

## RESEARCH

# Biallelic *CYP24A1* variants presenting during pregnancy: clinical and biochemical phenotypes

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## Abstract

**Introduction:** Inactivating mutations in *CYP24A1*, encoding vitamin D-24-hydroxylase, can lead to an accumulation of active vitamin D metabolites and consequent hypercalcaemia. Patient (infantile and adult) presentation is varied and includes mild-severe hypercalcaemia, hypercalciuria, nephrocalcinosis and nephrolithiasis. This study aimed to characterize the clinical and biochemical phenotypes of a family with two *CYP24A1* missense variants.

**Methods:** The proband and seven family members underwent detailed clinical and biochemical evaluation. Laboratory measurements included serum calcium, intact parathyroid hormone (iPTH), vitamin D metabolites and urine calcium and creatinine.

**Results:** The proband presented during the second trimester of a planned pregnancy with flu-like symptoms. Laboratory tests showed elevated adjusted calcium of 3.27 (upper reference limit (URL): 2.30) mmol/L, suppressed iPTH (<6 ng/L), elevated 25(OH)D (264 (URL: 55) nmol/L) and elevated 1,25(OH)D (293 (URL: <280) pmol/L). Ionized calcium was 1.55 (URL: 1.28) mmol/L. Sanger sequencing revealed two heterozygous missense variants in the *CYP24A1*: p.(Arg439Cys), R439C and p.(Trp275Arg), W275R. The proband’s brother and sister had the same genotype. The brother had intermittent hypercalcaemia and hypervitaminosis D. Only the sister had a history of nephrolithiasis. The proband’s daughter and two nephews were heterozygous for the R439C variant. The proband and her brother frequently had elevated 25(OH)D:24,25(OH)<sub>2</sub>D ratios (>50) during follow-up.

**Conclusions:** W275R is a new pathogenic *CYP24A1* mutation in compound heterozygotic form with R439C in this family.

## Key Words

- ▶ vitamin D
- ▶ *CYP24A1* mutation
- ▶ hypercalcaemia
- ▶ hypervitaminosis D
- ▶ genetic mutation
- ▶ phenotype

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## Introduction

Vitamin D is a fat soluble vitamin (1) essential for calcium and phosphate homeostasis, bone metabolism (2) and possibly the prevention of chronic diseases such as cardiovascular disease and cancer (3). Vitamin D is obtained when 7-dehydrocholesterol in the skin is exposed to UV B (UVB) radiation (vitamin D<sub>3</sub>) and from diet and/or dietary supplements (primarily vitamin D<sub>2</sub> but also vitamin D<sub>3</sub>) (4). Vitamin D<sub>3</sub>, by itself, is not an active substance and must first undergo a series of reactions in the liver and kidneys to generate the active form (5). In the liver, vitamin D<sub>3</sub> is hydroxylated to form the prohormone 25-hydroxycholecalciferol D (25(OH)D<sub>3</sub>) catalysed primarily by *CYP2R1* with *CYP27A1* possibly contributing (6). 25(OH)D<sub>3</sub> is then transported to the proximal tubules of the kidney bound to vitamin D binding protein where it is hydroxylated to form the active 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), catalysed by *CYP27B1*. Finally, 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are catabolised by *CYP24A1*, a key physiological regulator, to form 24,25-dihydroxyvitamin D<sub>3</sub> (24,25(OH)<sub>2</sub>D<sub>3</sub>) and 1,24,25-trihydroxyvitamin D<sub>3</sub> (1,24,25(OH)<sub>3</sub>D<sub>3</sub>) (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article), the first step toward the final inactive end product, calcitroic acid, which is excreted (7). Vitamin D<sub>2</sub> undergoes similar metabolism and catabolism to vitamin D<sub>3</sub> through similar pathways (6).

Loss-of-function mutations in *CYP24A1* inhibit the breakdown of 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>2</sub> leading to an accumulation of active vitamin D metabolites and consequent hypercalcaemia (8), nephrocalcinosis and nephrolithiasis (9). Therefore, mutations in *CYP24A1* should be considered as a potential cause of vitamin D-mediated parathyroid hormone (PTH)-independent hypercalcaemia. To date, cases of both adults and children with *CYP24A1* mutations and hence increased sensitivity to vitamin D loading have been reported. Classically, infants tend to present with hypercalcaemia (10), whereas older children and adults usually present with nephrolithiasis, hypercalcaemia and suppressed PTH. We recently identified a 32-year-old pregnant female (G<sup>1</sup>P<sup>0</sup>) who presented with symptomatic hypercalcaemia, hypercalcaemia, hypervitaminosis D and suppressed intact PTH (iPTH). Symptom onset occurred at 17 weeks of gestation coinciding with trimester 2 of pregnancy. DNA sequencing identified two *CYP24A1* variants.

This study aimed to characterize the clinical and biochemical phenotypes of a family with heterozygous

and compound heterozygous variants in *CYP24A1* following identification of two variants in the proband and to describe the proband's unique presentation.

## Methodology

Ethical approval for this study was granted by the Research Ethics Committee, Galway University Hospitals (Reference No – C.A. 1927; approval date 13-02-2018). Following informed written consent, DNA samples from the proband and the proband's family were examined using targeted exon PCR of all coding exons of *CYP24A1* followed by Sanger sequencing. Clinical and biochemical phenotypes were correlated with *CYP24A1* sequence information in the proband, her parents, siblings ( $n=2$ ), her daughter and two nephews.

