Vitamin D metabolites and binding protein predict preeclampsia in women with Type 1 diabetes: a cohort study

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Abstract

Objective: Preeclampsia (PE) occurs about four times more frequently in women with than without diabetes. Vitamin D is essential for healthy pregnancy. We investigated detailed measures of maternal plasma 25-hydroxyvitamin D (25(OH)D), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), and vitamin D binding protein (VDBP) to define associations with PE in women with Type 1 diabetes (T1DM). Design and setting: A multicentre prospective study in women at ˜12, ˜22 and ˜32 weeks' gestation ('Visits' (V) 1, 2, and 3, respectively). Population: We studied 23 T1DM women who subsequently developed PE, 24 who remained normotensive, and 19 non-diabetic, normotensive women (reference controls). Diabetic women were complication-free at V1, and all study visits preceded PE onset. Main Outcome Measures: Total, bioavailable, and free concentrations of 25(OH)D and $1,25(OH)_2D$; and VDBP. Results: 25(OH)D deficiency was more frequent in diabetic than non-diabetic women (69% vs 22%, $p<0.05$), but no measure of 25(OH)D predicted PE. In contrast, higher $1,25(OH)_2D$ concentrations at V2 (total and bioavailable: $p<0.01$; free: $p<0.05$) and V3 (bioavailable: $p<0.05$; free: $p<0.01$) were associated with subsequent PE in T1DM women, as were lower concentrations of VDBP at V3 ($p<0.05$) and elevated ratios of $1,25(OH)_2D$/VDBP (V2, V3: $p<0.01$) and $1,25(OH)_2D$/25(OH)D (V3, $p<0.05$). Significance persisted after adjustment for covariates. Conclusions:In women with T1DM, concentrations of active vitamin D were higher, and VDBP lower, in the second and third trimesters in those who developed PE than in those who did not. Active vitamin D may serve as a new marker for PE risk, and could be implicated in pathogenesis.

Abbreviations:

BMI, Body Mass Index;

DM+PE+, women with type 1 diabetes who subsequently developed PE;

DM+PE-, women with type 1 diabetes who did not develop PE;

DM-, non-diabetic, normotensive women;

eGFR, Estimated glomerular filtration rate;

HbA1c, glycated haemoglobin;

MAMPED, Markers And Mechanisms for PreEclampsia in Type 1 Diabetes;

MAP, Mean Arterial Pressure;

PE, preeclampsia;

T1DM, Type 1 diabetes mellitus;

uNGALcc, urinary neutrophil-gelatinase associated lipocalin (creatinine corrected);

VDBP, Vitamin D Binding Protein;

1,25$(OH)_2$D, 1,25-dihydroxyvitamin D;

25(OH)D, 25-hydroxyvitamin D.

Adequate levels of vitamin D are essential for bone health, immune function, proliferation and differentiation of cells, inflammation, insulin secretion and action, and vascular health.[1]Vitamin D deficiency is common worldwide, involving genetic, lifestyle and geographical factors.[1, 2] Vitamin D metabolism is markedly altered during pregnancy. Specifically, for reasons not fully understood, the active metabolite 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) increases 2-3 fold in the first trimester, reaching concentrations that would normally be toxic, and continues to increase as pregnancy advances.[3-5] Associations of active vitamin D levels with preeclampsia (PE) in the presence of maternal diabetes have not been investigated.

In the general population, the serum concentration of 25-hydroxyvitamin D (25(OH)D), the prohormone and precursor of 'active' 1,25$(OH)_2$D, is considered the principal metric to assess vitamin D status: deficiency and insufficiency are defined as <20 and <32 ng/mL (50 and 80 nmol/L), respectively.[6] Vitamin D deficiency is associated with poor pregnancy outcomes for both mother and child.[2, 7-20]

PE is a multisystem disorder defined by hypertension and proteinuria or other end-organ dysfunction, with onset after 20 weeks' gestation in a previously normotensive woman.[21] Women with type 1 diabetes (T1DM) have a markedly increased risk for PE (˜20% vs ˜5% in the general population).[22] Vitamin D deficiency is associated with abnormal placentation, altered angiogenesis, immune dysfunction, insulin secretion and action, adverse lipid profiles, and inflammation: problems that are also associated with diabetes.[2, 4, 10, 12-16, 18, 23] Vitamin D deficiency may also be implicated in PE.[8, 12]

We previously reported associations between PE and concentrations of fat-soluble vitamins and antioxidant pro-vitamins in women with type 1 diabetes.[24] In that study, we performed only one measure of vitamin D status, 25(OH)D measured by HPLC. Women with T1DM were more likely to be vitamin D deficient than the non-diabetic group, but almost all diabetic women were deficient, and concentrations did not differ significantly according to subsequent PE status.[24] Extending that work, we now investigate whether total, bioavailable, or free forms of 25(OH)D, its active metabolite 1,25$(OH)_2$D, Vitamin D Binding Protein (VDBP) concentrations, and relevant ratios are associated with the risk for subsequent PE in women with T1DM. As before, we include a group of healthy, normotensive non-diabetic pregnant women to obtain normal reference values.

