Abstract

Introduction
The genetics underlying the idiopathic hypercalciuria leading to calcium-containing renal stones remains elusive. The discovery of rare monogenic tubulopathies, often leading to hypercalciuria, has increased our understanding of tubular physiology and pathophysiology. However, insights into idiopathic calcium stone formation have not been gained from these disorders. The aim of this study is to examine CYP24A1 mutations in cohorts of patients with calcium nephrolithiasis.

Materials and Methods
We examined two cohorts of stone-forming patients for mutations in CYP24A1, which encodes the vitamin D24-hydroxylase enzyme. The first cohort had a biochemical phenotype of suppressed parathyroid hormone and high normal serum calcium, whilst the second cohort had a hypercalciuria phenotype. We did not identify bi-allelic sequence variants in CYP24A1 in our cohorts.

Results
In cohort 1, we identified 9 known sequence variants. In cohort 2 we identified 7 known sequence variants.

Conclusion
CYP24A1 mutations remain a rare cause of calcium nephrolithiasis and hypercalciuria.

Introduction
Renal stones have afflicted humans for many thousands of years. The oldest known stone (from approximately 4800 BCE) was found in an Egyptian grave in 1901. Today, renal stones pose a significant public health problem, with lifetime risk of forming a stone exceeding 5% in women and 12% in men. The direct and indirect costs are substantial, with the annual cost in the United States estimated to be in excess of $5 billion.

Genetic factors are suspected to be important in the aetiology of renal stone disease, based on evidence of familial clustering and data from twin studies, but so far the specific allelic variants contributing to nephrolithiasis susceptibility have been difficult to elucidate. Several rare monogenic disorders associated with hypercalciuria (urinary calcium excretion >6.2 mmol/24 h in females and >7.5 mmol/24 h in males) that cause stones have been identified. However, despite initial excitement, the genes involved in these disorders, such as CLCN5, have not proved to have a wider role in the more common ‘idiopathic’ renal stone disease. The genes involved in the majority of nephrolithiasis patients therefore remain unknown.

Calcium-containing stones account for over 80% of all renal stones, and thus much of the existing research into the genetics of nephrolithiasis has focused on candidate genes believed to be important in calcium regulation and excretion.

Genes involved in vitamin D-mediated calcium regulation have caused considerable interest. For example, the vitamin D receptor gene has been identified as a candidate gene for nephrolithiasis through linkage analysis. More recently, a novel cause of infantile hypercalcemia has been described involving mutations in the gene CYP24A1. CYP24A1 encodes the vitamin D24-hydroxylase enzyme, the enzyme responsible for the degradation of vitamin D from its active to inactive form. The enzyme also catalyses the reaction by which 25(OH) vitamin D, a precursor to the active form, is metabolised to an inactive product. It, thereby, acts as a major enzyme in the regulation of vitamin D.

CYP24A1 represents a candidate gene for calcium nephrolithiasis. Indeed, very recently a genetic analysis of two patients (a 9-year-old boy and a 38-year-old man) with elevated 1,25(OH)2 vitamin D, suppressed parathyroid hormone (PTH) and nephrocalcinosis or nephrolithiasis, respectively, were found to have bi-allelic mutations in CYP24A1. Furthermore, the predicted frequency of some potentially pathogenic sequence variants within this gene is high, suggesting these variants may be important genetic risk factors for stone formation. We therefore examined two cohorts of calcium stone formers with phenotypes that might be suggestive of an underlying defect in vitamin D metabolism mutations in CYP24A1.

Materials and Methods
Full ethical approval was obtained by the Newcastle and North Tyneside Research Ethics Committee and informed written consent were obtained from participants.

Recruitment of patients
Adult kidney stone formers (age range 18–80 years) attending for lithotripsy at the Newcastle upon Tyne
NHS Foundation Trust Hospital, UK were recruited following informed consent. Patients underwent a biochemical screen (serum biochemistry including calcium and PTH) and a spot urine estimation of urine calcium/creatinine ratio. Two cohorts of patients were selected from 703 renal calcium stone patients based on their biochemical phenotype. Cohort 1 consisted of 72 patients who displayed a high normal (corrected for albumin) serum calcium together with a low/low normal (suppressed) parathyroid hormone (PTH; level <31 pg/ml). Cohort 2 consisted of 94 patients with a tendency towards hypercalciuria as evidenced by a high or high normal calcium/creatinine ratio.