The proband (II.2) presented during the second trimester of a planned pregnancy with flu-like symptoms and was discovered following identification of asymptomatic hypercalcaemia on routine blood work. The proband (II.2), her father (I.1), mother (I.2), brother (II.1), sister (II.3), daughter (III.3) and two nephews (III.1, III.2) were recruited to this study. Detailed medical histories were recorded, and clinical examinations were performed. Family members had DNA samples tested for *CYP24A1* mutations by direct sequencing at the Northern Molecular Genetics Service, Newcastle upon Tyne, UK. Participants also had routine clinical biochemistry measured including calcium (Ca<sup>2+</sup>) (mmol/L), albumin (g/L), phosphate (mmol/L), vitamin D metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, 24,25(OH)<sub>2</sub>D), iPTH, fibroblast growth factor 23 (FGF-23), creatinine (estimated glomerular filtration rate (eGFR) using the CKD-Epidemiology Collaboration (CKD-EPI) formula (12)) and alkaline phosphatase (ALP) (U/L). Adult participants had urinary Ca<sup>2+</sup> measured (spot and/or 24-hour urine collections). II.3 was unable to attend in person and only had DNA analysis for the *CYP24A1* variants. II.1 and II.2 had serial measurements of Ca<sup>2+</sup>, 25(OH)D and iPTH.

## Measurement of vitamin D metabolites

Serum 25(OH)D concentrations were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) on the Agilent HPLC 1290; Agilent 6460 Triple quadrupole MS/MS in the Clinical Biochemistry Laboratory at Galway University Hospitals (ISO 15189:2012 standards) (13, 14). Vitamin D was reported

as total serum 25(OH)D concentration (the sum of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentrations). The limits of quantification for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> were 5 nmol/L and 8 nmol/L, respectively. Assay precision was ≤8.0% and bias was ≤5.0% at 25(OH)D<sub>2</sub> levels of 42.2 nmol/L and 94.4 nmol/L and 25(OH)D<sub>3</sub> levels of 39.9 nmol/L and 94.4 nmol/L. The Clinical Biochemistry Laboratory meets the performance targets set by the DEQAS (Vitamin D External Quality Assessment Scheme) advisory panel. Serum 24,25(OH)<sub>2</sub>D (total 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>) and serum 1,25(OH)<sub>2</sub>D (total 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>) were measured using LC-MS/MS on the Micromass Quattro Ultima Pt electrospray ionisation (ESI) tandem mass spectrometer (Waters Corp., Milford, MA, USA) at the Norfolk and Norwich University Hospital NHS Foundation Trust, Colney Lane, Norwich, UK (15). For serum 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>, the limits of quantification were 0.1 and 0.8 nmol/L, respectively. The inter-assay coefficients of variability (CVs) for both serum 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> were between 7.9 and 11% (15). For 1,25(OH)<sub>2</sub>D, the limit of quantification was 10 pmol/L with CVs of 8.0–13.0%.

## Results

### Clinical history: II.2 and III.3

The proband (II.2) presented during a planned pregnancy and was followed for 4 years. Of white European ethnicity, the proband was healthy, active and took over the counter (OTC) vitamin D supplements (1000 units per day; Protego Vitamina D 100 food supplement and/or Mama DHA food supplement) for 2 months prior to conception. Past medical history included mitral valve prolapse (21 years previous), surgery for deviated nasal septum, migraine and abnormal liver function tests secondary to isotretinoin use for acne. She was an ex-smoker for 5 years (5 pack per year history). There was no alcohol consumption up to or during pregnancy. Previous laboratory tests were within normal range (adjusted (adj.) Ca<sup>2+</sup> (reference interval (RI): 2.15–2.55 mmol/L). Serum 25(OH)D concentrations or other vitamin D metabolites were not previously measured.

At 17 weeks of gestation, the proband attended her first antenatal visit. Clinical examination was unremarkable (blood pressure (BP) 122/78 mmHg). Electrocardiograph (ECG) was normal. Routine blood tests revealed elevated adj. Ca<sup>2+</sup> (3.27 mmol/L

(trimester-specific upper reference limit (URL): <2.30 mmol/L) with an ionized Ca<sup>2+</sup> of 1.44 mmol/L (trimester-specific URL: 1.25 mmol/L), suppressed iPTH <6 ng/L (RI: 18–25 ng/L), elevated cholecalciferol (25(OH)D) 264 nmol/L (URL: 55 nmol/L) and elevated calcitriol (1,25(OH)<sub>2</sub>D) 293 pmol/L (URL: <280 pmol/L) with evidence of hypercalciuria (Table 1). While undergoing an extensive workup for hypercalcaemia of pregnancy, the proband was treated aggressively with i.v. fluids with a resultant fall in Ca<sup>2+</sup>. Relevant negative laboratory indices included: parathyroid hormone-related protein (PTHrP) <1.0 pmol/L (RI: 0–1.8 pmol/L), lactate dehydrogenase (LDH) 100 U/L (RI: 135–214 U/L), urine protein: creatinine ratio (PCR), serum protein electrophoresis (SPEP), serum free light chains (SFLC), urine protein electrophoresis (UPEP), vitamin A 1.54 μmol/L (RI: 0.0–3.84 μmol/L) and calcitonin 1.0 ng/L (RI: 0.0–10.0 ng/L). Spot urine Ca<sup>2+</sup>: creatinine molar ratio and 24-hour urine Ca<sup>2+</sup> were elevated at 0.82 mmol/mmol (RI: 0.25–0.75 mmol/mmol) and 9.85 mmol/24 h (RI: 0.30–6.9 mmol/24 h), respectively. Chest radiography, pelvis and thyroid echocardiography were unremarkable. Of note, abdominal ultrasound showed no evidence of urinary stone disease or nephrocalcinosis. The OTC vitamin D supplements which II.2 was taking prior to admission were examined and quantitated at the Public Analyst Laboratory Cork, Ireland (accredited service: ISO 17025). HPLC was the methodology used to determine the vitamin D content – Protego Vitamina D 100 food supplement, vitamin D<sub>3</sub>, label 25 μg/capsule, result of analysis 27.5 μg/capsule; Mama DHA food supplement vitamin D<sub>3</sub> label 25 μg/2 capsule, result of analysis 16.8 μg/2 capsule.

