Genetic variants of calcium and vitamin D metabolism in kidney stone disease

Sarah A. Howles D.Phil., F.R.C.S.(Urol), Akira Wiberg B.M.B.Ch., Michelle Goldsworthy Ph.D., Asha L. Bayliss Ph.D., Emily Grout R.G.N., Chizu Tanikawa Ph.D., Yoichiro Kamatani M.D., Ph.D., Chikashi Terao M.D., Ph.D., Atsushi Takahashi Ph.D., Michiaki Kubo M.D., Ph.D., Koichi Matsuda M.D., Ph.D., Rajesh V. Thakker M.D., F.R.C.P., F.R.S., Benjamin W. Turney D.Phil., F.R.C.S.(Urol), and Dominic Furniss D.M., F.R.C.S.(Plast).

Nuffield Department of Surgical Sciences, University of Oxford, United Kingdom (S.A.H., M.G., E.G., B.W.T.), Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, United Kingdom (A.W., D.F.), Academic Endocrine Unit, Radcliffe Department of Medicine, University of Oxford, United Kingdom (S.A.H., M.G., A.B., R.V.T.), Laboratory of Genome Technology, Human Genome Centre, University of Tokyo, Japan (C.T.), RIKEN Centre for Integrative Medical Sciences, Kanagawa, Japan (Y.K., C.T., A.T., M.K.), Laboratory of Clinical Genome Sequencing, Department of Computational Biology and Medical Sciences, University of Tokyo, Japan (K.M.).

Drs Sarah A. Howles and Akira Wiberg contributed equally to this article.

Dr Benjamin W. Turney and Prof. Dominic Furniss contributed equally to this article.

Address correspondence to Dr. Howles at the Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM), Churchill Hospital, Oxford, OX3 7LE, United Kingdom, or at sarah.howles@nds.ox.ac.uk

BACKGROUND

Kidney stone disease (nephrolithiasis) is a major clinical and economic health burden with a multifactorial etiology and heritability of ~45-60%. To identify common genetic variants associated with nephrolithiasis we performed genome-wide association studies (GWAS) and meta-analysis in British and Japanese populations.

METHODS

GWAS and trans-ethnic meta-analysis of 12,123 kidney stone cases and 416,928 controls was performed. Genotype-phenotype correlations were established in a validation cohort of kidney stone patients. Biological pathways were studied *in vitro* in HEK293 cells.

RESULTS

Twenty loci associated with nephrolithiasis were identified, ten of which are novel. One such locus is associated with *CYP24A1* and is predicted to affect vitamin D metabolism. Five loci, *DGKD, DGKH, WDR72, GPIC1,* and *BCR,* are predicted to influence calcium-sensing receptor (CaSR) signaling. The *CYP24A1*-associated locus, correlated with serum calcium concentration and number of kidney stone episodes in a validation cohort of nephrolithiasis patients. In addition, the *DGKD*-associated locus correlated with urinary calcium excretion in the validation cohort. Moreover, DGKD knockdown was shown to impair CaSR-signal transduction *in vitro*, an effect that was rectified by the calcimimetic cinacalcet, thereby supporting the role of DGKD in CaSR signaling.

CONCLUSIONS

Our study identified ten novel loci associated with kidney stone disease; six loci are predicted to influence calcium-sensing receptor and vitamin D metabolism pathways. These findings indicate that genotyping may help to inform risk of incident kidney stone disease prior to vitamin D supplementation and facilitate precision-medicine approaches, by targeting CaSR signaling or vitamin D activation pathways in patients with recurrent kidney stones.

Introduction

Kidney stone disease is a major clinical and economic health burden, affecting ~20% of men and ~10% of women by 70 years of age[1]. Kidney stones commonly cause debilitating pain, and an obstructing ureteric stone in the context of renal infection can be life threatening. The prevalence of this disorder is increasing and the United States is predicted to spend over $5 billion per year by 2030 on its treatment[2]. Unfortunately, up to 50% of individuals will experience a second kidney stone episode within 10 years of their initial presentation[3]. Furthermore, nephrolithiasis sufferers are at increased risk of chronic kidney disease and renal function decline is more commonly observed in individuals requiring repeated surgical intervention[4].

The etiology of kidney stone disease is multifactorial and the contribution of genetic factors well recognized. Thus, a strong family history of urolithiasis, including a parent and a sibling, results in a standard incidence ratio (SIR) for stone formation of >50 in contrast to a SIR of 1.29 in spouses[5]. Furthermore, twin studies have reported a heritability of >45% and >50% for stone disease and hypercalciuria, respectively[6,7]. In a minority of cases, a well-known monogenetic disorder accounts for the observed heritable phenotype, however, for the majority, a polygenetic predisposition accounts for familial risk.

Three genome-wide association studies of nephrolithiasis have been published, identifying five loci associated with disease (*CLDN14*, *RGS14-SLC34A1*, *INMT-FAM188B-AQP1*, *DGKH*, and *ALPL*) along with a suggestive association at a *CASR* locus, and association with a rare missense variant in *TRPV5*[8-10]. In addition, variants in *UMOD* were found to be associated with risk of nephrolithiasis in a GWAS of

chronic kidney disease[11]. However, only two of these loci (*CLDN14* and *RGS14-SLC34A1*) have been replicated and no trans-ethnic studies have been undertaken. To increase understanding of the common genetic factors contributing to risk of nephrolithiasis, we studied associations of genetic variants with nephrolithiasis in 12,123 stone formers and 416,928 controls of Japanese and White-British ancestry.

Methods

Study participants

Genome-wide studies were undertaken using UK Biobank and Biobank Japan resources[12,13]. In the UK Biobank, kidney stone cases were identified using ICD-10 and OPCS codes, patients were excluded if they were recorded to have a disorder predisposing to nephrolithiasis. Following quality control, 6,536 cases and 388,508 controls were identified (Tables S1-3 in the Supplementary Appendix). The UK Biobank has approval from the North West Multi-Centre Research Ethics Committee (11/NW/0382, study ID 885). Biobank Japan patients had a diagnosis of nephrolithiasis confirmed by enrolling physicians and patients were excluded if there was a history of bladder stones. Controls were identified from four population-based cohorts[14-16]. In total, 5,587 cases and 28,870 controls were identified (Table S4 in the Supplementary Appendix). Ethical committees at each Japanese institute approved the project.

