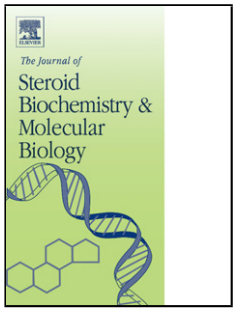

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GESTATIONAL HYPERCALCEMIA: PREVALENCE AND BIOCHEMICAL PROFILE

I. Schoenmakers[1], I. Piec[1], S. Baban[1], L. Barebring[2,], D.Green[1], C.J. Washbourne[1], J.C.Y. Tang[1], W.D. Fraser[1], H. Augustin[2]

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Highlights:

- Population prevalence of gestational hypercalcemia was 1.7% in the third trimester.
- Hyperparathyroidism and vitamin D toxicity were excluded as main causes.
- No cases with profiles suggestive of mutations in the CYP24A1 gene were found.
- Hypercalcemic women had a relatively high serum $1,25(OH)_2D$ concentration despite an appropriately suppressed PTH, suggestive of abnormal gestational adaptions.

ABSTRACT

Gestational hypercalcemia is associated with an increased risk of maternal, fetal and neonatal morbidity and mortality. Hypercalcemia may develop during pregnancy in individuals who were previously asymptomatic. The increased sensitivity during pregnancy may be related to physiological, gestational alterations in vitamin D and calcium metabolism and may be influenced by gene variants. The prevalence is unknown.

We investigated the prevalence of hypercalcemia in trimester 3 (T3) in a population representative prospective cohort study (n=1832) in South-West Sweden. Women with serum albumin (Alb) adjusted calcium (Ca_{Alb}) ≥ 2.65 mmol/L in T3 (n=30) were matched to normo-calcemic controls, and markers of calcium and vitamin D metabolism were investigated in trimester 1 (T1) and T3. Serum concentrations of Ca, phosphate (P), Magnesium (Mg), Alb and creatinine (Cr), parathyroid hormone (PTH; T3 only), vitamin D metabolites (total 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$, and free 25(OH)D) were analysed in T1 and T3. Ca_{Alb} (Payne; inter-laboratory difference: UEA=0.15+0.9*UGOT; UEA 2.54 = UGOT 2.65) and estimated glomerular filtration rate (eGFR; modified 4-variable MDRD) and vitamin D metabolites ratios (VMR) were calculated. Normally and non-normally distributed data were presented as mean (SD) or median (95%CI). Group differences in relationships between vitamin D metabolites and with PTH were investigated with multiple regression analyses.

Hypercalcemia in T3 was found in 1.7% of women. PTH concentrations suggestive of primary hyperparathyroidism was found in 1 woman and none had 25(OH)D or $24,25(OH)_2D$ concentrations in the toxicity range or suggestive of mutations in the CYP24A1 gene. Ca_{Alb} was significantly higher in hypercalcemic cases compared to controls in T1 (2.44 (2.30-2.80) vs 2.37 (2.25-2.49) mmol/L) and T3 (2.63 (2.52-2.78) vs 2.46 (2.31-2.58) mmol/L). Serum P was higher among cases than controls in T3 (1.12 (0.16) vs 1.07 (0.18) mmol/L) but not in T1 (1.12 (0.18) and 1.12 (0.16) mmol/L). PTH in T3 was lower in cases (1.6 (1.6-2.8) vs 2.3 (2.1-2.8) pmol/L) but $1,25(OH)_2D$ concentrations were similar. There were no significant group differences in serum 25(OH)D, free 25(OH)D, $24,25(OH)_2D$, Mg, Alb, Cr and eGFR. Regression analyses did not show significant differences between cases and controls in relationships between vitamin D metabolites and with PTH, except for the free 25(OH)D-PTH relationship and a higher free:total 25(OH)D ratio in cases at T1.

In conclusion, most common causes of hypercalcemia were excluded in the majority of women. Hypercalcemic women had a relatively high serum $1,25(OH)_2D$ concentration despite an appropriately suppressed PTH, suggestive of abnormal gestational adaptions.

Keywords:

Pregnancy; hypercalcemia; vitamin D metabolites; free 25 hydroxy vitamin D; case-control study

Introduction

Hypercalcemia is found in 0.2-4% of community-dwelling people and hospital patients (1). The most common causes are malignancy and primary hyperparathyroidism and less commonly, vitamin D toxicity and hypocalciuria (1, 2). During pregnancy, hypercalcemia may develop in individuals who were previously asymptomatic (1-3). This may be due to genetic predisposition. The increased susceptibility during pregnancy may be related to physiological, pregnancy induced alterations in vitamin D and calcium metabolism (4), including an increase in $1,25(OH)_2D$ concentration and intestinal calcium absorption and renal excretion. Placental vitamin D metabolism may also play a role. In addition, to meet the recommended dietary vitamin D intake, many women are taking vitamin D supplements during pregnancy, which increases vitamin D exposure. Gestational hypercalcemia is associated with an increased risk of maternal, fetal and neonatal morbidity and mortality, including maternal hypertension and renal impairment, fetal growth restriction and neonatal hypocalcemia (2). The prevalence of gestational hypercalcemia is unknown and in most countries, screening for hypercalcemia is not part of antenatal care (https://www.nice.org.uk/guidance/cg62; (2).