During pregnancy, the proband was treated with a combination of i.v. fluids, a diet low in calcium and vitamin D and oral corticosteroids. II.2 required numerous prolonged hospital admissions to maintain Ca<sup>2+</sup> levels within normal limits. The baby (III.3) had regular fetal scans with occipitofrontal diameter, head circumference, abdominal circumference and fetal weight within normal limits. At 36<sup>+3</sup> weeks of gestation, II.2 developed a headache with a BP of 177/80 mmHg and commenced on labetalol 200 mg bd. At 36<sup>+4</sup> weeks of gestation, BP increased to 190/100 mmHg with clonus on clinical examination. Pre-eclampsia was diagnosed. She was given betamethasone and BP was managed with the addition of hydralazine peripartum.

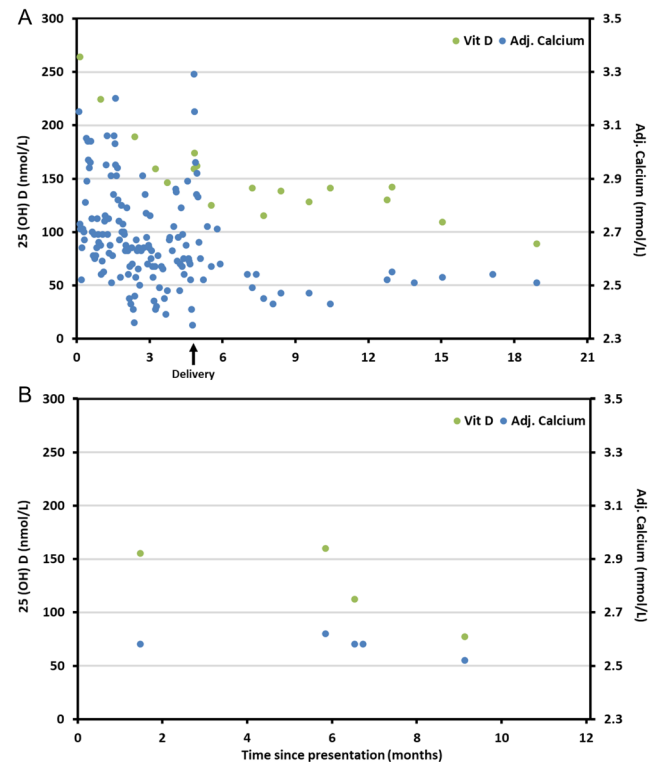
The baby (III.3) was delivered at 36<sup>+4</sup> weeks of gestation by emergency C-section. III.3's ionized Ca<sup>2+</sup> was just above the upper limit of normal (1.35 mmol/L (RI: 1.15–1.33 mmol/L)). The baby was

**Table 1** Demographics, clinical, biochemical and genetic analyses on first contact.

Parameter	II.2 <sup>a</sup>	RI <sup>b</sup>	II.2 <sup>c</sup>	I.1	I.2	II.1	II.3	RI <sup>d</sup>	III.1 <sup>e</sup>	III.2 <sup>e</sup>	III.3 <sup>e</sup>
Age (years)	32	-	33	58	58	36	32	-	2	7	1
Gender (F/M)	F	-	F	M	F	M	F	-	M	M	F
Mutation present A±B	A/B	-	A/B	A/A	B	A/B	A/B	-	A	A	A
Total Ca <sup>2+</sup> (mmol/L)	<b>3.15</b>	2.05–2.25	2.45	2.47	2.41	2.51	n/a	2.15–2.55	2.68	2.44	2.45
Adj. Ca <sup>2+</sup> (mmol/L)	<b>3.27</b>	2.10–2.30	2.41	2.42	2.37	2.46	n/a	2.18–2.55	n/a	n/a	n/a
Albumin (g/L)	36	26–45	47	48	47	48	n/a	39–51	46	50	42
Phosphate (mmol/L)	1.04	0.87–1.48	0.93	1.37	1.2	1.1	n/a	0.87–1.45	1.61	1.46	1.81
iPTH (ng/L)	<6	18–25	15.5	54.9	37.6	18	n/a	15–65	23.5	21.8	46.3
25(OH)D (nmol/L)	<b>264</b>	25–55	105	57	35	<b>148</b>	n/a	75–125	141	81	93
1,25(OH) <sub>2</sub> D (pmol/L)	<b>293</b>	72–280	83	125	94	<b>143</b>	n/a	55–139	n/a	n/a	n/a
24,25(OH) <sub>2</sub> D (nmol/L)	n/a	-	3.3	n/a	n/a	2.1	n/a	1.3–13.5 (13)	n/a	n/a	n/a
25(OH)D:24,25(OH) <sub>2</sub> D ratio	n/a	-	<b>32</b>	n/a	n/a	<b>70</b>	n/a	7–23 (13)	n/a	n/a	n/a
1,25(OH) <sub>2</sub> D:24,25(OH) <sub>2</sub> D ratio	n/a	-	25	n/a	n/a	<b>68</b>	n/a	11–62	n/a	n/a	n/a
FGF-23 (RU/mL)	n/a	-	<b>299</b>	45	<b>129</b>	<b>155</b>	n/a	<100	n/a	n/a	n/a
Creatinine (µmol/L)	<b>90</b>	35–71	<b>94</b>	<b>111</b>	82	102	n/a	F: 49–90 M: 60–110	28	42	24
eGFR (mL/min/1.73 m <sup>2</sup> )	63	60–120	<b>59</b>	63	68	72	n/a	60–120	n/a	n/a	n/a
ALP (U/L)	51	25–126	35	72	55	68	n/a	35–104	367	235	263
<sup>f</sup> Ur. Ca <sup>2+</sup> :creatinine molar ratio	<b>0.82</b>	0.25–0.75	n/a	0.21	0.21	n/a	n/a	0.25–0.75	n/a	n/a	n/a
Ur. Ca <sup>2+</sup> (mmol/24h)	<b>9.85</b>	0.3–6.9	n/a	n/a	n/a	<b>17.4</b>	n/a	2.5–7.5	n/a	n/a	n/a