Genetic analysis

In the UK Biobank, genome-wide association testing was undertaken across 547,011 genotyped SNPs and ~8.4 million imputed SNPs with a minor allele frequency (MAF) ≥0.01 and Info Score≥0.9, using a linear mixed non-infinitesimal model

implemented in BOLT-LMM v2.3[17]. Japanese samples were excluded if SNP call rate was <0.99, MAF < 1%, or Hardy–Weinberg equilibrium $p<1.0 \times 10^{-6}$. Age and sex were used as covariates in British and Japanese populations. Trans-ethnic meta-analysis was performed using the summary statistics from UK and Japanese GWAS data sets. An info threshold of >0.5 was used to prune the Japanese GWAS SNPs prior to performing a fixed-effects meta-analysis using GWAMA[18], using ~5million SNPs common to both datasets. Population Attributable Risk (PAR) at each locus was calculated using data from the UK Biobank population.

In Silico Analyses

Candidate genes were identified based on FUMA positional mapping, functional annotation, and biological plausibility. Expression across different tissue types was assessed using MAGMA gene-property analysis implemented in FUMA, and enriched gene ontologies investigated with GENE2FUNC tool using 54 positionally mapped genes with unique Entrez IDs and gene symbols[19,20].

Functional characterisation of the Calcium-Sensing Receptor Pathway

Scrambled or *DGKD* targeted siRNA was transfected into HEK293 cells stably expressing calcium-sensing receptors (CaSRs) and luciferase under the control of a serum-response element (SRE) (HEK-CaSR-SRE cells). Knockdown was confirmed with quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. The effects of DGKD knockdown on intracellular CaSR-induced mitogen-activated protein kinase (MAPK) signaling response was assessed by alterations in SRE, a downstream mediator of MAPK signaling. The ability of 5nM cinacalcet, an

allosteric activator of the CaSR, to rectify observed alterations in the CaSR-signaling was investigated.

Genotype-Phenotype Correlations

Clinical data was collected from a cohort of UK-based kidney stone patients and SNP genotyping undertaken. Patients were excluded from genotype-phenotype correlations if they were known to suffer from disorders predisposing to nephrolithiasis. The project was approved under the Oxford Radcliffe Biobank research tissue bank ethics (09/H0606/5+5).

Results

Twenty genetic loci associated with kidney stone disease

A total of 20 genetic loci were identified to associate with nephrolithiasis. Ten of these were initially identified from the UK Biobank discovery cohort (Table S5 in the Supplementary Appendix) and another 10 from a subsequent trans-ethnic meta-analysis with Japanese GWAS summary statistics (submitted for publication), including four novel loci identified independently in the Japanese GWAS (*GCKR, SAYSD1-KCNK5, BCAS3-TBX2-C17orf82,* and *PKN1-PTGER1-GIPC1*) (Table 1, Fig. 1, and Fig. S1 in the Supplementary Appendix).

In silico analysis of the 20 loci implicated 54 genes associated with kidney stone disease and tissue expression analysis revealed a striking overexpression of these genes in the kidney cortex. There was enrichment for gene ontologies associated with transmembrane ion transport, renal function, and calcium homeostasis, including "response to vitamin D" (Figures S2-3 and Table S7 in the Supplementary Appendix).

PAR for each increased-risk allele ranged from 1.3% to 22.3% (Table S6 in the Supplementary Appendix) and the highest PAR was identified with a *CYP24A1*-associated locus. In addition, five of the identified loci, with PAR scores between 5.9-7.9%, were predicted to influence CaSR signaling. These loci were selected for further analysis.

CYP24A1 risk allele correlates with serum calcium concentration and stone recurrence

rs17216707 on chromosome 20 is located ~38kb upstream of *CYP24A1*, a gene that encodes cytochrome P450 family 24 subfamily A member 1 (CYP24A1). CYP24A1 metabolises active 1,25-dihydroxyvitamin D to inactive 24,25-dihydroxyvitamin D and loss-of-function mutations cause the autosomal recessive condition infantile hypercalcaemia type 1 (OMIM 126065)[21]. We postulated that the *CYP24A1* increased-risk allele might associate with decreased CYP24A1 activity, leading to perturbations of calcium homeostasis and mimicking an attenuated form of infantile hypercalcaemia type 1. Therefore, associations of rs17216707 with serum calcium, phosphate, parathyroid hormone (PTH), and 25-hydroxyvitamin D concentrations were sought in a validation cohort of kidney stone formers. 1,25-hydroxyvitamin D levels were unavailable. Associations of genotype with urinary calcium excretion and number of kidney stone episodes were also examined. Reference ranges for 24-hour urinary calcium excretion differ for men and women[22], and associations with urinary calcium excretion were therefore examined separately.

Individuals homozygous for the *CYP24A1* increased-risk allele rs17216707 (T) had a significantly increased mean serum calcium concentration when compared to those with one reduced-risk allele rs17216707 (C), consistent with a recessive effect (mean serum calcium 2.36mmol/l (TT) *vs.* 2.32mmol/l (TC)) (Table 2). Furthermore, rs17216707 (T) homozygotes had more kidney stone episodes than heterozygous individuals (mean number of stone episodes 4.0 (TT) *vs.* 2.4 (TC), p=0.0003) and there was a significant correlation across genotypes (TT *vs.* TC *vs.* CC) with number of stone episodes (p=0.0024) (Table 2). No correlation was found between rs17216707 genotype and serum phosphate, PTH, 25-hydroxyvitamin D concentration or urinary calcium excretion. These findings support our hypothesis that the rs17216707 increased-risk allele is associated with a relative hypercalcaemia and demonstrate increased stone recurrence rates in these individuals.