Recently, inactivating mutations or gene variants of the cytochrome P450 family 24 subfamily A member 1 (CYP24A1) gene have been identified as a cause of hypercalcemia. Variants in CYP24A1, the gene coding for the 24-hydroxylase enzyme lead to low concentrations of the catabolic product 24, 25 di-hydroxy vitamin D ($24,25(OH)_2D$), elevated 1, 25 di-hydroxy vitamin D ($1,25(OH)_2D$) and Fibroblast Growth Factor 23 (FGF23) and consequently hypercalcemia (5-8). The phenotype tends to be heterogeneous suggesting that variations may occur in several parts of the gene with differential effects on CYP24A1 activity. Gestational alterations in metabolism and vitamin D supply may trigger the consequences of otherwise asymptomatic gene variants in vitamin D metabolic pathways. The prevalence of mutations or important variants in the CYP24A1 gene are unknown (1).

The aim of this study was to assess the prevalence of hypercalcemia in late pregnancy in a population representative prospective cohort study. In a nested case-control study, we investigated potential causes of hypercalcemia and differences in vitamin D metabolism in early and late pregnancy.

Materials and methods

Study participants

The GRAVID study is a prospective cohort study, conducted in the Västra Götaland region in the South West of Sweden, at a latitude 57-58 °N. During autumn 2013 to spring 2014, pregnant women were recruited from gestational week 4 when registering with one of the participating antenatal care units. The only exclusion criterion was a pregnancy exceeding 16 weeks at inclusion. The GRAVID cohort is population representative and multi-ethnic. The primary outcome was the association between vitamin D status (serum 25 hydroxy vitamin D) and the risk of gestational complications, with a focus on pre-eclampsia. Data were collected during two routine visits to antenatal care units and from medical records as previously described (9-11). In total 2125 women were included of which 1827 provided a blood sample in trimester 1 (T1), before gestational week 16 (with majority of samples collected between week 8-12) and in trimester 3 (T3) after gestational week 31, (with the majority of samples collected between week 32-35).

Case-control study of gestational hypercalcemia

The current study is retrospective, explorative secondary analyses and utilized banked serum samples collected in T3 to investigate the prevalence of hypercalcemia in the full cohort. Cases, i.e., individuals with an albumin (Alb) adjusted calcium (Ca) concentration (Ca_{Alb}) ≥ 2.65 mmol/L were matched to a normo-calcemic control. In this nested case-control study, markers of vitamin D metabolism were analyzed in T1 and T3 in order to investigate causes of hypercalcemia and identify endocrine profiles suggestive of variants in the CYP24A1 gene. Matching was conducted on the following criteria: firstly for parity (± 1), single or multifetal pregnancy, maternal continent of birth (North Europe, Continental Europe, America, Asia or Africa), gestational age at sampling in T3 (±14 days) and at delivery (±21 days), season of conception (November-April or May-October); secondary matching was performed on maternal age (±10 years) and BMI (±10 kg/m^2) at T1.

Ethics

Ethical approval was obtained from the Regional Ethics Committee in Gothenburg (Dnr 897-11, T439-13) and the UEA Faculty of Medicine and Health Sciences Research Ethics Committee (2017/18 – 149). All procedures were conducted in line with the Declaration of Helsinki. Written informed consent was obtained from all participating women. Study information and consent forms were available in 9 languages and interpreters were consulted when required.

Sample collection, processing and biochemical analyses

Non-fasting venous blood samples were collected in serum gel tubes. Blood was allowed to clot for 30-120 min and serum was separated and stored at -70°C until analyses. For screening of T3 samples, serum total Ca and Alb were analyzed by standard colorimetric methods at the Sahlgrenska University Hospital in collaboration with University of

Gothenburg (UGOT). Samples for the nested case-control study were sent to University of East Anglia (UEA) on dry ice and stored at -70°C. Serum concentrations of total Ca, Alb , phosphate (P), creatinine, and magnesium were all measured by photometric assays (COBAS c501, Roche Diagnostics, Germany) and intact parathyroid hormone (PTH) (T3 only) by electro-chemilumicescent immunoassay (ECLIA)(COBAS e601, as before). The inter assay CVs were <10%. Serum $1,25(OH)_2D_3$ was measured by ECLIA (DiaSorin LIAISON XL, Saluggia, Italy) and had an inter assay CV of <10%. Free 25(OH)D concentrations were measured by ELISA (DIASource Immunoassays, Louvain-la Neuve, Belgium). The lower limit of detection was 4.8 pmol/L and the intra-assay variation was <15%. Vitamin D binding protein concentrations were measured by an ImmunDiagnostik AG kit (Bensheim, Germany), but inter and intra- assay performance did not meet Good Laboratory Practice quality criteria and resulted in irreproducible and improbable data and were therefore not reported.