Research Design and Methods

Study design and participants

Participants in the current study are a subset of the "Markers And Mechanisms for PreEclampsia in Type 1 Diabetes" (MAMPED) cohort. MAMPED design, participant characteristics, and inclusion/exclusion criteria have been described previously.[24-30] The overall goal of MAMPED was to identify early markers and potential mechanisms for PE in the context of pregnancy complicated by maternal T1DM. Briefly, it was a longitudinal, prospective pregnancy study of 151 women with T1DM and 24 non-diabetic women enrolled in the first trimester and followed until delivery. The study was conducted in Norway, Australia, and the United States, and participants were predominantly Caucasian (86%). All were free of hypertension,

proteinuria and microalbuminuria at enrolment (urinary albumin:creatinine ratio <30mg/g at the first study visit when gestational age was 9-16 weeks)). PE was defined as new-onset hypertension (>140/90 mmHg) and proteinuria (>300 mg/24 h) after 20 weeks' gestation. Clinical data and specimens (plasma, urine) were collected at three visits: first trimester (V1: gestation 12.2 ± 1.9 wks (mean±SD)), mid-second trimester (V2: 21.6 ± 1.5 wks), and early third trimester (V3: 31.5±1.7 wks). Samples were stored at -80°C until analysis. The study was conducted according to Declaration of Helsinki guidelines and approved by the Institutional Review Boards at all participating institutions. Written informed consent was obtained from all participants.

In accordance with the original MAMPED design, type 1 diabetic women with PE were compared with a matched group (by age, diabetes duration, and parity) without PE. For this report, these subsets comprised 23 (of the original 26) who developed PE (DM+PE+; three were unavailable through sample attrition), and 24 who remained normotensive (DM+PE-; from an original matched subset of 26). We also included 19 non-diabetic, non-PE women (DM-) as 'reference controls'. All study visits occurred prior to the onset of PE.

Medication usage was recorded at V1. All with diabetes were taking insulin. A majority were taking folic acid at V1 (DM+PE+: 70%; DM+PE-: 71%; DM-: 42%, p>0.05), but overall, a minority took vitamin supplements (DM+PE+: 39%; DM+PE-: 50%; DM-: 32%, p>0.05). Use of vitamin supplements did not differ by presence of diabetes, PE outcome, or vitamin D deficiency.

Laboratory measures

Vitamin D is highly lipophilic, and in plasma is almost entirely protein-bound. VDBP is the principal carrier of both 25(OH)D and 1,25$(OH)_2$D (85-90% of total): a smaller amount of each metabolite (10-15%) is bound to albumin (a lower affinity carrier), and [?]1% exists unbound in the 'free', biologically active form.[31, 32] 'Bioavailable' vitamin D is the sum of unbound (free) and albumin-bound: it is considered another useful estimate of biologically active vitamin D because the affinity of vitamin D for albumin is so low.[31-35]

Plasma 25(OH)D concentration was measured using the DiaSorin Corporation 25-hydroxyvitamin D ^{125}I RIA Kit (Stillwater, MN, USA). No dilution was required. The intra- and inter-assay coefficients of variation were [?]10%. 1,25$(OH)_2$D was measured by Quantitative Chemiluminescent Immunoassay at ARUP laboratories, Salt Lake City, UT, after dilution 6.25-fold. VDBP was measured using the human Vitamin D BP Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA), per manufacturer's protocol: plasma samples were diluted 10,000-fold, assayed in duplicate, and intra- and inter-assay coefficients of variation were 3% and 7%, respectively. Operators were masked to clinical status and sample order throughout and all samples from an individual were in the same assay run. Circulating albumin levels were measured at the Department of Clinical Biochemistry, Royal Victoria Hospital, Belfast, Northern Ireland. The levels of free and bioavailable 25(OH)D and 1,25$(OH)_2$D were calculated from total measured 25(OH)D, 1,25$(OH)_2$D, VDBP and albumin concentrations using the following equations[31, 32, 36]:

$$\text{Calculated free [D]} = \frac{\text{Total [D]}}{1 + (K_{\text{Alb}} \times [\text{Albumin}]) + (K_{\text{VDBP}} \times [\text{VDBP}])}$$

$$\text{Bioavailable [D]} = (K_{\text{Alb}} \times [\text{Albumin}] + 1) \times \text{calculated free [D]}$$