Molecular analysis
Mutational analysis of CYP24A1 was performed using exon PCR, mutational screening of amplicons by CEL1 digest and direct sequencing. Oligonucleotide primer sequences are listed in Table 1. For the DNA screening of control patients, 92 samples were obtained from blood donor (Caucasian healthy control) panels. Sequence variants were identified using Mutation Surveyor V4.0 software and cross-referenced to dbSNP.

In silico analysis of mutations
Online in silico analyses were performed when sequence variants were identified. These included MutationTaster (www.mutationtaster.org), SIFT (http://sift.jcvi.org/) and PolyPhen (http://genetics.bwh.harvard.edu/). PyMOL molecular visualisation system was used to model the protein structure of CYP24A1.

Results
The biochemical phenotypic characteristics of the 72 stone-forming patients in cohort 1 are shown in Table 2. The main features of these adult patients include a high normal serum calcium together with a relatively suppressed PTH. Unfortunately, data are not available for serum vitamin D metabolites in these patients. This cohort did not have prominent hypercalciuria, with a mean urinary calcium/creatinine ratio of 0.49 mmol/mmol creatinine. Cohort 2 consisted of an additional 94 patients with evidence of hypercalciuria as determined by a spot urine analysis for calcium/creatinine ratio. The mean urinary calcium/creatinine ratio was raised at 0.72 mmol/mmol creatinine. Where available, 24 h urine collections for calcium were performed and confirmed the hypercalciuria evident on the spot urine samples. In this cohort, serum calcium was within normal range (mean 2.3 mmol/L) and PTH was also within normal limits (Table 3).

In cohort 1, we identified nine known sequence variants (Table 4). All variants had a low minor allele frequency (MAF) except

Figure 1: Overview of vitamin D metabolism and role in calcium regulation. Vitamin D is either ingested or is synthesised by the body in response to sunlight exposure. It undergoes a series of hydroxylations in the liver and kidneys to convert it to its biologically active form 1,25(OH)₂ vitamin D. 1,25(OH)₂ vitamin D regulates serum calcium levels by increasing calcium absorption from the intestine, increasing bone turnover and decreasing calcium excretion from the kidneys. When serum calcium levels are high parathyroid hormone (PTH) is released and increases the conversion of vitamin D to this active form. CYP24A1 catalyses the hydroxylation of 1,25(OH)₂ vitamin D, and its precursor 25(OH) vitamin D, to inactive forms for excretion. This figure is adapted from reference.
for two; a common synonymous single-nucleotide polymorphism (SNP) (rs2296241) and an intronic SNP (rs4809960). In cohort 2, we identified seven known sequence variants (Table 5), all with a low MAF, and a single novel heterozygous non-synonymous sequence change was identified in one patient (c.576G>C, E192D), together with a homoyzous synonymous change in the same patient (p.A184A). The E192D variant was not detected in a panel of 96 healthy controls. The glutamic acid residue at position 192 is conserved in chicken and mouse but not in lower organisms. MutationTaster and PolyPhen both suggest that the amino acid change in E192D is benign and the algorithm SIFT also predicted that the substitution would be tolerated. Structural modelling of CY24A1 protein demonstrates that the E192

Table 1. Oligonucleotide primer sequences.

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Sequence</th>
<th>Reverse primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP24A1 Exon1_1</td>
<td>AGGGCATGCTCTGTCTCC</td>
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<td>CCCTCTTGGCTCCTTTTCC</td>
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<td>ATGTCGGGAGGGGTTT</td>
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<td>CYP24A1 Exon6</td>
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<tr>
<td>CYP24A1 Exon12_3</td>
<td>TTGGATCTGCTGTGAGG</td>
<td>CYP24A1 Exon12_3</td>
<td>TTGTGTGATAGGGCTTGG</td>
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</table>

Table 2. Biochemical Phenotype of cohort 1.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Mean serum calcium ± SD (mmol/L) (NR 2.12-2.55)</th>
<th>Mean urine calcium creatinine ratio ± SD (NR &lt; 0.60 mmol/mmol creatinine)</th>
<th>Mean serum PTH ± SD (pg/mL) (NR 11-54 pg/mL)</th>
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</thead>
<tbody>
<tr>
<td>72</td>
<td>2.45 ± 0.12 (Range 2.25-2.95)</td>
<td>0.49 ± 0.3</td>
<td>22 ± 7 (Range 4-30)</td>
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</table>