Serum concentrations of $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$ were simultaneously quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Micromass Quattro Ultima Pt, Waters) as described previously (5, 12, 13). Serum C3-epi $25(OH)D_3$ (3-epi 25(OH)D) was analysed separately using protein precipitation followed by quadrupole tandem mass spectrometry. In short, 100 µl of sample was combined with 100 µL of 0.1 mol/L ZnSO4 (2.88g ZnSO4 in 100ml in acetonitrile and 0.1% formic acid) and then spiked with 200 µL of 3-epi $25(OH)D_3$-[13C5] internal standard from IsoSciences (Pennsylvania, U.S.) in acetonitrile, mixed well, and allowed to precipitate at 4°C. The contents were centrifuged and the supernatant collected. Analyses were performed using a Flux Instruments HPLC module interfaced with a Micromass Quattro Ultima Pt (Waters, U.K.) quadrupole mass spectrometer. Separations were performed on a Restek pentafluorophenyl (2.7µm, 100 x 2.1mm) column in gradient mode (Mobile phase A-water and 0.1% formic acid, Mobile Phase B- methanol and 0.1% formic acid). The inter-assay coefficients of variation were <15% for all vitamin D metabolites. The LOQ in serum and standard solutions was 0.1 nmol/L for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ and 1.5 nmol/L for C3-epi $25(OH)D_3$. Assay performance was traceable to NIST standards (NIST 972A)(5, 12, 13).

All analyses were performed in singleton, except free 25(OH)D, which was performed in duplicate. Assay performance was monitored using kit and in-house controls and under strict standardisation according to Good Laboratory Practice. External quality assurance was obtained through participation in DEQAS (www.deqas.org) and NEQAS for PTH and was consistently within acceptable limits.

Derived variables

Albumin adjusted calcium (Ca_{Alb}) was calculated according to Payne. There were inter-laboratory differences in the measured concentrations of calcium and albumin and therefore in the resulting calculated concentration of Ca_{Alb} and the laboratory specific reference range. A conversion algorithm was derived from regression analyses

(UEA=0.15+0.9*UGOT). The measured UGOT value of Ca_{Alb} = 2.65 mmol/L therefore equates to 2.54 mmol/L as measured at UEA. UEA data are reported, unless stated otherwise. Estimated glomerular filtration rate (eGFR) was calculated according to the modified 4-variable modification of diet in renal disease (MDRD) equation without race (14). Serum 25(OH)D concentrations are given as the sum of $25(OH)D_3$ and $25(OH)D_2$. The following Vitamin D metabolites ratios (VMR) were calculated: 25:1,$25(OH)_2D$, 25:24,$25(OH)_2D$ and 25:free 25(OH)D and presented in molar:molar concentrations (5, 15).

Statistical analyses

Data were tested for normality, converted to natural logs if required and retested for normality. Since natural logs conversion did not consistently result in normally distributed data, parametric and non-parametric tests were applied, as appropriate. Differences between cases and controls and changes between T1 and T3 were tested by paired T-tests, the Wilcoxon Signed Ranks Test for numeric data, or Chi square test for categorical data. Differences between cases and the full cohort were tested by unpaired T-tests for continuous data or the Chi square test for categorical data. Normally distributed data were presented as mean and SD and non-normally distributed as median and 95% CI, categorical data as percentage (%).

To assess group differences in the relationships between vitamin D metabolites and between vitamin D metabolites and PTH, multiple regression analyses were conducted with the inclusion of group (hypercalcemic/control) and an interaction term (group*independent variable) as co-variates. This approach , together with the VMRs, is used in the differential diagnosis of individuals suspected to carry gene variants in the vitamin D hydroxylation enzymes. Analyses were conducted separately for T1 and T3 data. No correction for gestational week at the time of blood sampling was required as these did not differ between cases and controls. All analyses were conducted in SPSS Statistics version 25 (Armonk, New York: IBM Corp).

Results

Prevalence of gestational hypercalcemia and characteristics of hypercalcemic women

A total of 1827 women were screened for hypercalcemia in T3, of which 31 (1.7%) had a (UGOT measured) Ca_{Alb} concentration ≥ 2.65 mmol/L (UEA equivalent ≥ 2.54 mmol/L). N=30 could be matched to controls for further analyses. Matching failed in 1 woman because of high BMI in combination with a multi-fetal pregnancy.

Compared to the full GraviD cohort (n=2046), hypercalcemic women had a higher BMI and were more likely to be obese (27% vs 10%) . In addition, they were more likely to develop

gestational hypertension and to give birth to a small for gestational age child. There were no differences in birth weight (Table 1).