Calcium-sensing receptor pathway

The previously reported association between the CaSR-associated intronic SNP rs7627468 and nephrolithiasis was confirmed ($p=3.5x10^{-5}$)[9]. In addition, five of the identified loci are linked to genes that are predicted to influence CaSR signaling. rs13003198 is ~6kb upstream of *DGKD* which encodes diacylglycerol kinase delta (DGKD); rs1037271 is an intronic variant in *DGKH,* encoding diacylglycerol kinase eta (DGKH). DGKD and DGKH phosphorylate diacylglcerol (DAG), a component of the intracellular CaSR signaling pathway which induces CaSR-mediated membrane ruffling and activates protein kinase C signaling cascades including MAPK[23,24] (Fig. 2). rs578595 is an intronic variant in *WDR72* that encodes WD repeat domain 72 (WDR72) and rs3760702 is ~300bp upstream of *GIPC1* that encodes Regulator of G-protein signaling 19 Interacting Protein 1 (GIPC1). Both WDR72 and GIPC1 are

thought to play a role in clathrin-mediated endocytosis, a process known to be central in sustained intracellular CaSR signaling[24-27] (Fig. 2). rs13054904 is an intronic variant in *RSPH14,* which encodes Radial Spoke Head 14 Homolog (RSPH14), a protein with an unclear function. However, it is ~110kb upstream of *BCR,* a gene encoding a RAC1 (Rac Family Small GTPase 1) GTPase-activating protein known as Breakpoint Cluster Region (BCR)[28]. RAC1 activation is induced by CaSR ligand binding and mediates CaSR-induced membrane ruffling[23] (Fig. 2).

***DGKD* risk allele correlates with urinary calcium excretion**

Gain-of-function mutations in components of the CaSR-signaling pathway result in autosomal dominant hypocalcaemia (ADH), which is associated with hypercalciuria and nephrolithiasis[29,30]. We therefore hypothesized that loci linked to CaSR-signaling associate with enhanced CaSR signal transduction resulting in a biochemical phenotype mimicking an attenuated form of ADH.

The PAR for the *DGKD* and *DGKH*-associated SNPs were highest amongst the CaSR-associated loci (7.0% and 7.9%, respectively). Furthermore, *DGKD* and *DGKH* may influence both membrane ruffling and MAPK CaSR-signaling pathways (Fig.2). Therefore associations of rs838717 (top *DGKD*-associated SNP in the UK Biobank GWAS, Table S4 in the Supplementary Appendix, linkage disequilibrium with rs13003198 r^2=0.53) and rs1170174 (top *DGKH*-associated SNP in the UK Biobank GWAS, Table S4 in the Supplementary Appendix, linkage disequilibrium with rs13003198 r^2=0.3) with biochemical phenotypes and episodes of stone recurrence were investigated.

The *DGKD* increased-risk allele rs838717 (G) associated with increased 24-hour urinary calcium excretion in male stone formers (mean 24-hour urinary calcium excretion 7.27mmol (GG) *vs.* 4.54mmol (AA), p=0.0055) (Table 2) consistent with enhanced CaSR-signal transduction. No correlations were observed between genotype and serum calcium, phosphate, PTH, 25-hydroxyvitamin D concentrations or number of stone episodes. The *DGKH* increased-risk allele rs1170174 (A) did not associate with biochemical phenotype or stone recurrence. However the sample size of homozygotes was small (AA, n=6) and prior to Bonnferonni correction, a suggestive association was detected (mean 24-hour urinary calcium excretion 8.14mmol (AA) *vs.* 5.09mmol (GG), p=0.0503).

DGKD knockdown results in impaired signal transduction via the CaSR

DGKD, which had an associated allele that correlated with an increased urinary calcium excretion, was selected for further *in vitro* characterisation. HEK-CaSR-SRE cells were treated with scrambled or *DGKD* targeted siRNA and intracellular MAPK responses to alterations in extracellular calcium concentration ($[Ca^{2+}]_o$) assessed. Reduction in DGKD expression was confirmed by qRT-PCR and western blot analysis (Fig. 2A-C). SRE responses were significantly decreased in cells with reduced DGKD expression (DGKD-KD maximal response = 5.28 fold change, 95% confidence interval (CI) = 4.77-5.79) compared to cells with baseline DGKD expression (wild-type (WT) maximal response = 7.20 fold change, 95% CI = 6.46-7.93, p=0.0065). Cinacalcet, rectified this loss-of-function (DGKD-KD + 5nM cinacalcet, maximal response = 7.62 fold change, 95% CI = 5.98-9.27)(Fig. 2D-E). Thus, reduced DGKD expression impairs CaSR signaling via the MAPK pathway, an

effect that can be rectified with cincalcet. These findings provide *in vitro* evidence for the role of DGKD in CaSR-mediated signal transduction.

Discussion

Our study, which represents the largest kidney stone GWAS to date and integrates data from 12,123 stone formers and 416,928 controls from British and Japanese ancestries, has identified 10 novel genetic loci to be associated with nephrolithiasis and confirmed the association of 10 previously reported loci. The PAR for each increased-risk allele ranged from 1.3% to 22.3%, indicating that these common variants are important in the pathogenesis of nephrolithiasis at a population level. The genes implicated by our GWAS were disproportionately expressed in the renal cortex, with enrichment for biological pathways and gene ontologies involving solute transport, renal physiology and calcium homeostasis.

Our findings highlight the role of vitamin D catabolism in kidney stone formation. Thus we identified a locus ~38kb upstream of *CYP24A1* (rs17216707) and demonstrated that genotype at this locus associated with serum calcium concentration and stone recurrence episodes in a cohort of kidney stone patients. Biallelic loss-of-function mutations in *CYP24A1* are a rare cause of hypercalcaemia and vitamin D supplementation in these patients is reported to cause nephrocalcinosis due to an impaired ability of CYP24A1 to catabolise 1,25-dihydroxyvitamin D via 24-hydroxylation[21]. Thus, we hypothesize that individuals carrying the *CYP24A1* increased-risk allele may have a reduced activity of the 24-hydroxylase enzyme and therefore increased sensitivity to vitamin D. Patients with loss-of-function CYP24A1 mutations have been successfully treated with inhibitors of vitamin D synthesis

including fluconazole, and similar therapies may be useful in this subset of recurrent kidney stone formers[31].

Cholecalciferol (vitamin D) in combination with calcium supplementation is recognised to increase risk of kidney stone formation[32]. However, the role of cholecalciferol supplementation in hypercalciuria and risk of stone formation is not established[33]. An association between kidney stones and serum 25-hydroxyvitamin D in the range 20-100ng/ml has not been detected[34] and studies of the risk of incident kidney stone formation due to vitamin D intake in three populations have reported an increased risk in only one population[35]. Our findings suggest that a specific subset of individuals may be at risk of kidney stones and hypercalciuria from vitamin D supplementation.