Table 3. Biochemical Phenotype of cohort 2.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Mean serum calcium ± SD (mmol/L) (NR 2.12-2.55)</th>
<th>Mean urine calcium creatinine ratio ± SD (NR &lt; 0.60 mmol/mmol creatinine)</th>
<th>Mean serum PTH ± SD (pg/mL) (NR 11-54 pg/mL)</th>
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<tbody>
<tr>
<td>94</td>
<td>2.3 ± 0.09 (Range 2.08-2.58)</td>
<td>0.72 ± 0.24</td>
<td>33 ± 14 (Range 18-57)</td>
</tr>
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</table>

NR, Normal range; SD, Standard deviation.
Discussion

Recent studies have identified mutations in CYP24A1 to be a novel cause of hypercalcaemia\textsuperscript{13,20,21}. Currently, more than 10 loss-of-function mutations in CYP24A1 have been described with this phenotype, supported by evidence of \textit{in vitro} and \textit{in vivo} loss of enzyme function.

Schlingman et al.\textsuperscript{13} identified homozygous and compound heterozygous mutations in CYP24A1 in eight hypercalcaemic infants with an autosomal recessive pattern of inheritance. Transfection experiments, which provided \textit{in vitro} evidence of loss of enzyme function, were also carried out.

This was confirmed by findings by Dauber et al.\textsuperscript{20} who identified a homozygous CYP24A1 mutation in one child with idiopathic infantile hypercalcaemia and demonstrated decreased enzyme activity \textit{in vivo}. However, CYP24A1 mutations were not identified in 27 other children with idiopathic infantile hypercalcaemia, implying that CYP24A1 mutations remain a rare cause of infantile hypercalcaemia.

A third study by Tebben et al.\textsuperscript{21} describes a 44-year-old Caucasian male with compound heterozygous splice junction mutations in CYP24A1 with a clinical phenotype of intermittent hypercalcaemia, hypercalciuria and renal stones. Two children of the proband, each with single heterozygous mutations, also displayed a clinical phenotype, with hypercalcaemia in infancy and nephrocalcinosis. Other family members with heterozygous and compound heterozygous CYP24A1 mutations also had a biochemical phenotype (of elevated 1,25(OH)\textsubscript{2} vitamin D concentrations) but no clinical phenotype. This family indicates that considerable phenotypic heterogeneity may exist with CYP24A1 mutations.

While these fascinating studies have provided new insights into the pathophysiology of idiopathic hypercalcaemia, amino acid is spatially distant from the other reported mutations and the active enzyme site (Figure 2). Unfortunately, additional family samples to look for segregation of this variant were not available.

![Figure 2](image_url)

\textbf{Figure 2:} Functionally inactivating CYP24A1 mutations. The locations of previously identified functionally inactivating mutations in CYP24A1 are highlighted in yellow in (a) an anterior view and (b) a posterior view of the enzyme structure. They can be seen to cluster around the substrate access channel close to important substrate binding residues (blue). Amino acid 192 is highlighted in red. Image generated using PyMOL molecular visualisation system.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Location of change</th>
<th>MAF in this cohort</th>
<th>Reported MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6022998</td>
<td>Intronic</td>
<td>0.055</td>
<td>n/a</td>
</tr>
<tr>
<td>rs2296241</td>
<td>p.A184A</td>
<td>0.125</td>
<td>0.47</td>
</tr>
<tr>
<td>rs4809960</td>
<td>Intronic</td>
<td>0.25</td>
<td>0.184</td>
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<td>rs6022998</td>
<td>Intronic</td>
<td>0.007</td>
<td>0.001</td>
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<td>rs2274130</td>
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<td>0.377</td>
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<td>Intronic</td>
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<td>rs4809957</td>
<td>UTR-3</td>
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<tr>
<td>rs6022987</td>
<td>UTR-3</td>
<td>0.007</td>
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infantile hypercalcaemia and collectively confirm mutations in CYP24A1 as a cause of hypercalcaemia, it was unclear, until recently, whether mutations in CYP24A1 might contribute to the phenotype of patients with ‘idiopathic’ nephrolithiasis. Several findings suggest that they could.

First, the pedigree described by Tebben et al.\textsuperscript{21} includes an adult (detailed above) with active renal stone disease, who was asymptomatic throughout childhood. Second, in response to the initial report describing CYP24A1 mutations in idiopathic infantile hypercalcaemia\textsuperscript{13}, Streeten et al. describe an adult patient with hypercalcaemia, nephrolithiasis and bone disease who was found to have a homozygous mutation in CYP24A1. His initial presentation was at the age of 19 years, with nephrolithiasis in the context of only mild hypercalcaemia and hypercalciuria\textsuperscript{22}.