$$\text{The percentage of free [D]} = \frac{\text{Free [D]}}{\text{Total [D]}}$$

$$[\text{D}] = 25(\text{OH})\text{D or } 1,25(\text{OH})_2\text{D}$$

$$[\text{Albumin}] = \text{serum albumin in g/L} \div 66,430 \text{ g/mol}$$

$$[\text{VDBP}] = \text{serum VDBP in g/L} \div 58,000 \text{ g/mol}$$

Note that the affinity constants for albumin (25(OH)D: $K_{Alb}=6\times10^5$ M^{-1}; $1,25(OH)_2D$: $K_{Alb}=5.4x10^4$ M^{-1}) are substantially lower than those for VDBP (25(OH)D: $K_{VDBP}=7x10^8$ M^{-1}; $1,25(OH)_2D$: $K_{VDBP}=4x10^7M^{-1}$).[31, 32]

The amount of each vitamin D metabolite bound to VDBP was calculated by subtracting bioavailable from total vitamin D. Using this information, it was possible to calculate VDBP saturation for 25(OH)D and $1,25(OH)_2D$.

Statistical analysis

As pre-defined in MAMPED, primary analysis compared DM+PE+ vs. DM+PE-. Secondary analyses compared 'uncomplicated' T1DM (DM+PE-) with non-diabetic women (DM-). Results were expressed as means +- SD (Tables 1 & 2) or SEM (Figures 1-2). Groups were compared using unpaired Student's t tests for continuous measures and χ^2 test for categorical measures; unpaired tests were used because of differential sample attrition. Analyses of repeated measures used Friedman's test. Logistic regression, with and without covariate adjustments, was used to estimate the probability of women with T1DM developing PE based on clinical characteristics and biomarker values. The following covariates were selected based on differences at the time of visit, and/or their known associations with vitamin D metabolism: BMI, HbA1c, and total adiponectin.[37] All tests were two-tailed, with $p<0.05$ considered significant. Statistical analyses were performed using SPSS software, version 22 (IBM Corp, Armonk, NY).

Results

Maternal characteristics: Table 1 shows the baseline clinical characteristics of all women. There were no significant differences in age, ethnicity, smoking, gravidity, parity, duration of T1DM, systolic and diastolic blood pressure, mean arterial pressure (MAP), total cholesterol, LDL-cholesterol, triacylglycerol, and gestational age per visit between DM+PE+ and DM+PE-. However, at the initial study visit, HbA1c, Body Mass Index (BMI) and total daily insulin dose were significantly higher in DM+PE+ than DM+PE-, and HDL-cholesterol was significantly lower. There were no significant differences between the two normotensive groups at V1, except as expected, HbA1c was higher in women with diabetes.

25(OH)D deficiency was not associated with subsequent PE in women with T1DM: Vitamin D insufficiency and deficiency are defined as <32 and <20 ng/mL, respectively.[6]As shown in Figure 1, a majority (97%) of all women fell below the 'normal' level of vitamin D throughout pregnancy. Women with T1DM were more likely to be 25(OH)D deficient (DM+PE+: 73%; DM+PE-: 65%) than non-diabetic women (22%) at the first visit (p=0.009); however there were no significant differences in any measure of 25(OH)D during pregnancy between the DM+PE+ and DM+PE- groups. Total 25(OH)D was lower in DM+PE- than in DM- groups at the beginning of pregnancy (V1, p=0.020; V2, p=0.034), but neither bioavailable nor free 25(OH)D differed by diabetes status at any visit.

Higher second and/or third trimester $1,25(OH)_2D$ (total, bioavailable, free) are associated with subsequent PE in women with T1DM: As shown in Figure 2, in the DM+PE+ vs the DM+PE- group, total $1,25(OH)_2D$ was higher at V2 (p=0.005), and bioavailable and free $1,25(OH)_2D$ were higher at V2 and V3 (bioavailable: V2, p=0.005; V3, p=0.031; free: V2, p=0.007 & V3, p=0.009). In the DM+PE- vs. the DM- group, all measures of $1,25(OH)_2D$ were lower at V2 (total, p=0.002; bioavailable, p=0.004; free, p=0.018). Total $1,25(OH)_2D$ significantly increased as pregnancy advanced in DM+PE+ ($p<0.001$) and DM+PE- (p=0.007).

Using logistic regression, data were analysed without and with covariates to assess the effectiveness of $1,25(OH)_2D$ as a biomarker of PE. At V2, without covariates and in women with T1DM only, every 1 pg/mL increase in total $1,25(OH)_2D$ increased the odds of developing PE by 3% (OR: 1.03 (1.01-1.05), p=0.012), while every 1pg/mL increase in bioavailable $1,25(OH)_2D$ increased the odds for PE by 28% (OR: 1.28 (1.06-1.54), p=0.010). Likewise, at V3, every 1 pg/mL increase in bioavailable $1,25(OH)_2D$ increased

the odds for PE by 18% (OR: 1.18 (1.00-1.39), p=0.047). Covariate analyses including BMI, HbA1c and total adiponectin did not affect significance.