A or B, heterozygous; A, p.(Arg439Cys); A/A, homozygous; A/B, compound heterozygous, <sup>a</sup>17/40 weeks of gestation; B, p.(Trp275Arg); <sup>b</sup>specific for trimester 2 of pregnancy; <sup>c</sup>indicates results from sample collected 3 months postpartum; <sup>d</sup>non-pregnant adult; <sup>e</sup>all values for P6, P7 and P8 are within age-defined RIs; F, female; <sup>f</sup>spot/random urine sample; I.1, father; I.2, mother; II.1, brother; II.2, proband; II.3, sister; III.1, nephew; III.2, nephew; III.3, proband's daughter; M, male; n/a, not available; RI, reference interval.

alert with normal vitals (afebrile, heart rate 150 (100–159) b.p.m, respiratory rate 50 (31–60) breaths per minute, oxygen 98 (>95)%. Placental histology, on microscopic examination of villi, pointed to segmental areas of impaired maternal blood flow within the placenta. However, this did not appear to have compromised the overall placental function in relation to fetal growth and oxygenation. Post-delivery, there was a spike in II.2's adj. Ca<sup>2+</sup> (3.29 (RI: 2.17–2.51) mmol/L) and ionized Ca<sup>2+</sup> (1.35 (RI arterial: 1.15–1.28)mmol/L) (Fig. 1A). This was aggressively treated with i.v. fluids and i.v. hydrocortisone followed by an oral prednisolone taper with a resultant normalisation of adj. Ca<sup>2+</sup>. Post-partum, BP remained difficult to control and required an increase in labetalol and the addition of nifedipine and methyldopa with a resultant normalisation in BP. Antihypertensives were tapered over time and eventually stopped – BP remained normal. II.2 developed an acute kidney injury post-delivery (eGFR decreased from a baseline 63 mL/min/1.73 m<sup>2</sup> to 44 mL/min/1.73 m<sup>2</sup>). Kidney function returned to baseline following aggressive hydration. Work-up for secondary hypertension revealed normal aldosterone:renin ratio and plasma metanephrines. Computerized tomography (CT) of thorax, abdomen and pelvis did not reveal any potential source of hypercalcaemia. At 3 months post-partum, the proband's 24,25(OH)<sub>2</sub>D and 25(OH)D measured 3.3 nmol/L



**Figure 1** 25(OH)D and adj. Ca<sup>2+</sup> trends post presentation in the (A) proband (II.2) and (B) brother (II.1).

(RI: 1.1–13.5 (15)) and 105 nmol/L, respectively, providing for an elevated 25(OH)D:24,25(OH)<sub>2</sub>D ratio of 32 (RI: 7–23 (15)). While acknowledging that the magnitude of the 25(OH)D:24,25(OH)<sub>2</sub>D ratio was below currently published cut-offs (>50 and mostly >80), indicative of CYP24A1 mutations (16), the dual measurements (absolute 25(OH)D and 24,25(OH)<sub>2</sub>D concentrations) together with the abnormal 25(OH)D:24,25(OH)<sub>2</sub>D ratio warranted further investigation. As substrate depletion, bone disorders or pathological conditions associated with an increased concentration of FGF23 were not clinically implicated, follow-up examinations focused on 24-hydroxylase under activity and possible genetic abnormalities of CYP24A1. Sequencing of the proband's DNA was carried out which revealed two CYP24A1 genetic variants: R439C and W275R. The baby (III.3) was heterozygous for the known pathogenic mutation R439C.

II.2 is being followed closely in our outpatient department. II.2 has been advised to apply sunscreen daily, be cautious regarding sun exposure and keep hydrated. A dietician has prescribed a low calcium/vitamin D diet. The trends in II.2's 25(OH)D and adj. Ca<sup>2+</sup> are presented in Fig. 1A.

### Clinical history: II.1, III.1 and III.2

The proband's brother (II.1) presented as part of our familial screening program and was compound heterozygous for

CYP24A1 variants: W275R and R439C. At his initial visit, II.1 had normal adj. Ca<sup>2+</sup> (2.46 mmol/L), 24,25(OH)<sub>2</sub>D (2.1 nmol/L) and iPTH (18 ng/L) and elevated 25(OH)D (148 nmol/L), 25(OH)D:24,25(OH)<sub>2</sub>D ratio of 70 (RI: 7–23 (15)), 1,25(OH)<sub>2</sub>D (143 pmol/L) and 1,25:24,25(OH)<sub>2</sub>D ratio of 68 (RI:11–62) (Table 2). The trends in II.1s 25(OH)D and adj. Ca<sup>2+</sup> over the following 12 months are presented in Fig. 1B. II.1 had intermittent hypercalcaemia with adj. Ca<sup>2+</sup> between 2.51 and 2.65 mmol/L. Like II.2, he was advised to apply sunscreen daily, be cautious regarding sun exposure, keep hydrated and have a low calcium/vitamin D diet. His children III.1 and III.2 are heterozygous for the R439C mutation.

### Clinical history: I.1, I.2 and II.3

Following enrolment in our study, the proband's sister (II.3) was identified as being compound heterozygous with a history of nephrolithiasis during childhood for which no cause was identified. Following identification of her mutations, she presented with hypercalcaemia and nephrolithiasis during pregnancy to a hospital in a different country. The recent identification of her genotype facilitated rapid management. II.2's mother (I.2) was heterozygous for the W275R variant and her father (I.1) was homozygous for the R439C mutation. Baseline laboratory indices were not indicative of hypercalcaemia or hypervitaminosis D (Table 1).