Five of the identified loci are linked to genes that are predicted to influence CaSR signaling: *DGKD, DGKH, WDR72, GIPC1,* and *BCR*. The CaSR is a G-protein coupled receptor that is highly expressed in the parathyroid and kidneys and has a central role in calcium homeostasis, stimulating release of PTH in response to alterations in serum calcium concentration[36]. PTH enhances bone resorption, urinary calcium reabsorption, and renal synthesis of 1,25-dihydroxyvitamin D from 25-dihydroxyvitamin D, leading to increased intestinal calcium absorption[36]. The CaSR also increases renal calcium reabsorption in a PTH-independent manner[36]. Mutations in the CaSR and its signaling partner, G-protein subunit Gα11 cause ADH type 1 (OMIM 601198) and ADH type 2 (OMIM 615361), respectively[29,30]. ADH patients are mildly hypocalcemic with a PTH that is inappropriately reduced or in the normal range. Approximately 10% of ADH patients experience hypercalciuria and are

therefore at risk of kidney stones[30,37]. Furthermore, treatment with active metabolites of vitamin D may cause marked hypercalciuria and nephrolithiasis[29,37]. *In vitro* expression analysis of ADH-associated CaSR and Gα11 mutations demonstrate a gain-of-function in intracellular signaling in response to alterations in $[Ca^{2+}]_o$ via pathways including intracellular calcium ions or MAPK[29,30,38].

The *DGKD* increased-risk allele was found to associate with urinary calcium excretion in male stone formers but not in female stone formers. This is probably due to a lack of power as a result of the small sample size of female stone formers, however it is interesting to note that heritability of stone disease is lower in women than in men[6]. Furthermore, no correlations were identified with serum calcium concentrations despite this being the prominent phenotype in ADH patients; the reasons for this are unclear but may relate to differential tissue expression patterns of DGKD or the specificity of DGKD effects on intracellular CaSR-signaling pathways.

Reduced expression of DGKD was shown to result in decreased CaSR-mediated intracellular signaling via the MAPK pathway *in vitro*, an effect that was rectified with cinacalcet. This suggests that the *DGKD* increased-risk allele may associate with a relative increase in DGKD expression thereby enhancing CaSR-mediated signal transduction. Calcilytics, including NPS-2143 and ronacaleret, rectify enhanced CaSR-mediated signaling *in vitro* and biochemical phenotypes in mouse models of ADH[38-40] and these compounds may therefore represent novel, targeted therapies for recurrent stone formers carrying CaSR-associated increased-risk alleles.

In conclusion, our study has identified 20 loci linked to kidney stone formation, 10 of which are novel, and revealed that vitamin D metabolism pathways and enhanced CaSR-signaling contribute to the pathogenesis of kidney stone disease. Our findings suggest a role for genetic testing to identify individuals in whom vitamin D supplementation should be used with caution and to facilitate a precision-medicine approach for the treatment of recurrent kidney stone disease, whereby targeting of the CaSR-signaling or vitamin D metabolism pathways may be beneficial in the treatment of a subset of patients with nephrolithiasis.

References

1. Scales CD, Smith AC, Hanley JM, Saigal CS, Urologic Diseases in America Project. Prevalence of Kidney Stones in the United States. Eur Urol 2012;62(1):160–5.

2. Antonelli JA, Maalouf NM, Pearle MS, Lotan Y. Use of the National Health and Nutrition Examination Survey to calculate the impact of obesity and diabetes on cost and prevalence of urolithiasis in 2030. Eur Urol 2014;66(4):724–9.

3. Pearle MS, Goldfarb DS, Assimos DG, et al. Medical management of kidney stones: AUA guideline. The Journal of Urology. 2014;192(2):316–24.

4. Gambaro G, Croppi E, Bushinsky D, et al. The Risk of Chronic Kidney Disease Associated with Urolithiasis and its Urological Treatments: A Review. The Journal of Urology 2017;198(2):268–73.

5. Hemminki K, Hemminki O, Försti A, Sundquist K, Sundquist J, Li X. Familial risks in urolithiasis in the population of Sweden. BJU Int 2018;121(3):479–85.

6. Goldfarb DS, Avery AR, Beara-Lasic L, Duncan GE, Goldberg J. A Twin Study of Genetic Influences on Nephrolithiasis in Women and Men. Kidney Int Rep 2018;

7. Hunter DJ, Lange M de, Snieder H, et al. Genetic contribution to renal function and electrolyte balance: a twin study. Clin Sci 2002;103(3):259–65.

8. Thorleifsson G, Holm H, Edvardsson V, et al. Sequence variants in the CLDN14 gene associate with kidney stones and bone mineral density. Nat Genet 2009;41(8):926–30.

9. Oddsson A, Sulem P, Helgason H, et al. Common and rare variants associated with kidney stones and biochemical traits. Nat Commun 2015;6:7975.

10. Urabe Y, Tanikawa C, Takahashi A, et al. A genome-wide association study of nephrolithiasis in the Japanese population identifies novel susceptible Loci at 5q35.3, 7p14.3, and 13q14.1. PLoS Genet 2012;8(3):e1002541.

11. Gudbjartsson DF, Holm H, Indridason OS, et al. Association of variants at UMOD with chronic kidney disease and kidney stones-role of age and comorbid diseases. PLoS Genet 2010;6(7):e1001039.

12. Collins R. What makes UK Biobank special? Lancet 2012;379(9822):1173–4.

13. Hirata M, Nagai A, Kamatani Y, et al. Overview of BioBank Japan follow-up data in 32 diseases. J Epidemiol 2017;27(3S):S22–8.

14. Tsugane S, Sobue T. Baseline Survey of JPHC Study Design and Participation Rate. J Epidemiol 2001;11(6sup):24–9.

15. Hamajima N, J-MICC Study Group. The Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) to detect gene-environment interactions for cancer. Asian Pac J Cancer Prev 2007;8(2):317–23.

16. Kuriyama S, Yaegashi N, Nagami F, et al. The Tohoku Medical Megabank Project: Design and Mission. J Epidemiol 2016;26(9):493–511.

17. Loh P-R, Tucker G, Bulik-Sullivan BK, et al. Efficient Bayesian mixed-model analysis increases association power in large cohorts. Nat Genet 2015;47(3):284–90.

