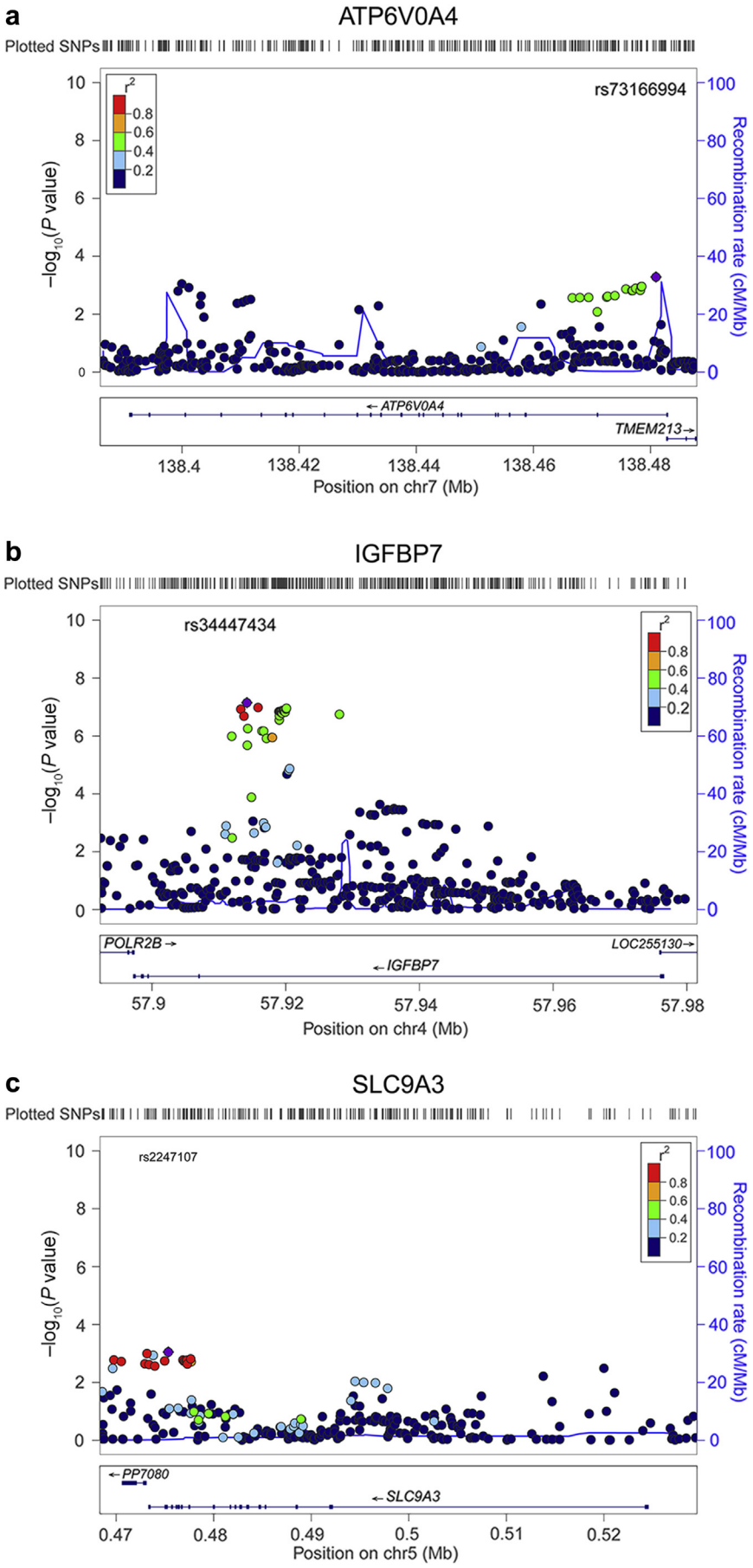
In this model, most of the SNPs in *IGFBP7* and 1 SNP in *ATP6V0A4* remained suggestively associated with urine pH. Several additional SNPs in *IGFBP7* (a total of 24 SNPs altogether) and 1 SNP in *GLS* were also suggestively associated with urine pH. As in the model 1 analysis, the lowest meta-analysis *P* value was observed for rs34447434 in the *IGFBP7* gene ($P = 4.2 \times 10^{-6}$). The maximal effect size (in pH units) for each additional minor allele of the identified SNPs was −0.08 for *IGFBP7*, −0.05 for *ATP6V0A4*, and −0.18 for *GLS*. Similar to model 1, all suggestively associated SNPs were located in gene intron regions.