**Table 2** Biochemical analyses and vitamin D metabolite profiling during follow-up: proband (II.2) and brother (II.1).

Parameter	Proband (II.2)					II.1		Reference interval
	F					M		
Month/year	<sup>b</sup> 05/16	04/17	06/17	11/18	05/19	05/16	07/17	-
Total Ca <sup>2+</sup> (mmol/L)	2.45	2.50	2.53	<b>2.59</b>	2.54	2.51	<b>2.65</b>	2.15–2.55
Adj. Ca <sup>2+</sup> (mmol/L)	2.41	2.51	2.49	<b>2.55</b>	<b>2.52</b>	2.46	<b>2.60</b>	2.17–2.51
Albumin (g/L)	47	44	47	47	46	48	48	39–51
Phosphate (mmol/L)	0.93	1.10	1.19	<b>1.47</b>	1.21	1.10	1.16	0.87–1.45
iPTH (ng/L)	15.5	<b>&lt;6.0</b>	<b>12.3</b>	<b>10.2</b>	<b>9.6</b>	18.0	<b>14.6</b>	15–65
25(OH)D (nmol/L)	105	94	104	106	89	<b>148</b>	<b>136</b>	75–125
1,25(OH) <sub>2</sub> D (pmol/L)	83	124	135	101	124	<b>143</b>	<b>192</b>	55–139
24,25(OH) <sub>2</sub> D (nmol/L)	3.3	1.3	1.7	2.5	1.3	2.1	2.6	1.3–13.5
25(OH)D:24,25(OH) <sub>2</sub> D ratio	<b>32</b>	<b>72</b>	<b>61</b>	<b>42</b>	<b>68</b>	<b>70</b>	<b>52</b>	7–23
1,25(OH) <sub>2</sub> D:24,25(OH) <sub>2</sub> D ratio	25	<b>95</b>	<b>79</b>	40	<b>95</b>	<b>68</b>	<b>74</b>	11–62
FGF-23 (RU/mL)	<b>299</b>	n/a	<b>216</b>	n/a	n/a	<b>155</b>	<b>240</b>	<100
Creatinine (µmol/L)	90	75	76	72	87	102	108	F: 49–90 M: 60–110
eGFR (mL/min/1.73 m <sup>2</sup> )	63	>90	88	>90	74	72	75	60–120
ALP (U/L)	51	46	48	37	43	68	72	35–104
<sup>c</sup> Ur. Ca <sup>2+</sup> :creatinine molar ratio	<b>0.82</b>	n/a	<b>1.32</b>	n/a	n/a	n/a	n/a	0.25–0.75
Ur. Ca <sup>2+</sup> (mmol/24h)	<b>9.85</b>	n/a	n/a	n/a	n/a	<b>17.4</b>	n/a	2.5–7.5

<sup>a</sup>compound heterozygous (A/B): A, p.(Arg439Cys); B, p.(Trp275Arg); eGFR was calculated using the Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) formula; F, female; M, male; <sup>b</sup>3 months post-partum (not breastfeeding); n/a, not available; <sup>c</sup>spot/random urine sample.

### Genetic testing

Two heterozygous *CYP24A1* missense variants were identified in the proband (II.2). R439C is a known pathogenic mutation resulting in a C to T transition in exon 10, c.1315C>T; p.(Arg439Cys) and was previously shown to be a loss of function mutation by site directed mutagenesis (16). W275R is a novel missense variant resulting in a T to C transition in exon 6, c.823T>C; p.(Trp275Arg) first identified by our group (15). The W275R variant is not previously reported in ClinVar (NIH Clinical Genomic Resource) or LOVD (Leiden Open Variation database) and is present at very low frequency in gnomAD and Exome Aggregation consortium (ExAC) public databases. Investigations into the pathogenicity of W275R show that the substitution occurs in a highly conserved amino acid within the protein domain of Cytochrome P450. An online genetic variant tool predictor (17) was used to grade the evidence accumulated for W275R. Variant classification was based on the American College of Medical Genetics (ACMG) Guidelines (18) and the Association for Clinical Genomic Science (ACGS) Best Practice Guidelines for Variant Classification (19). Predicted pathogenicity using *in silico*

evidence (MutationTaster, Polyphen and SIFT) support a deleterious effect on the *CYP24A1* protein (Fig. 2). Based on current evidence, the W275R variant is classified as likely pathogenic (class 4). Stronger evidence from functional studies or new cases will be required to upgrade its pathogenicity. Sanger sequencing of parental bloods confirmed the inheritance of both mutations in *trans* in the proband. The probands father (I.1) is homozygous for R439C and her mother (I.2) is heterozygous for the W275R mutation. Segregation analysis in other family members was performed. The family pedigree is presented in Fig. 3. Participants demographics, clinical and biochemical analyses on first contact and genotype are presented in Table 1. Of note, only the probands sister had a history of nephrolithiasis (II.3).

### Follow-up 25(OH)D:24,25(OH)<sub>2</sub>D ratio determinations in the proband (II.2) and brother (II.1)

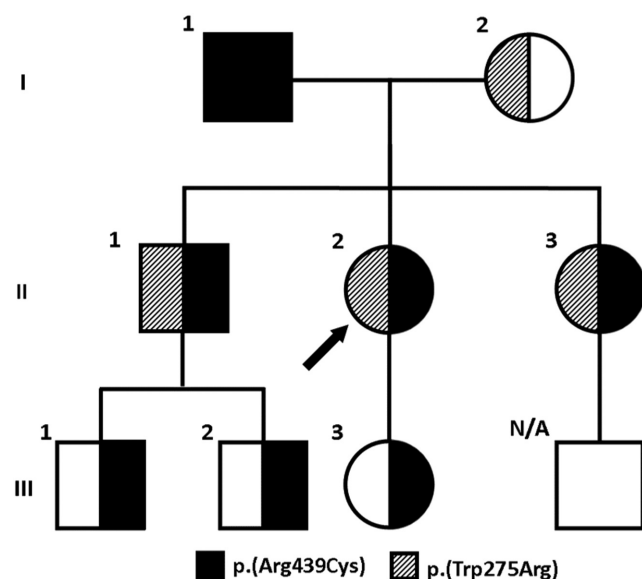
A total of five separate 25(OH)D:24,25(OH)<sub>2</sub>D ratio determinations were performed on serum samples collected from the proband. The first was carried out at