18. Mägi R, Morris AP. GWAMA: software for genome-wide association meta-analysis. BMC Bioinformatics 2010;11(1):288.

19. de Leeuw CA, Mooij JM, Heskes T, Posthuma D. MAGMA: generalized gene-set analysis of GWAS data. PLoS Comput Biol 2015;11(4):e1004219.

20. Watanabe K, Taskesen E, van Bochoven A, Posthuma D. Functional mapping and annotation of genetic associations with FUMA. Nat Commun 2017;8(1):1826.

21. Schlingmann KP, Kaufmann M, Weber S, et al. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. N Engl J Med 2011;365(5):410–21.

22. Curhan GC, Willett WC, Speizer FE, Stampfer MJ. Twenty-four-hour urine chemistries and the risk of kidney stones among women and men. Kidney Int 2001;59(6):2290–8.

23. Schlam D, Canton J. Every day I“m rufflin”: Calcium sensing and actin dynamics in the growth factor-independent membrane ruffling of professional

phagocytes. Small GTPases 2017;8(2):65–70.

24. Gorvin CM, Rogers A, Hastoy B, et al. AP2σ Mutations Impair Calcium-Sensing Receptor Trafficking and Signaling, and Show an Endosomal Pathway to Spatially Direct G-Protein Selectivity. Cell Rep 2018;22(4):1054–66.

25. Wang S-K, Hu Y, Yang J, et al. Critical roles for WDR72 in calcium transport and matrix protein removal during enamel maturation. Mol Genet Genomic Med 2015;3(4):302–19.

26. Shang G, Brautigam CA, Chen R, Lu D, Torres-Vázquez J, Zhang X. Structure analyses reveal a regulated oligomerization mechanism of the PlexinD1/GIPC/myosin VI complex. Elife 2017;6:213.

27. Nesbit MA, Hannan FM, Howles SA, et al. Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3. Nat Genet 2012;45(1):93–7.

28. Smith KR, Rajgor D, Hanley JG. Differential regulation of the Rac1 GTPase-activating protein (GAP) BCR during oxygen/glucose deprivation in hippocampal and cortical neurons. J Biol Chem 2017;292(49):20173–83.

29. Pearce SH, Williamson C, Kifor O, et al. A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. N Engl J Med 1996;335(15):1115–22.

30. Nesbit MA, Hannan FM, Howles SA, et al. Mutations affecting G-protein subunit α11 in hypercalcemia and hypocalcemia. N Engl J Med 2013;368(26):2476–86.

31. Sayers J, Hynes AM, Srivastava S, et al. Successful treatment of

hypercalcaemia associated with a CYP24A1 mutation with fluconazole. Clin Kidney J 2015;8(4):453–5.

32. Wallace RB, Wactawski-Wende J, O'Sullivan MJ, et al. Urinary tract stone occurrence in the Women's Health Initiative (WHI) randomized clinical trial of calcium and vitamin D supplements. Am J Clin Nutr 2011;94(1):270–7.

33. Malihi Z, Wu Z, Stewart AW, Lawes CM, Scragg R. Hypercalcemia, hypercalciuria, and kidney stones in long-term studies of vitamin D supplementation: a systematic review and meta-analysis. Am J Clin Nutr 2016;104(4):1039–51.

34. Nguyen S, Baggerly L, French C, Heaney RP, Gorham ED, Garland CF. 25-Hydroxyvitamin D in the range of 20 to 100 ng/mL and incidence of kidney stones. Am J Public Health 2014;104(9):1783–7.

35. Ferraro PM, Taylor EN, Gambaro G, Curhan GC. Vitamin D Intake and the Risk of Incident Kidney Stones. The Journal of Urology 2017;197(2):405–10.

36. Hannan FM, Babinsky VN, Thakker RV. Disorders of the calcium-sensing receptor and partner proteins: insights into the molecular basis of calcium homeostasis. J Mol Endocrinol 2016;57(3):R127–42.

37. Piret SE, Gorvin CM, Pagnamenta AT, et al. Identification of a G-Protein Subunit-α11 Gain-of-Function Mutation, Val340Met, in a Family With Autosomal Dominant Hypocalcemia Type 2 (ADH2). J Bone Miner Res 2016;31(6):1207–14.

38. Gorvin CM, Hannan FM, Howles SA, et al. Gα11 mutation in mice causes

hypocalcemia rectifiable by calcilytic therapy. JCI Insight 2017;2(3):e91103.

39. Hannan FM, Walls GV, Babinsky VN, et al. The Calcilytic Agent NPS 2143 Rectifies Hypocalcemia in a Mouse Model With an Activating Calcium-Sensing Receptor (CaSR) Mutation: Relevance to Autosomal Dominant Hypocalcemia Type 1 (ADH1). Endocrinology 2015;156(9):3114–21.

40. Babinsky VN, Hannan FM, Ramracheya RD, et al. Mutant Mice With Calcium-Sensing Receptor Activation Have Hyperglycemia That Is Rectified by Calcilytic Therapy. Endocrinology 2017;158(8):2486–502.

Acknowledgements This work was supported by grants from Kidney Research UK (RP_030_20180306) to S.A.H., A.W., M.G., B.W.T., and D.F, National Institute for Health Research (N.I.H.R) Oxford Biomedical Research Centre to R.V.T, and the Wellcome Trust (204826/z/16/z) to S.A.H, and M.G. S.A.H. is a N.I.H.R Academic Clinical Lecturer. A.W is a MRC Clinical Research Training Fellow. R.V.T. has Senior Investigator Awards from the Wellcome Trust (106995/z/15/z) and N.I.H.R. (NF-SI-0514-10091). We acknowledge the contribution to this study made by the Oxford Centre for Histopathology Research and the Oxford Radcliffe Biobank, which are supported by the NIHR Oxford Biomedical Research Centre.