Sensitivity Analysis for Diabetes

After excluding participants in cohort 1 with diabetes from the analysis (and participants with missing data for diabetes), the directions of effect for the suggestively associated SNPs remained consistent, but the *P* values were generally attenuated. Approximately half of the SNPs in *ATP6V0A4* and the SNP in *RHCG* that met criteria for suggestive association in model 1 remained suggestively associated in the sensitivity analysis. Only 1 SNP in *SLC9A3* and no SNPs in *IGFBP7* retained suggestive association in the sensitivity analysis for model 1 (Table 3). In model 2, 1 SNP in *IGFBP7* and the SNP in *GLS* remained suggestively associated in the sensitivity analysis (Table 4). Importantly, in addition to true attenuation of the effects of the SNPs on urine pH after accounting for diabetes, the loss of suggestive associations might also be due, in part, to the reduced cohort 1 sample size in this sensitivity analysis (n = 585 after exclusions).

DISCUSSION

The present study expands our understanding of the genetic factors that regulate urine pH. Because


a
ATP6V0A4
Plotted SNPs
rs73166994
r2
0.8
0.6
0.4
0.2
−log10(P value)
Recombination rate (cM/Mb)
ATP6V0A4
TMEM213
138.4
138.42
138.44
138.46
138.48
Position on chr7 (Mb)
b
IGFBP7
Plotted SNPs
rs34447434
POLR2B
LOC255130
IGFBP7
57.9
57.92
57.94
57.96
57.98
Position on chr4 (Mb)
c
SLC9A3
Plotted SNPs
rs2247107
PP7080
SLC9A3
0.47
0.48
0.49
0.5
0.51
0.52
Position on chr5 (Mb)

adjusting for net alkali, which is a major determinant of systemic acid base balance, could partially mask some genetic contributions, we modeled our SNP investigations with and without alkali adjustments. In the first analysis (adjusted for age, sex, and BMI only), SNPs in 4 candidate genes (*ATP6V0A4*, *IGFBP7*, *RHCG*, and *SLC9A3*) were identified to be highly associated with urine pH (Table 3). When net gastrointestinal alkali absorption was added in the second model, many of the SNPs in *IGFBP7* and 1 SNP in *ATP6V0A4* remained suggestively associated with urine pH effects as great as 0.08 pH units for each polymorphism (Table 4). One previously unidentified SNP in *GLS* became suggestively associated with adjusted urine pH. Lastly, when we excluded participants with diabetes from model 1, all of the SNPs in *IGFBP7* and half of the SNPs in *ATP6V0A4* and *SLC9A3*, were no longer associated with urine pH. This result could reflect the previously known link between diabetes and urine pH, but might also be a function of reduced sample size. Because an appreciation of these common polymorphisms might deepen our understanding of the pathogenesis of diseases associated with variable urine pH, a brief discussion of these genes and the polymorphisms identified through this candidate gene analysis is appropriate.