Third, Dauber et al.\textsuperscript{20} also reported that the mother of their proband, who had a heterozygous mutation in CYP24A1, suffered from nephrolithiasis.

This evidence for CYP24A1 variants contributing to nephrolithiasis was recently strengthened by a report detailing two patients with bi-allelic mutations in CYP24A1\textsuperscript{15}. First, a 9-year-old boy with hypercalciuria, increased 1,25(OH)\textsubscript{2} vitamin D and medullary nephrocalcinosis was found to have compound heterozygous mutations (c.428_430del; p.E143del, previously reported by Schlingman\textsuperscript{13} together with c.443T>C; p.L148P). Each heterozygous change segregated from his mother and father, respectively. The second patient was described as a 38-year-old man, with recurrent (calcium phosphate) renal stones since the age of 25 and hypercalcaemia, hypercalciuria, suppressed PTH and elevation of 1,25(OH)\textsubscript{2} vitamin D. He also had bi-allelic mutations, a c.1226T>C, p.L409S change inherited from his mother and the c.428_430del; p.E143del mutation inherited from his father. Metabolic studies in both patients confirmed reduced enzyme function with undetectable activity of CYP24A1\textsuperscript{15}.

The hypothesis of CYP24A1 mutations contributing to renal calculi is also supported by observations from a CYP24A1 knockout mouse model\textsuperscript{23}. The CYP24A1 knockout mice show high perinatal mortality (about 50%), believed to be due to hypercalcaemia. However, the mice that survive the perinatal period develop normally indicating that there is capacity in alternate pathways of vitamin D disposal or down-regulation of vitamin D activation in later life. Despite this, in response to vitamin D supplementation, the mice developed nephrocalcinosis. This raises the possibility that humans with CYP24A1 variants could also be clinically asymptomatic under normal conditions but develop nephrolithiasis in response to high vitamin D intake.

Considering this evidence, implicating CYP24A1 variants in calcium stone formation/nephrocalcinosis with specific biochemical phenotypes\textsuperscript{15} could CYP24A1 be contributing to adult onset nephrolithiasis in the ‘idiopathic’ hypercalciuria population?

We set out to determine whether variants in CYP24A1 were a common cause of nephrolithiasis by screening a large group of adult stone formers for mutations in the CYP24A1 gene. This was a reasonable strategy given both the previously reported cases and the noted frequency of potentially pathogenic non-synonymous sequence variants reported in dbSNP\textsuperscript{15}. Two cohorts of renal stone-forming patients from the Northeast of England with metabolic phenotypes potentially consistent with loss of CYP24A1 function were screened. The cohorts included patients with high normal serum calcium levels and low/low normal parathyroid hormone levels and hypercalciuria. We did not identify pathogenic mutations were in these cohorts of calcium stone forming patients. A single novel heterozygous change was observed in one patient with hypercalciuria, but we conclude that this is likely to be a rare variant of unknown significance. We did not find any patients with bi-allelic mutations. Based on these data, CYP24A1 mutations do not therefore appear to be a common cause of nephrolithiasis in the populations we have studied. CYP24A1 mutations leading to renal stone formation/nephrocalcinosis in adult patients remain a rare, autosomal recessive disease. We hypothesise that as the clinically relevant effects of CYP24A1 mutations may be sensitive to vitamin D intake, defects in the enzyme may account for clinical renal stone disease in populations with different dietary and sunlight exposures. Refining the biochemical phenotype by...
measuring 25(OH) vitamin D levels as well as 1,25 and 24,25 vitamin D would help to identify patients with potential CYP24A1 mutations. A phenotype of normal serum 25(OH) vitamin D combined with elevated 1,25 (OH)2 vitamin D and decreased 24,25 (OH)2 vitamin D would be very suggestive. Osteopenia may also be an important clinical manifestation of this disease.

Conclusion
On the basis of our screen, CYP24A1 mutations are unlikely to be a prominent cause of idiopathic hypercalciuria and nephrolithiasis. However, CYP24A1 mutations should be considered in stone-forming patients with suppressed PTH, hypercalciuria and hypercalcaemia and increased 1,25 vitamin D levels and vigilance is required to identify such patients.

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References
19. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.