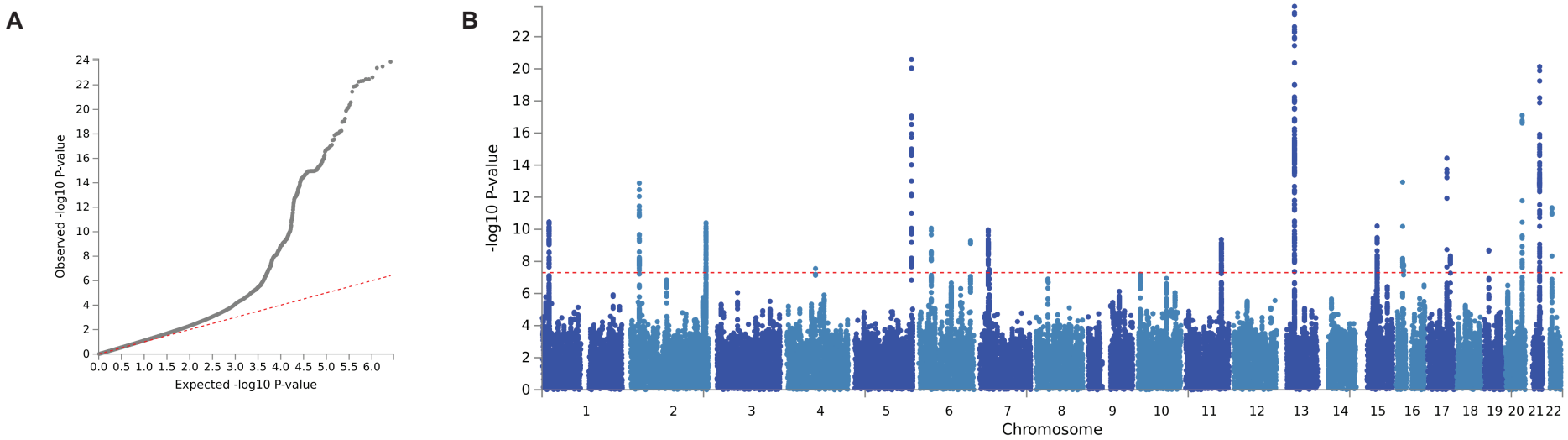

Figure 1. Results of trans-ethnic genome-wide association study in kidney stone disease. A trans-ethnic meta-analysis of kidney stone disease was performed for 12,123 patients with kidney stone disease and 416,928 controls from the UK Biobank and BioBank Japan. Panel A is a quantile-quantile plot of observed *vs.* expected p-values. The λ_{GC} demonstrated some inflation (1.0957), but the LD score regression (LDSC) intercept of 0.9997, with an attenuation ratio of 0.0075 indicated that the inflation was largely due to polygenicity and the large sample size. Panel B is a Manhattan plot showing the genome-wide p values (-log10) plotted against their respective positions on each of the autosomes. The horizontal red line shows the genome-wide significance threshold of 5.0×10^{-8}.

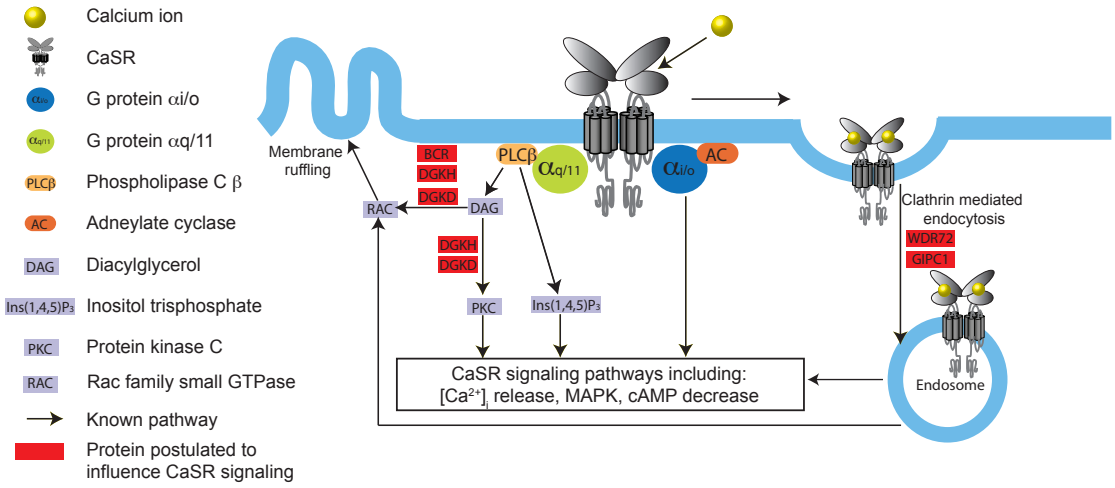

Figure 2. Schematic model for CaSR signaling

Ligand binding of calcium ions (yellow) by the G protein coupled receptor CaSR (gray) results in G protein-dependent stimulation via $G\alpha_{q/11}$ (green) or $G\alpha_{i/o}$ (blue) causing stimulation of intracellular signaling pathways including intracellular calcium ($[Ca^{2+}]_i$) release, MAPK stimulation or cAMP reduction. $G\alpha_{q/11}$ signals via inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG leads to protein kinase C (PKC) stimulation along with RAC activation, which results in membrane ruffling. Following calcium ion binding the CaSR is internalized via clathrin-mediated endocytosis where signaling continues via the endosome. Proteins postulated to influence CaSR-signaling and their potential sites of action are shown in red.