SNPs from genes that encoded 3 major proteins that could alter urine pH (*ATP6V0A4*, *SLC9A3*, *RHCG*) were identified within the analysis of model 1. There were 2 likely explanations for this. First, although these SNPs had some association with urine pH, their effects were likely surpassed by the effect of diet and gastrointestinal function, at least under normal physiological conditions. Second, due to bicarbonate backleak, the proximal tubule was unable to lower luminal fluid pH below ~6.8. We suspected that net proton excretion in this area (and hence, genetic changes in protein expression) did not play a major role in urine pH regulation,[26] but remained essential for acid-base homeostasis. *SLC9A3* codes for the NHE3 and is located on the apical membrane in the proximal tubule, TAL, and intestinal epithelial cells.[27,28] NHE3 is the major apical transporter required for sodium and bicarbonate reabsorption in the proximal tubule, and its absence in animal studies has resulted in reduced bicarbonate and fluid absorption in the proximal convoluted tubules, reduced blood pressure, and severe intestinal absorptive defects.[28] In addition, NHE3 has a critical role in proximal tubule ammonium secretion through Na^+/NH_4^+ exchange.[29,30] Our finding of associations among 3 SNPs in *SLC9A3* with urine pH could account for differences in human urine pH, perhaps through variations in urinary ammonium excretion. Further studies are needed to investigate this.

The gene *ATP6V0A4* codes for the a4 subunit of H−adenosine triphosphatase, a multi-subunit protein present on the apical membrane of the proximal tubule and within collecting duct intercalated cells that mediates H^+ secretion and bicarbonate reabsorption.[31] Mutations in its a4 subunit in the proton-translocating V_0 domain produce autosomal recessive, severe, early-onset distal renal tubular acidosis and occasionally hearing loss.[32] Under physiologic conditions, variations in regulation of collecting duct H−adenosine triphosphatase subcellular localization and activity are a critical mechanism through which urine pH is determined.[33,34] Eight of the 9 SNPs identified on *ATP6V0A4* had no significant or suggestive associations with urine pH when the model included net alkali absorption. Thus, subtle variations in hydrogen ion secretion may not be critical determinants of urine pH under physiologic conditions, at least in comparison to dietary acid load.

The gene *RHCG* encodes for the protein Rhesus C glycoprotein (Rhcg), a member of the NH_3 transporter family and an orthologue of NH_3-specific transporters expressed in plants, bacteria, yeast, and essentially all multicellular organisms.[35,36] Rhcg is expressed specifically in both the apical and basolateral plasma membranes of distal epithelial and collecting duct cells[35–37] and transports the specific molecular form of ammonia, NH_3, across lipid bilayers. One SNP in *RHCG*, rs74032414, was suggestively associated with urinary pH (Table 3). Our data confirmed what was seen in global- and collecting duct−specific *RHCG* gene deletion studies in which urinary NH_3 excretion was decreased.[38,39] Urine pH was more alkaline in the global *RHCG* deletion study, whereas collecting duct or intercalated cell deletion caused greater sensitivity of urine pH to systemic acid loads.[40] Our human data corroborated these findings and suggested that

Figure 1. Meta-analysis results for model 1 (adjusted for age, sex, and body mass index [BMI]) for genes with multiple single nucleotide polymorphisms (SNPs) meeting criteria for suggestive association. (a) *ATP6V0A4*, (b) *IGFBP7*, and (c) *SLC9A3*. Left *y*-axis: –log10 (*P* value) from association between SNPs and urine pH, adjusted for age, sex, and BMI, and accounting for sibship; right *y*-axis: SNP recombination rate based on 1000 Genomes 2012 EUR reference panel (hg19); *x*-axis: chromosomal location and gene regions; each dot within the plot represents an individual SNP; r^2 color code: the color of each dot represents the degree of linkage disequilibrium of that particular SNP to the most strongly associated SNP meeting all criteria for suggestive association (purple diamond).

variation in *RHCG* expression could cause changes in urine pH.