Vitamin D intakes from food sources (fatty fish, milk, yoghurt/sourmilk and margarine; (11)) in the full cohort and hypercalcemic women were similar in both trimesters (Table 1).

Markers of calcium and vitamin D metabolism in hypercalcemic cases and matched controls

Serum Ca_{Alb} was significantly higher in cases compared to controls in both trimesters (Fig 1). This was determined by significantly higher serum calcium concentrations (not shown); serum albumin did not differ between cases and controls in either T1 or T3 and were within the reference range (Table 2). Already in T1, three women had a serum Ca_{Alb} in the hypercalcemic range. In two of these women, PTH concentrations in T3 were elevated with low normal 25(OH)D and 24,25$(OH)_2$D concentrations (see below). The third woman had a high-normal concentration of 25(OH)D (>160 nmol/L), but this was not in the toxic range (> 225 nmol/L). All three women had 1,25$(OH)_2$D concentrations in the mid-range of values observed in this study in both T1 and T3. Serum phosphate was higher in cases in T3, but not in T1 (Table 2). There were no differences between cases and controls in serum magnesium, creatinine and eGFR.

Serum PTH in T3 was lower in cases than in controls. PTH concentrations could not measured in T1. PTH concentrations suggestive of primary hyperparathyroidism (> 6.9 pmol/L) was found in only one of the cases and borderline in another. Serum 1,25$(OH)_2$D concentrations were similar but the concentration range was wider in cases than in controls (Fig 1). The majority of observed concentrations in both cases and controls were above the published references ranges in healthy adults (36-144 pmol/L (16)) but were within those observed in pregnant women using the same assay (17, 18). Serum 25(OH)D concentrations did not differ between cases and controls and no 25(OH)D concentrations in the toxicity range were found (Fig 1). There were also no differences between cases and controls in serum free 25(OH)D and 3-epi 25(OH)D and 24,25$(OH)_2$D concentrations at T1 and T3.

Similar to earlier studies in pregnant and non-pregnant women, multiple regression analyses showed that total 25(OH)D significantly and strongly predicted 24,25$(OH)_2$D. Serum 25(OH)D also significantly predicted free 25(OH)D and 3-epi 25(OH)D, but not 1,25$(OH)_2$D and PTH (T3 only). Group (hypercalcemic cases/control) and the interaction term (group*independent variable) were non-significant in all of these models, indicating relationships did not differ between the hypercalcemic cases and control group (Table 3). Relationships were similar in both trimesters. Replacing total 25(OH)D with free 25(OH)D as the independent variable did not materially change these findings, except for PTH. Free 25(OH)D significantly predicted PTH (Table 3). There were group differences in the relationship between free 25(OH)D and PTH, where no relationship was observed in the

hypercalcemic group, there was a significant negative relationship in the control group. Serum PTH in T3 did not predict $1,25(OH)_2D$ or $24,25(OH)_2D$ (Table 4). Serum $24,25(OH)_2D$ did not predict $1,25(OH)_2D$ in either trimester.

No differences in VMRs between cases and controls were found except for a higher total:free 25(OH)D ratio in cases in T1. Concentrations of $24,25(OH)_2D$ and VMRs of 25(OH)D:$24,25(OH)_2D$ outside the reference range established in non-pregnant women and suggestive of gene variants in CYP24A1 were not found (Table 2).

Changes of markers of calcium metabolism and vitamin D metabolism from trimester 1 to 3

In controls, serum total Ca decreased during pregnancy, but this was not observed in cases (not shown). Serum Alb showed the expected gestational decrease in both groups. As a result, serum Ca_{Alb} significantly increased in both groups, but more so in cases compared to controls. Serum magnesium and creatinine decreased and eGFR increased in both groups as expected during the course of pregnancy (Table 2).

In both groups, serum $1,25(OH)_2D$ concentrations significantly increased, serum 25(OH)D and $24,25(OH)_2D$ concentrations remained unaltered and free 25(OH)D decreased. These changes are according to expected gestational changes (Fig 1). The VMR of 25(OH)D: $1,25(OH)_2D$ decreased significantly only in cases and the 25(OH)D: free 25(OH)D ratio increased in controls but remained unchanged in cases (Fig 1, Table 2). $1,25(OH)_2D$: $24,25(OH)_2D$ increased from T1 to T3 in both cases and controls (Table 2).

Discussion

The prevalence of gestational hypercalcemia was 1.7% in the third trimester in this population representative cohort of pregnant women residing in South West Sweden. In our subsequent nested case-control study, we did not find primary hyperparathyroidism and vitamin D toxicity as the main causes of hypercalcemia. In hypercalcemic women, PTH was suppressed but concomitant $1,25(OH)_2D$ concentrations were similar to controls. Serum concentrations of vitamin D metabolites did not suggest abnormalities in the activity of the catabolic enzyme CYP24A1 as a cause hypercalcemia in any of the women.