Gene	<i>CYP24A1</i>
Variant description: HGVS	NM_000782.4:c.823T>C p.(Trp275Arg)
Variant location: GRCh37 (hg19)	Chr20(GRCh38):g.54164473A>G
Variant classification: (Pathogenic/Likely Pathogenic/Uncertain Significance/Likely Benign/Benign)	Likely Pathogenic (Class 4)

Evidence for variant classification (ACMG code – level)
<ul style="list-style-type: none"> <li>The p.(Trp275Arg) variant is located in a highly conserved nucleotide and in a moderately conserved amino acid (across 12 species). It is located in the protein domain of cytochrome P450 (PM1).</li> <li>The p.(Trp275Arg) variant is present at very low frequency in Exome Aggregation Consortium (ExAC) and gnomAD public database (PM2).</li> <li>The Trp275Arg variant is detected <i>in trans</i> with a pathogenic variant (Arg439Cys) in a recessive disorder (PM3).</li> <li>Multiple lines of computational (<i>in-silico</i>) evidence support a deleterious effect on the gene. MutationTaster suggests the amino acid change is disease causing, Polyphen suggests it is probably damaging and SIFT predicts deleterious (PP3).</li> <li>The patient’s phenotype is highly specific for a disease with a single genetic aetiology. Biochemical evidence from the patient’s 25(OH)D: 24,25(OH)<sub>2</sub>D ratio is consistent with inactivating <i>CYP24A1</i> mutations <i>in vivo</i> [15, 16] (PP4).</li> </ul>

**Figure 2**

Evidence for *CYP24A1* c.823T>C; p.(Trp275Arg) variant classification. The ACMG utilises a series of evidence criteria in support of a pathogenic (P) or benign (B) classification. The different types of evidence (functional, genetic, population, *in-silico* etc) are stratified according to the level of evidence (supporting, moderate, strong or very strong) and a pathogenicity classification (pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign or benign) assigned according to a set of ‘combined criteria’ provided in the guidelines (19). PM, moderate evidence of pathogenicity; PP, supporting evidence of pathogenicity. The level of evidence is then categorised numerically: PM1, located in functional domain; PM2, absent from controls; PM3, recessive disorder and present in *trans* with a pathogenic mutation; PP4, functional biochemical evidence; PP3, *in silico* evidence supporting pathogenicity.



**Figure 3**

Family pedigree showing inheritance of *CYP24A1* mutations. Arrow indicates the position of the proband in the family. I.1, father; I.2, mother; II.1, brother; II.2, proband; II.3, sister; III.1, nephew; III.2, nephew; III.3, proband's daughter.

3 months post-partum and the remaining four over the following 3 years (Table 2). Of note, ratios in two of these five analyses were 32 and 42, respectively, and inconsistent with current criteria for biallelic *CYP24A1* mutations (16). However, the remaining three 25(OH)D:24,25(OH)<sub>2</sub>D ratio results varied between 61 and 72 (Table 2) and are in keeping with published decision thresholds for biallelic *CYP24A1* mutations (16). Furthermore, the proband's brother (II.1), who harbours the same *CYP24A1* genotype, had 25(OH)D:24,25(OH)<sub>2</sub>D ratios measured on two occasions and both results were consistent with published cut-offs indicative of biallelic *CYP24A1* mutations (16). Biochemical results, together with the population data and *in silico* assessment (see Fig. 2), support the contention that this novel W275R variant is likely a mutation and not a polymorphism.

## Discussion

This study reports two *CYP24A1* mutations R439C and W275R identified in a pregnant female during the second trimester. The proband's father (I.1) was homozygous for the R439C pathogenic mutation but did not have hypercalcaemia, which may be due to the variable penetrance of this mutation together with external factors that mitigate the development of hypercalcaemia, such as dietary calcium intake, vitamin D levels, volume

status and so on. Family members who were compound heterozygous for R439C and W275R mutations had hypercalcaemia and elevated concentrations of vitamin D metabolites. Only one family member had nephrolithiasis. The proband first presented during pregnancy, suggesting that patients with *CYP24A1* mutations can maintain normal Ca<sup>2+</sup> levels during steady state but may develop hypercalcaemia with the challenge of pregnancy when 1,25(OH)<sub>2</sub>D levels are physiologically elevated. Variable presentations and severity of *CYP24A1* mutations have been reported with only eight cases reported during the perinatal period (Table 3) (11, 16, 20, 21, 22, 23, 24, 25).

Vitamin D is essential for calcium homeostasis and bone metabolism (2). Calcium homeostasis is altered in normal pregnancy (24). In healthy women, total 1,25-(OH)<sub>2</sub>D levels were more than double early in pregnancy due to increased production of renal and placental 1- $\alpha$  hydroxylases (26) and remained elevated until the end of pregnancy. In addition, the absorption of calcium from the intestine more than doubles and bone resorption occurs to provide calcium for breast milk (27). Hypercalcaemia is exacerbated in pregnant women with inactivating *CYP24A1* mutations and is normally associated with 1,25(OH)<sub>2</sub>D levels in the upper normal range or slightly raised and concentrations of 25(OH)D that can be low, normal or mildly elevated (28). Vitamin D supplements recommended during pregnancy have the potential to aggravate hypercalcaemia in women with loss-of-function *CYP24A1* mutations. The vitamin D supplements taken by the proband during pregnancy likely explain the markedly elevated 25-hydroxyvitamin D concentration observed at presentation. Castanet *et al.* reported that a child who received higher dose vitamin D supplements (1900 U/day) as a baby developed hypercalcaemia, hypercalciuria and resultant nephrocalcinosis compared to his brother who only received 400 U/day – both had a homozygous *CYP24A1* mutation (29) – suggesting that vitamin D dose may have an effect. Careful consideration should be given to the dosage of vitamin D supplementation and to monitoring adj. Ca<sup>2+</sup> and vitamin D levels in pregnant females.