Twelve SNPs within *IGFBP7* were suggestively associated with urine pH (Table 3), and the association of most of these SNPs remained suggestive in a model that adjusted for net alkali absorption (Table 4). IGF-binding protein 7 is expressed in almost all epithelial cells[41,42] and resides in the IGPBP polypeptide hormone family with structural homology to insulin. Members of this family have a number of systemic effects, including insulin-like stimulation of peripheral glucose, activation of IGFs, regulation of IGF bioavailability, and even IGF-independent effects such as tumor suppression through cell cycle effects.[43,44] Protein products of *IGFBP7* deviate from its IGF 1-6 family members by having a low affinity for IGFs but a relatively high affinity for insulin.[45] Because of its ability to interfere with insulin action, higher systemic levels of IGFBP-7 have been hypothesized to promote the development of insulin resistance and even diabetic vascular complications, which is a concept supported by 4 small cohort studies in noninsulin-dependent diabetics.[14,46–48] Although none of these studies evaluated urinary pH in their patients, a plausible link exists between the two. *In vitro* studies have shown that insulin stimulates renal ammoniagenesis from the substrate L-glutamine and through activation of the NHE3 in the proximal tubule.[15] Maalouf *et al*. found the degree of insulin resistance to be inversely related to 24-hour urine pH after adjustment for age, sex, creatinine clearance, urine sulfate and BMI.[49] Thus, effects on renal ammoniagenesis could underlie the association between *IGFBP7* and urinary pH. Further studies are needed to verify this association.

The present study had both strengths and potential weaknesses. The strengths included the use of multiple well-characterized genetic databases, the use of well-characterized 24-hour urine collections to assess urine pH, thereby avoiding diurnal variations in urine acidification, and the performance of meta-analysis to allow combination of data from the different databases. The population studied was European American, so results will require replication before generalization to other populations. Both diabetics and nondiabetics were included in the study. Although some SNP associations were attenuated when people with diabetes were excluded from this analysis, at least 1 SNP in *ATP6V0A4* (model 1), *RCHG* (model 1), *SLC9A3* (model 1), *GLS* (model 2), and *IGFBP7* (model 2) remained associated, which suggested that similar genetic factors might function to affect urine pH in both diabetics and nondiabetics. Study weaknesses included the inability to determine the specific molecular mechanism by which each of these SNPs altered expression, activity, or regulation of their encoded proteins. Data on medications that might influence urine pH (such as citrate/bicarbonate salts and allopurinol) were not available for all participants, so we were not able to fully account for medication effects on urine pH. We also lacked complete data on kidney stone composition in the cohorts, and thus we were not able to tie urinary pH to kidney stone risk. In addition, little *in vitro* or *in vivo* functional data regarding the effect of genetic variation on protein function were available for some genes of interest, such as *GLS* (glutaminase), which is predominantly expressed in the kidney and converts glutamine to glutamate, a step necessary in NH_3 production.[50] Some SNP associations in the joint analysis were highly suggestive in only 1 of the 2 cohorts, that is, for model 1, 2 genes (*SLC9A3* and *RHCG*) had highly suggestive SNPs in the cohort 2 (NHSI/NHSII/HPFS), but the same SNPs were only marginally suggestive ($P < 0.1$) in cohort 1 (GENOA). Reasons for these differing results might include subtle differences in the genetic background of these cohorts (different geographic locales or ethnic mix) or differences in nongenetic factors (diet, medications, environment) that could also vary day to day. Finally, most of the known mutations that influence stone formation are found in exons. However, with improved technology, genetic variation in introns and intergenic regions has been shown to be linked to a variety of diseases, that is the most recent genome-wide association study for kidney stones showed the only SNPs with genome-side significance were in introns ($P = 6 \times 10^{-10}$ and 2×10^{-8}).[51]

Nevertheless, this is one of the first studies to provide evidence for genetic influence of urinary pH in the general population under physiologic conditions, evaluating polymorphisms in genes encoding proteins involved in renal acid-base and NH_3 metabolism. The most strongly associated genes encode for proteins involved in urine acidification (*SLC9A3* and *ATP6V0A4*), NH_3 metabolism (*GLS*, *RHCG*), or insulin binding (*IGFBP7*). Identification that these SNPs associate with urine pH suggests that multiple genetic factors may contribute to the pathogenesis of conditions associated with variable urine pH.

DISCLOSURE

All the authors declared no competing interests.

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