The prevalence of hypercalcemia of 1.7%, which is higher than reported in the general population (1) and in other pregnant women (2). Rey suggested that this may partly caused by under-diagnosis of gestational hypercalcemia, as no routine antenatal or general screening is performed. In addition, clinical symptoms, particularly when hypercalcemia is mild, are not very specific and similar to common pregnancy ailments, such as nausea, constipation and fatigue (2).

Consistent with earlier reports, we found a higher proportion of gestational hypertension and children born small for gestational age in the hypercalcemic group (3, 8, 19) compared

to the full cohort. Despite this and the increased risk of neonatal hypocalcemia associated with maternal hypercalcemia**,** monitoring of plasma calcium is not part of antenatal care in most countries. Data regarding the use of medication to reduce blood pressure were not available for this cohort. However, the most common types of anti-hypertensive medication in Sweden are beta blockers (Labetalol) and calcium antagonists and these are associated with a reduced serum calcium rather an increase and were therefore an unlikely cause of hypercalcemia (personal communication Maria Bullarbo)(20).

PTH is known to be decreased during pregnancy (21), while the plasma concentration of PTH related peptide (PTHrP) increases, which is produced by the placenta and fetal tissues and the mammary gland. PTH and PTHrP have partly overlapping effects. However, PTH secretion and plasma concentrations remain responsive to plasma calcium (22), whereas PTHrP concentrations may not (21). In the current study, we observed lower serum concentrations of PTH in hypercalcemic women compared to controls. Despite this, $1,25(OH)_2D$ concentrations were similar. This is possibly driven by PTHrP. However, this needs to be confirmed once an assay suitable for the measurement of PTHrP in concentrations found during normal pregnancy becomes available, agian. An alternative explanation for the relatively high concentrations of $1,25(OH)_2D$ in hypercalcaemic women is an altered placental vitamin D metabolism. Placental $1,25(OH)_2D$ and $24,25(OH)_2D$ production and catabolism are not thought to be regulated by PTH. These metabolites mostly have an auto- or paracrine effect within the placenta (23, 24) and are normally not released into the circulation. The placenta has however been suggested to be a major site of CYP24A1 expression and therefore catabolism of $1,25(OH)_2D$ and 25(OH)D and is potentially a source of circulating $24,25(OH)_2D$. Altered placental vitamin D metabolism and plasma concentrations of vitamin D metabolites have been reported in women with pre-eclampsia (25).

Serum $1,25(OH)_2D$ concentrations were above published reference range in non-pregnant adults (36-144 pmol/L (16)) in the majority of cases and controls. In the first trimester of pregnancy, $1,25(OH)_2D$ increases to approximately 2-3 fold higher concentrations than in non-pregnant women until delivery (21). Values observed in the current study were within ranges reported in another study with pregnant women using the same assay (17). Also in non-pregnant, healthy women, values over the reference range were frequently found with this assay (15). There are considerable differences in $1,25(OH)_2D$ assay performance and these need to be considered in the interpretation of data (26).

Concentrations of $24,25(OH)_2D$ and VMRs of $25:24,25(OH)_2D$ outside the reference range established in non-pregnant women, suggestive of gene mutations in CYP24A1, were not found in the current study. It is however possible that placental production of $24,25(OH)_2D$ may mask this. Therefore, concentration intervals as applied in non-pregnant individuals might need to be validated in pregnant women with and without known abnormalities in enzyme activity levels of CYP24A1. Earlier publications suggest that the relationship

between 25(OH)D and 24,25$(OH)_2$D does not substantially change during the course of a healthy pregnancy (27), despite considerable changes in vitamin D metabolism. This is expected to be different in women with CYP24A1 mutations.

The investigation of interrelationships of vitamin D metabolites and those with PTH did not show pronounced differences between cases and controls. Similar to earlier studies in pregnant and non-pregnant women, total 25(OH)D was highly related to 24,25$(OH)_2$D, free 25(OH)D and 3-epi 25(OH)D. There was no significant relationship with 1,25$(OH)_2$D and PTH since the latter are under strict metabolic control, whereas the plasma 25(OH)D concentration is not strictly regulated and is influenced by vitamin D supply and catabolism (28). Total and free 25(OH)D had similar relationships with other vitamin D metabolites. However, the total:free 25(OH)D ratio was higher in cases compared to controls in trimester 1 and there was an, unexpected, highly significant relationship between free 25(OH)D and PTH in the 3rd trimester in controls, which was absent in cases. The involvement of the free fraction of 25(OH)D in pregnancy complications needs further investigation.