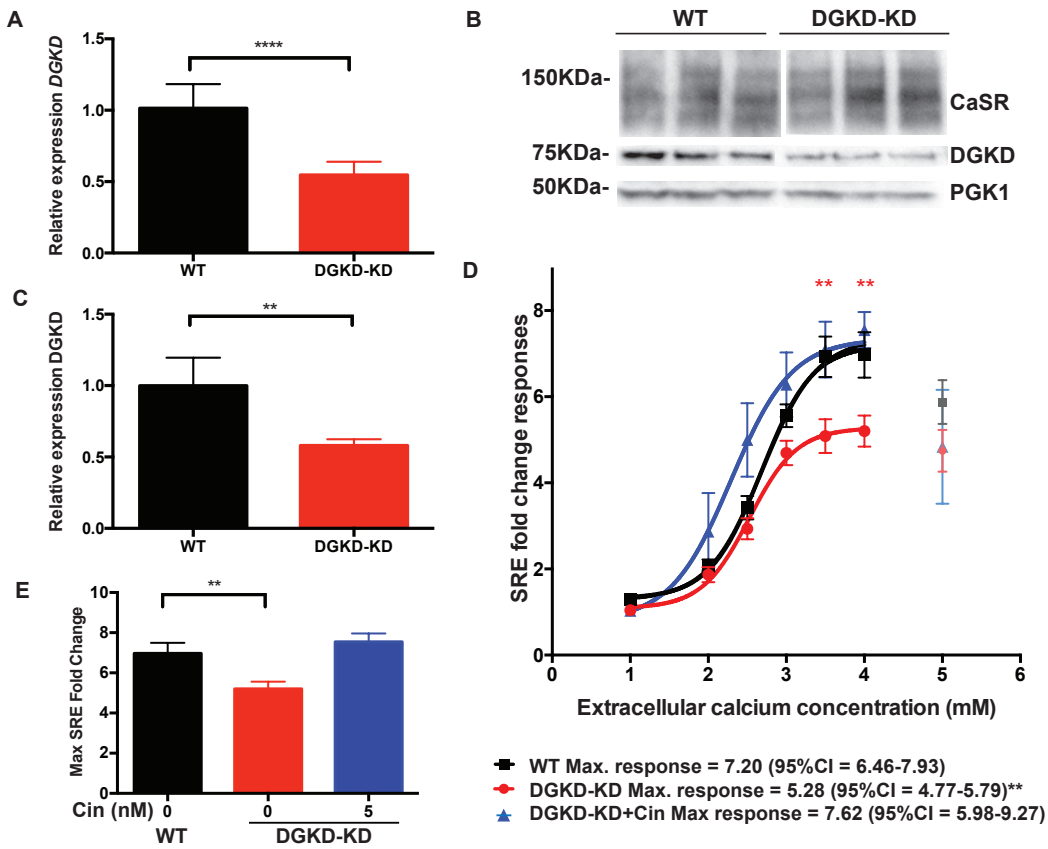

Figure 3. CaSR-mediated SRE responses following DGKD knockdown and effect of cinacalcet treatment in HEK-CaSR-SRE cells. Panel A shows relative expression of *DGKD*, as assessed by quantitative real-time PCR of HEK-CaSR-SRE cells treated with scrambled (WT) or *DGKD* (DGKD-KD) siRNA and used for SRE experiments. Samples were normalized to a geometric mean of four housekeeper genes: *PGK1*, *GAPDH*, *TUB1A*, *CDNK1B*. n=8. Panel B shows a representative western blot of lysates from HEK-CaSR cells treated with scrambled or *DGKD* siRNA and used for SRE experiments. PGK1 was used as a loading control. Panel C shows the relative expression of DGKD, as assessed by densitometry of western blots from cells treated with scrambled or *DGKD* siRNA demonstrating a ~50% reduction in expression of DGKD following treatment with *DGKD* siRNA. Samples were

normalized to PGK1. All cells expressed CaSR. n=6. Panel D shows SRE responses of HEK-CaSR-SRE cells in response to changes in extracellular calcium concentration. Cells were treated with scrambled (WT) or *DGKD* (DGKD-KD) siRNA. The responses ± SEM of a minimum of 4 independent transfections are shown. Treatment with *DGKD* siRNA led to a reduction in maximal response (red line) compared to cells treated with scrambled siRNA (black line). This loss-of-function could be rectified by treatment with 5nM cinacalcet (blue line). Post desensitization points were not included in the analysis (grey, light red, and light blue). Panel E demonstrates the mean maximal responses with SEM of cells treated with scrambled siRNA (WT, black), *DGKD* siRNA (DGKD-KD, red) and *DGKD* siRNA incubated with 5nM cinacalcet (blue). Statistical comparisons of maximal response were undertaken using F test. Students T tests were used to compare relative expression. Two-way ANOVA was used to compare points on dose response curve with reference to WT. Data are shown as mean ±SEM with **$p<0.01$, ****$p<0.0001$.

Table 1. SNPs significantly associated with kidney stone disease at trans-ethnic meta-analysis