As highlighted by our case (II.2), investigation of non-PTH mediated hypercalcaemia remains challenging, especially in pregnancy. The commonest causes (90%) of hypercalcaemia are hyperparathyroidism and malignancy (30). The other 10% of cases result from hypercalcaemia related to vitamin D (granulomatous disease and vitamin D intoxication), endocrine disorders such as thyrotoxicosis, medications (thiazide diuretic, milk-alkali syndrome and vitamin A), renal (acute renal failure, chronic renal failure

**Table 3** Clinical, biochemical and genetic findings in eight reported cases of maternal hypercalcaemia due to CYP24A1 pathogenic variants.

Case Report	Molin 2015 (16)	Dinour 2015 (20)	Shah 2015 (11)	Woods 2016 (23)	Hedberg 2019 (24)	McBride 2019 (21, 22)	MacDonald 2019 (25)
Genotype	Compound heterozygous p.E322A/p.L409S	Homozygous p.E143del	Compound heterozygous p.E143del/p.R396W	Homozygous p.S334Vf*9	Compound heterozygous p.R396W/p.L148P	Compound heterozygous p.E143del/p.K351Nfs*21	Homozygous p.E143del
Mutation							
Age/years	NS	35	28	20	23	27	Mid 20s
Gestation week/PP	32	32	14	1–5 days PP	38	5–7 days PP	13
Ethnicity	NS	Moroccan	NS	Mexican	Swedish	Caucasian	NS
PMHx	NS	Renal stones	Renal stones	Hypertension	Renal stones	Renal stones	Renal calculus
Medullary nephrocalcinosis	NS	Yes	Yes	NS	Yes	Yes	NS
Symptoms	NS	Weakness and confusion	Fatigue and constipation	Altered mental status and acute pancreatitis	Epigastric pain and HTN	Symptomatic hypercalcaemia	Symptomatic hypercalcaemia
Calcium (mmol/L)	3.3	3.00 to 3.68	2.38 to 3.05	3.50	4.29	3.63	2.90
PTH (ng/L)	Suppressed	<3	<3	<6	5	5.6	6.6
25(OH)D (nmol/L)	NS	70	159–185 <sup>a</sup>	113–133	98	77	116
1,25(OH) <sub>2</sub> D (pmol/L)	441	214	422–509	396–468	187	250	380
24,25(OH) <sub>2</sub> D (nmol/L)	NS	NS	1.85 <sup>a</sup>	Not detected	NS	NS	0.1
25(OH)D:24,25(OH) <sub>2</sub> D ratio	NS	NS	86 <sup>a</sup>	NS	NS	NS	594
Ur calcium:creatinine ratio	NS	NS	NS	NS	NS	0.22	2.09
24-h Ur calcium (mmol/day)	NS	10.53	9.43	6.91	15.5	NS	NS
Vitamin D supplements	NS	750 IU/day	NS	Yes	NS	250 IU/day	50,000 IU/month
Medication/over the counter	NS	Calcium bicarbonate	Prenatal vitamin	Amlodipine	Citalopram	NS	NS

<sup>a</sup>Values used to derive 25(OH)D to 24,25(OH)<sub>2</sub>D ratio. HTN: hypertension; NS: not stated; PP: post-partum.



or renal transplant) or other rarer causes such as *CYP24A1* mutations. The choice of imaging modality is limited in pregnancy as ionizing radiation poses risks to both mother and fetus (31). Therefore, initial investigations are typically biochemical and haematological. PTH related peptide (PTHrP) is raised in humoral hypercalcaemia of malignancy (32). In the context of low PTHrP, measurement of  $1,25(\text{OH})_2\text{D}$  can be used to screen for vitamin D-mediated hypercalcaemia which can be seen with lymphoma. If both PTHrP and  $1,25(\text{OH})_2\text{D}$  levels are low, hypercalcaemia secondary to osteolytic metastases should be considered (32). There is often a considerable delay in obtaining PTHrP or  $1,25(\text{OH})_2\text{D}$  results as they are usually only performed in specialised centres. When there is clinical suspicion of malignancy, imaging (typically CT) or invasive procedures, such as bone marrow biopsy, can be required to identify the source. Delaying such investigations (a risk benefit analysis is required in pregnancy) contributes to anxiety and stress in the pregnant female.

The utility of the ratio of serum  $25(\text{OH})\text{D}$  to  $24,25(\text{OH})_2\text{D}$  concentration has been promulgated as a useful biochemical screening tool to assess vitamin D catabolic status. Persistent hypercalcaemia together with a suppressed PTH, elevated  $1,25(\text{OH})_2\text{D}$  level and a raised  $25(\text{OH})\text{D}:24,25(\text{OH})_2\text{D}$  ratio are consistent with a *CYP24A1* loss-of-function mutation (33). Molin *et al.* suggest that a  $25(\text{OH})\text{D}:24,25(\text{OH})_2\text{D}$  ratio of  $>50$  and usually  $>80$  is indicative of inactivating *CYP24A1* mutations *in vivo* (16). Previous work reveals that patients with biallelic *CYP24A1* pathogenic variants have a significantly elevated ratio, whether they exhibit hypercalcaemia or not (16). In the current study, the proband had  $25(\text{OH})\text{D}:24,25(\text{OH})_2\text{D}$  ratios measured on five occasions and in two, the ratio while above the reference interval of 23, were well below published cut-offs indicative of biallelic *CYP24A1* mutations. We can only surmise that the observed fluctuations in the ratio occurred in tandem with the proband adhering to medical advice: minimizing unprotected/direct exposure to sunlight and avoiding diets rich in vitamin D or vitamin D supplements. Identifying *CYP24A1* variants, as in II.3 pre-pregnancy, facilitates a more nuanced approach to the management of hypercalcaemia.