Limitations of this study were the relatively mild hypercalcemia observed (Ca_{Alb} 2.65-2.82 mmol/L). PTH measurements were not available in T1. There was also a lack of available sample types to enable the measurement of PTHrP and FGF23. For PTHrP, sample collection and processing protocols are critical and there is currently no commercial assay available that quantifies PTHrP in the concentration range found in healthy people, including during pregnancy. Current FGF23 assays are not suitable for serum samples. However, serum phosphate concentrations were within or close to the normal range for all cases and controls and there were no differences in 24,25$(OH)_2$D. Based on biochemical profiles in other, mostly non pregnant individuals, this suggests that neither a pathological elevation in PTHrP or FGF23 may be likely explanations for the findings in the hypercalcemic women in this study. This needs to be confirmed in further studies. We could not report values for DBP due to poor assay performance. No urine samples were collected to enable a fuller investigation of the potential involvement of the kidney, i.e. to investigate hypocalciuria (29). Also, we based screening on the measurement of total calcium and albumin concentrations in serum and derived Ca_{Alb} from these data. Measurement of ionized calcium is considered to be a more sensitive marker of disorders in calcium metabolism (29) but was not part of the sample collection protocol since samples were collected in antenatal care centers without facilities for immediate laboratory measurements (9). We could also not explore alternative explanations, including abnormalities (both high and low) in the rate of bone turnover leading to high release of calcium and phosphate from bone or reduced uptake of these minerals. The known increase in bone remodeling during pregnancy is highly variable between women and may determine the disposition for gestational abnormalities in calcium metabolism (4).

In conclusion, the population prevalence of gestational hypercalcemia was 1.7% in the third trimester among women residing in the South-West of Sweden. Primary

hyperparathyroidism, vitamin D toxicity were excluded as main causes of gestational hypercalcemia. Serum 24,25$(OH)_2$D concentrations suggestive of mutations in the CYP24A1 gene were not found. Although hypercalcemic women had a suppressed PTH, their serum 1,25$(OH)_2$D concentrations were relatively high, suggestive of abnormal gestational adaptions. Causes of hypercalcemia and increased 1,25$(OH)_2$D concentrations were unclear and primary causes other than those found in the general population should be investigated.

Author statement

The authors have no conflicts of interest to declare

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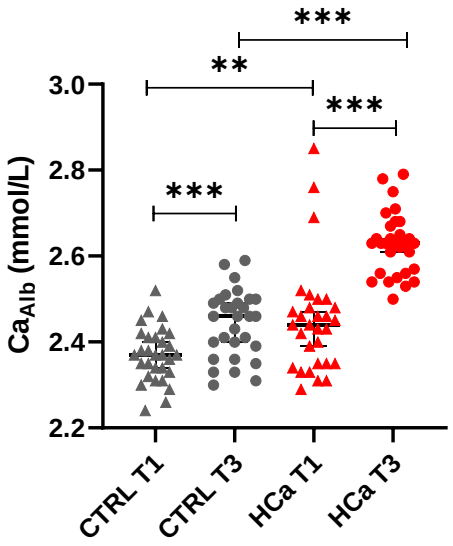
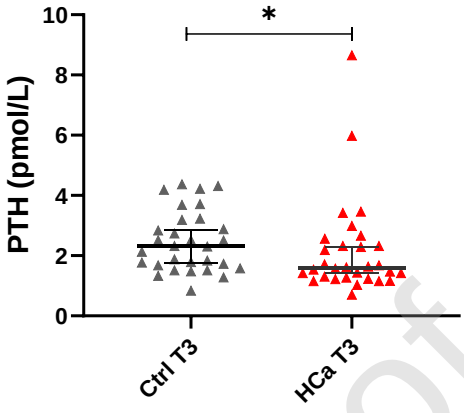
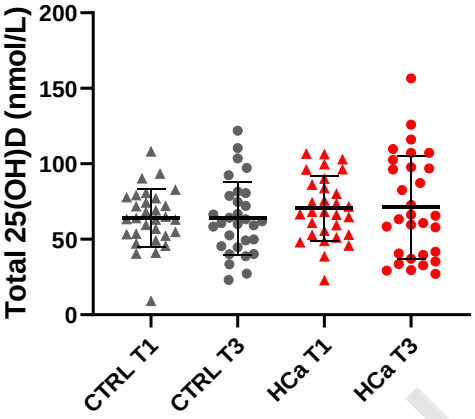
Figure Legend

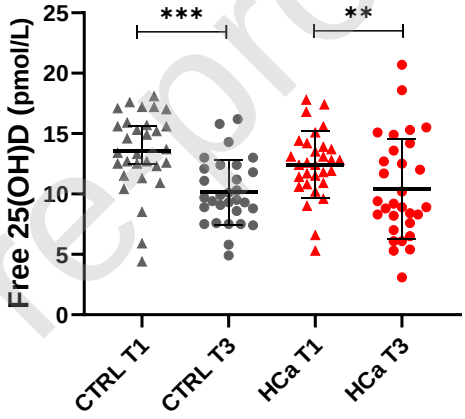
Figure 1: Serum concentrations albumin adjusted calcium concentration, parathyroid hormone and vitamin D metabolites in pregnant women with hypercalcemia and controls.