SNP					Discovery GWAS in UK Biobank				Replication GWAS in BioBank Japan				Meta-Analysis		
Chromosome	SNP	Position[a]	EA[b]	NEA[c]	EAF[d]	INFO[e]	OR[f]	P	EAF[d]	INFO[e]	OR[f]	P	OR[f]	P	Candidate genes
1	rs10917002	21836340	T	C	0.11	0.997	1.18 (1.12-1.25)	3.60×10^{-9}	0.38	0.998	1.09 (1.04-1.15)	5.83×10^{-5}	1.13 (1.09-1.17)	3.45×10^{-11}	*ALPL*
2	rs780093	27742603	T	C	0.38	1	1.08 (1.04-1.12)	3.60×10^{-5}	0.56	0.997	1.14 (1.09-1.18)	1.10×10^{-8}	1.10 (1.08-1.13)	1.31×10^{-13}	*GCKR*
2	rs13003198	234257105	T	C	0.39	0.997	1.10 (1.06-1.14)	6.50×10^{-8}	0.25	0.98	1.12 (1.06-1.18)	1.09×10^{-5}	1.11 (1.07-1.14)	3.89×10^{-11}	*DGKD*
4	rs1481012	89039082	G	A	0.11	0.994	1.12 (1.06-1.18)	4.30×10^{-5}	0.30	0.994	1.11 (1.05-1.17)	1.50×10^{-5}	1.11 (1.07-1.16)	2.79×10^{-8}	*ABCG2*
5	rs56235845	176798040	G	T	0.33	0.986	1.16 (1.12-1.20)	9.10×10^{-15}	0.31	0.87	1.18 (1.12-1.25)	1.88×10^{-11}	1.16 (1.13-1.20)	2.64×10^{-21}	*SLC34A1*
6	rs1155347	39146230	C	T	0.22	0.975	1.12 (1.07-1.17)	2.60×10^{-7}	0.16	0.925	1.16 (1.08-1.24)	1.33×10^{-6}	1.13 (1.09-1.17)	8.54×10^{-11}	*KCNK5*
6	rs77648599	160624115	G	T	0.03	0.992	1.33 (1.21-1.47)	5.50×10^{-9}	0.04	0.739	1.22 (1.06-1.44)	1.89×10^{-3}	1.30 (1.20-1.42)	5.39×10^{-10}	*SLC22A2*
7	rs12539707	27626165	T	C	0.30	0.999	1.13 (1.08-1.17)	6.30×10^{-10}	0.09	0.789	1.10 (1.01-1.21)	0.0268	1.12 (1.08-1.16)	1.09×10^{-10}	*HIBADH*
7	rs12666466	30916430	G	C	0.03	0.994	1.22 (1.11-1.34)	5.00×10^{-5}	0.12	0.989	1.17 (1.08-1.26)	2.80×10^{-6}	1.19 (1.12-1.26)	3.26×10^{-8}	*AQP1*
11	rs4529910	111243102	T	G	0.27	0.998	1.07 (1.02-1.11)	1.40×10^{-3}	0.59	0.999	1.12 (1.08-1.16)	3.94×10^{-7}	1.09 (1.06-1.12)	4.25×10^{-10}	*POU2AF1*
13	rs1037271	42779410	C	T	0.39	0.995	1.11 (1.07-1.15)	2.50×10^{-8}	0.55	0.936	1.20 (1.15-1.24)	7.49×10^{-15}	1.15 (1.12-1.18)	1.29×10^{-24}	*DGKH*
15	rs578595	53997089	C	A	0.46	0.996	1.09 (1.05-1.13)	2.50×10^{-6}	0.69	0.996	1.11 (1.06-1.15)	2.25×10^{-5}	1.09 (1.07-1.12)	6.26×10^{-11}	*WDR72*
16	rs77924615	20392332	A	G	0.20	0.980	1.13 (1.08-1.18)	1.80×10^{-8}	0.22	0.984	1.17 (1.10-1.24)	2.80×10^{-9}	1.14 (1.10-1.19)	1.14×10^{-13}	*UMOD*
16	rs889299	23381914	G	A	0.76	1	1.10 (1.05-1.14)	8.20×10^{-6}	0.66	0.895	1.09 (1.04-1.14)	9.39×10^{-4}	1.09 (1.06-1.13)	1.55×10^{-8}	*SCNN1B*
17	rs1010269	59448945	G	A	0.83	0.981	1.08 (1.03-1.14)	7.10×10^{-4}	0.56	0.87	1.17 (1.12-1.22)	4.82×10^{-11}	1.13 (1.10-1.17)	3.71×10^{-15}	*BCAS3*
17	rs4793434	70352537	G	C	0.50	0.993	1.09 (1.05-1.13)	1.50×10^{-6}	0.32	0.983	1.09 (1.04-1.15)	2.04×10^{-4}	1.09 (1.06-1.12)	4.52×10^{-9}	*SOX9*
19	rs3760702	14588237	A	G	0.33	0.994	1.08 (1.05-1.13)	1.40×10^{-5}	0.25	0.971	1.14 (1.08-1.20)	3.78×10^{-7}	1.09 (1.07-1.13)	1.98×10^{-9}	*GIPC1*
20	rs17216707	52732362	T	C	0.81	0.961	1.17 (1.12-1.22)	9.90×10^{-12}	0.92	0.766	1.24 (1.15-1.34)	5.90×10^{-6}	1.19 (1.14-1.23)	7.82×10^{-18}	*CYP24A1*
21	rs12626330	37835982	G	C	0.49	0.980	1.16 (1.12-1.20)	5.80×10^{-17}	0.39	0.981	1.12 (1.07-1.18)	2.77×10^{-7}	1.15 (1.12-1.18)	7.24×10^{-21}	*CLDN14*
22	rs13054904	23410918	A	T	0.26	0.999	1.15 (1.11-1.20)	3.30×10^{-12}	0.02	0.967	1.05 (0.91-1.26)	0.505	1.14 (1.10-1.19)	4.49×10^{-12}	*BCR*

[a]Based on NCBI Genome Build 37 (hg19). [b]The effect allele. [c]The alternate (non-effect) allele. [d]The effect allele frequency in the study population. [e]The imputation quality score. [f]Odds ratio (95% confidence intervals).

OR>1 indicative of increased risk with effect allele.

Table 2: Genotype-phenotype correlations in cohort of kidney stone formers

Variable	Normal range§	*CYP24A1* (rs17216707)			*DGKD* (rs838717)		
		TT	TC	CC	AA	AG	GG
Serum							
Calcium (mmol/l)	2.10-2.50	**2.36±0.01*** **(260)**	**2.32±0.01** **(109)**	2.34±0.02 (15)	2.34±0.01 (107)	2.35±0.01 (182)	2.36±0.01 (95)
Phosphate (mmol)	0.7-1.40	1.02±0.13 (274)	1.02±0.02 (111)	0.99±0.05 (14)	1.03±0.02 (114)	1.01±0.02 (193)	1.02±0.02 (92)
Parathyroid hormone (pmol/l)	1.3-7.6	5.06±0.18 (271)	5.59±0.32 (107)	5.27±0.64 (14)	5.34±0.32 (108)	5.38±0.23 (189)	4.72±0.23 (95)
25-hydroxy vitamin D (nmol/l)	>50	54.8±1.84 (227)	50.6±2.60 (89)	55.7±6.92 (10)	56.1±3.13 (88)	52.6±2.04 (157)	53.2±2.90 (81)
Urine							
Male patients 24hr calcium excretion (mmol)	<7.5	6.11±0.47 (77)	4.82±0.55 (33)	3.101.27 (3)	**4.54±0.45*** **(33)**	5.45±0.48 (57)	**7.27±0.91** **(25)**
Female patients 24hr calcium excretion (mmol)	<6.2	4.85±0.53 (31)	5.06±0.56 (15)	4.27±1.69 (2)	5.83±1.34 (9)	4.92±0.46 (27)	4.12±0.56 (12)
Number stone episodes	-	**4.0±0.4*** **(287)**	**2.4±0.2** **(19)**	2.4±0.4 (14)	3.5±0.41 (114)	3.8±0.43 (208)	2.8±0.26 (98)

Numbers of stone forming patients included in analysis are shown in parentheses. Serum calcium values are albumin-adjusted. All values are expressed as mean ±SEM. Students T-tests were used for comparisons between groups of parametric data, Mann-Whitney-U tests were used for comparison of non-parametric data (number of stone episodes). Anova tests was used for comparisons of multiple sets of parametric data, no significance was reached. Kruskall-Wallis tests were used for comparisons of multiple sets of non-parametric data (number of stone episodes), significance at p=0.0024 was reached for *CYP24A1* locus correlations. *Denotes significance on comparison to bold cohort within group at Bonferroni corrected threshold of $p<0.05/7 = 0.007$. §Normal ranges are from Nesbit et al[30] and Curhan et al[22].