Consensus regarding the management of hypercalcaemia secondary to *CYP24A1* pathogenic variants during pregnancy (or indeed hypercalcaemia during pregnancy due to other causes) do not currently exist. Based on our experience and reports in other case studies, for patients with known *CYP24A1* pathogenic

variants considering pregnancy, we advocate avoiding supplements containing vitamin D or calcium, avoiding calcium bicarbonate, remaining well hydrated, generous daily application of sunscreen and reduced exposure to sunshine. Referral to a dietician for advice on a low calcium and vitamin D diet is recommended. Close monitoring of adj.  $\text{Ca}^{2+}$ ,  $25(\text{OH})\text{D}$  and  $\text{iPTH}$  before, during and after pregnancy is essential. If  $\text{Ca}^{2+}$  remains elevated, aggressive hydration with i.v. fluids (saline) is recommended. There are several reports on the use of oral corticosteroids in managing hypercalcaemia due to *CYP24A1* pathogenic variants during pregnancy (22); they may be of benefit as they inhibit  $1-\alpha$ -hydroxylase conversion of  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$  thereby lessening intestinal calcium absorption (34). Calcitonin has been used cyclically (22) or as a single dose (23); calcitonin decreases hypercalcaemia by inhibiting osteoclast activity, enhances renal excretion of calcium (35) and does not cross the placenta (27). There are limited reports on the use of cinacalcet (36, 37) and bisphosphonates (38, 39, 40, 41, 42) during pregnancy or for treatment of hypercalcaemia of pregnancy due to other causes. Regarding bisphosphonates, Losada *et al.* reported that 20% of children of women treated with bisphosphonates close to or during pregnancy had congenital malformations (42); therefore, their use in pregnancy cannot be recommended.

Rifampicin (43), ketoconazole (44, 45, 46) and fluconazole (47) have been reported as successful treatments for hypercalcaemia due to *CYP24A1* mutations in the non-pregnant state. Rifampicin has been used to treat tuberculosis during pregnancy (48); however, there are no reports on its effectiveness or safety in treating hypercalcaemia during pregnancy. Depending on the dose and duration of treatment, ketoconazole and fluconazole are potentially teratogenic and should only be used if the benefit to the fetus clearly outweighs the risk (49). Regarding any pharmacotherapy, a risk benefit analysis must be undertaken on a patient-by-patient basis.

Most laboratory systems do not have the digital functionality to deal with appropriate RIs in pregnancy. At our institution, the RI for total  $\text{Ca}^{2+}$  is 2.18 to 2.55 mmol/L in males and non-pregnant females. In pregnancy, this changes to 2.10–2.30 mmol/L during the second trimester and 2.05–2.43 mmol/L during the third trimester. In non-pregnant females, RIs are typically compiled from the local population at each institution. This is not the case for RIs in pregnancy. Robust trimester specific RIs for PTH and vitamin D metabolites are not available. In the absence of appropriate RIs associated with laboratory results for each trimester, the physician could potentially

miss mild elevations in adj. Ca<sup>2+</sup> and, therefore, miss the opportunity to instigate appropriate management strategies at an early stage.

## Conclusions

In this family, only patients with the compound heterozygous mutations (R439C/W275R) had elevated Ca<sup>2+</sup> and vitamin D metabolites, while only the proband's sister had nephrolithiasis. The proband became symptomatic during pregnancy, suggesting that these mutations may be associated with a variable phenotype or incomplete penetrance, supporting the view that pregnancy can unmask an otherwise 'silent genetic disorder'. Hypercalcaemia due to *CYP24A1* mutations in pregnancy may be associated with late onset hypertension/pre-eclampsia and warrants further study to determine causation. Accurate variant classification is critical as interpretation impacts directly on both patient and family care/management. Hypercalcaemia due to *CYP24A1* mutations in pregnancy has also been associated with adverse maternal and baby outcomes in many of the reported cases and necessitates aggressive management. Our case highlights the difficulties associated with the investigation and management of hypercalcaemia in pregnancy and the importance of vitamin D profiling and genetic testing to investigate kindred of patients with known *CYP24A1* mutations. Of importance, for positive mother and baby outcomes, all laboratory systems should as a rule and not as an exception have the appropriate digital functionality to deal with RIs in pregnancy. Identifying even a mild elevation in Ca<sup>2+</sup> early in the gestation period can prevent or lessen the complications associated with hypercalcaemia for mother and baby.

### Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-20-0150>.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Ethical approval

Ethical approval was granted by the Clinical Research Ethics Committee, Galway University Hospitals (Reference No – C.A. 1927; approval date 13-02-2018).

### Guarantor

P M O S.

### Author contribution statement

T P G, J A S, M B and P M O S performed study design. T P G, C J, J A S, M B and P M O S conducted the study. T P G, C J, S A, L M B, D T O K, D B, M N I, M C D, J E G, J J M, T O B, J A S, M B and P M O S performed data collection. T P G, C J and P M O S performed data analysis. T P G, C J, S A, L M B, D T O K, D B, M N I, M C D, J E G, J J M, T O B, J A S, M B and P M O S performed data interpretation. T P G, C J and P M O S drafted the manuscript. D T O K, D B, M N I and J A S revised the manuscript content. T P G, C J, S A, L M B, D T O K, D B, M N I, M C D, J E G, J J M, T O B, J A S, M B and P M O S approved the final version of the manuscript. P M O S takes responsibility for the integrity of the data analysis.

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