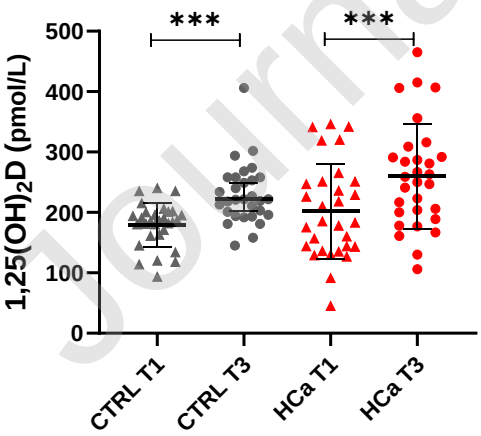
HCa: hypercalcemic cases, CTRL: controls, T1 and T3: trimester 1 and 3, respectively, Ca_{Alb}: albumin (Alb) adjusted calcium (Ca) concentration, PTH: parathyroid hormone, 25(OH)D: 25 hydroxy vitamin D, $24,25(OH)_2D$: 24, 25 di-hydroxy vitamin D, $1,25(OH)_2D$: 1, 25 di-hydroxy vitamin D. *:P<0.05; **:P<0.005; ***:P<0.0005.











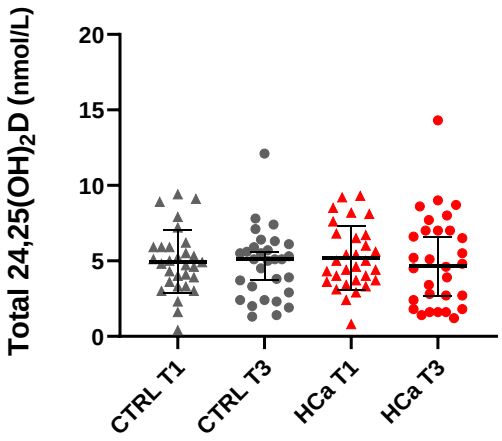


Table 1: Characteristics of the GRAVID cohort and hypercalcemic cases and controls.

Maternal characteristics	**Full Cohort** (n=2046)	**Cases** (n=30)	**Controls** (n=30)
Age (years)	31.3 (4.9)	33.2 (6.0)	30.4 (4.2)
Ethnicity-North EU (%)	74	81	81
Nulliparous (%)	41	44	37
BMI (kg/m^2)	23.6 (4.7)	27.0 (5.3) ^{G}	24.7 (3.5) ^{C}
Height (cm)	166.8 (6.3)	166.7 (7.1)	166.9 (5.2)
Gestational Hypertension (%)	8	29 ^{G}	13
Pre-eclampsia (%)	4	7	7
Vitamin D intake (µg/d)			
Trimester 1	2.4 (1.6)	2.2 (1.6)	2.4 (1.7)
Trimester 3	4.2 (2.2)	4.8 (3.0)	4.4 (2.7)
Infant characteristics			
Birth weight (g)	3560 (513)	3590 (739)	3351 (571)
SGA (%)	5	14 ^{G}	3
Premature <37 wks (%)	4	7	3

Data are given as mean (SD), unless given otherwise. Cases were matched to controls on maternal age (±10 years) and BMI (±10 kg/m^2) at trimester 1, parity (± 1), single or multifetal pregnancy, maternal origin, gestational age at trimester 3 (±14 days), gestational age at birth (±14 days). ^{C} indicates significantly differences between cases and controls and ^{G} between cases and the full cohort.

Table 2: Maternal serum concentrations of phosphate, magnesium and eGFR and Vitamin D metabolite ratio's in hypercalcemic cases and controls in trimester 1 and 3.

Maternal biochemistry	**Cases (N=30)**		**Controls (N=30)**	
	T1	T3	T1	T3
Phosphate (mmol/L)	1.12 (0.18)	1.18 (0.17)	1.12 (0.16)	1.07 (0.18)^{c}
Albumin (g/L)	36.5 (2.6)	27.3 (2.2)^{T1}	36.3 (2.7)	27.0 (1.9)^{T1}
Magnesium (mmol/L)	0.79 (0.05)	0.72 (0.05)^{T1}	0.78 (0.04)	0.74 (0.06)^{T1}
eGFR (ml/min)	121 (20)	133 (24)^{T1}	122 (116-137)	131 (128-154)^{T1}
3-epi 25(OH)D (nmol/L)	2.50 (2.14-3.03)	3.10 (2.43-5.03)	2.65 (2.35-3.31)	2.30 (2.14-3.71)
25(OH)D:1,25$(OH)_2$D (M:M)	365 (349-564)	265(247-403)^{T1}	384 (140)	312 (127)
25(OH)D:24,25$(OH)_2$D (M:M)	15.0 (14.4-16.8)	15.8 (15.5-18.1)	15.0 (13.3-16.6)	15.3 (14.2-17.5)
1,25$(OH)_2$D: 24,25$(OH)_2$D (M:M)	0.04 (0.03-0.07)	0.06 (0.06-0.010)^{T1}	0.04 (0.03-0.07)	0.05 (0.05-0.08)^{T1}
25(OH)D:free 25(OH)D (M:M*10^3)	5.98 (4.53-7.33)	7.08 (6.26-9.36)	5.11 (4.53-5.84)^{c}	6.96 (6.10-7.81)^{T1}

T1 and T3: trimester 1 and 3, respectively. Data are given as mean (SD) or median and 95% CI. $^{C:}$Different between cases and controls at the same time point; ^{T1}: Different between trimester 1 and 3 within group.

Table 3: Multiple regression analyses of interrelationships between vitamin D metabolites and parathyroid hormone.

Dependent variables[A]	25(OH)D[A] (nmol/L)			Free 25(OH)D[A] (pmol/L)		
Trimester 1	**R^2**	**β-coefficient (SE)**	**P value**	**R^2**	**β-coefficient (SE)**	**P value**
Free 25(OH)D (pmol/L)	0.235	0.082 (0.026)	*0.03*	-	-	-
3-epi25(OH)D (nmol/L)	0.360	0.034 (0.10)	*0.01*	0.137	0.059 (0.067)	0.381
$24,25(OH)_2D$ (nmol/L)	0.775	0.087 (0.010)	*0.000*	0.235	0.309 (0.106)	*0.005*
$1,25(OH)_2D$ (pmol/L)	0.045	0.401 (0.581)	0.493	0.046	-1.259 (3.474)	0.718
Trimester 3	**R^2**	**β-coefficient (SE)**	**P value**	**R^2**	**β-coefficient (SE)**	**P value**
Free 25(OH)D (pmol/L)	0.401	0.059 (0.021)	*0.006*			
3-epi 25(OH)D (nmol/L)	0.500	0.036 (0.015)	*0.024*	0.385	0.275 (0.159)	0.090
$24,25(OH)_2D$ (nmol/L)	0.883	0.081 (0.007)	*0.000*	0.446	0.419 (0.141)	*0.004*
$1,25(OH)_2D$ (pmol/L)	0.066	-0.078 (0.529)	0.883	0.050	1.918 (4.937)	0.699
PTH (pmol/L)[B]	0.241	-0.006 (0.003)	0.054	0.201	-0.092 (0.031)[G]	*0.004*

Multiple regression analyses of interrelationships between vitamin D metabolites and parathyroid hormone (PTH). [A]Dependent variables are given in the left hand column, independent variables were total 25 hydroxy (25(OH)D) or free 25(OH)D. Group (hypercalcemic/control) and an interaction term (group*independent variable) were included as co-variates to assess differences between groups in the relationships. Group and the group interaction term were not significant unless indicted by [G]; [G]Significant between- group difference. Explained variance (R^2) of the full model and the β- coefficient (SE) and P-value of the slope is given for independent variables listed. [B]Log transformed variable. For log transformed variables, the coefficient represents a 100% change in the predicted value for a unit change in the predictor value.

Table 4: Multiple regression analyses of interrelationships between vitamin D metabolites and parathyroid hormone and between parathyroid hormone and vitamin D metabolites.

Dependent variables[A]	24,25$(OH)_2D$ [A] (nmol/L)			lnPTH [A] (pmol/L)		
Trimester 1	**R^2**	**β-coefficient (SE)**	**P value**	**R^2**	**β-coefficient (SE)**	**P value**
1,25$(OH)_2D$ (pmol/L)	0.036	0.767 (5.475)	0.889	0.046	-1.259 (3.474)	0.718
Trimester 3	**R^2**	**β-coefficient (SE)**	**P value**	**R^2**	**β-coefficient (SE)**	**P value**
24,25$(OH)_2D$ (nmol/L)				0.171	2.015 (1.109)	0.075
1,25$(OH)_2D$ (pmol/L)	0.051	0.412 (5.831)	0.944	0.108	-8.968 (30.819)	0.772

Multiple regression analyses of interrelationships between vitamin D metabolites and PTH. [A]Dependent variables are given in the left hand column, independent variables were total 24,25 dihydroxy (24,25$(OH)_2D$ or the natural log of parathyroid hormone (lnPTH). Group (hypercalcemic/control) and an interaction term (group*independent variable) were included as co-variates to assess differences between groups in the relationships. Group and the group interaction term were not significant for any variable. Explained variance (R^2) of the full model and the β-coefficient (SE) and P-value of the slope is given for independent variables listed.