Ratios of total, bioavailable, and free 1,25(OH)$_2$D to corresponding 25(OH)D concentrations (Table 2): In the DM+PE+ vs the DM+PE- group, total, bioavailable and free 1,25(OH)$_2$D/25(OH)D (product:substrate) ratios were all higher at V3 (all p<0.05). There were no significant differences in these ratios at any stage of pregnancy between the DM+PE- and DM- groups, and there were no significant changes over time in any of the groups. For women with T1DM only, at V3, for every unit increase in total 1,25(OH)$_2$D/25(OH)D, the odds of developing PE increased by 17% (OR: 1.17 (1.01-1.35), p=0.037), however, this significance did not persist after covariate adjustment.

Lower VDBP and higher 1,25(OH)$_2$D/VDBP ratio are associated with subsequent PE in women with T1DM: As summarized in Table 2, in the DM+PE+ vs the DM+PE- group, VDBP was lower at V3 (p=0.032), and total 1,25(OH)$_2$D/VDBP and [1,25(OH)$_2$D bound to VDBP]/VDBP were both higher at V2 & V3 (all p<0.01). Total 25(OH)D/VDBP and ['25(OH)D bound to VDBP']/VDBP did not differ between DM+PE+ and DM+PE- at any study visit. In the DM+PE- vs the DM- group, total 1,25(OH)$_2$D/VDBP and ['1,25(OH)$_2$D bound to VDBP']/VDBP were lower at V2 (p=0.025 and p=0.018 respectively). VDBP significantly increased throughout pregnancy in all groups (all p<0.001). For women with T1DM only, for every 1 mg/dL increase in VDBP at V3, the odds of developing PE decreased by 8% (OR: 0.92 (0.85-1.00), p<0.05). At V2, for every unit increase in total 1,25(OH)$_2$D/VDBP, the odds of developing PE increased almost three-fold (OR: 2.71 (1.28-5.77), p=0.009). Likewise, at V3, for every unit increase in total 1,25(OH)$_2$D/VDBP, the odds of developing PE increased similarly (OR: 2.53 (1.21-5.29), p=0.013). Consideration of covariates had no effect.

Discussion

Main findings: This longitudinal study of pregnancy in T1DM is the first to report multiple detailed measures of vitamin D (total, bioavailable and free concentrations of 25(OH)D and 1,25(OH)$_2$D; VDBP; relevant ratios), and their associations with subsequent PE. We were surprised to find that at V2 and V3, elevated plasma 'active' 1,25(OH)$_2$D, low VDBP, and elevated 1,25(OH)$_2$D:VDBP ratios were associated with subsequent PE.

In contrast, 25(OH)D, the standard metric of vitamin D, did not predict PE; of note, however, insufficient or deficient levels were almost universal in our diabetic cohort. Reported associations of vitamin D deficiency in diabetic pregnancy include preterm birth, increased T1DM rates in offspring of women with T1DM, and poor glycaemic control.[11, 16, 19, 20, 23] Some studies of non-diabetic pregnant women have suggested associations between low total 25(OH)D and contemporaneous[9] or subsequent PE,[8, 11] perhaps limited to early-onset disease.[12] Our present finding of no significant association is consistent with our previous study (that used different methodology to measure 25(OH)D),[24] and with a study by Vestgaard *et al* ..[16] The present work extends those findings by showing that, like total 25(OH)D, neither bioavailable nor free forms of 25(OH)D were predictive of PE. Again, there is the caveat that our cohort did not contain vitamin D-sufficient women.

Maternal 'active vitamin D' (1,25(OH)$_2$D) concentrations are known to increase markedly throughout normal pregnancy compared with normal, non-pregnant values,[38] reaching levels that would be toxic in other circumstances. This phenomenon is recognized but not fully understood. Other studies of active vitamin D throughout pregnancy are sparse, and there is none in the context of maternal diabetes. A smaller longitudinal case-control study of PE in non-diabetic pregnancy (10 PE cases vs 40 controls) found no association between serum 1,25(OH)$_2$D in late second and early third trimesters and subsequent PE.[7] In contrast, we found that bioavailable and free 1,25(OH)$_2$D both predict PE at the second and early third trimesters, while total 1,25(OH)$_2$D predicted PE at the second trimester. The pregnancy-associated increase in active vitamin D is known to be accompanied by, but disproportionate to, an increase in VDBP, and a change in binding affinity has been suggested.[38, 39]High 1,25(OH)$_2$D is thought to reflect the calcium needs of the developing foetus, increasing calcium absorption and up-regulating trans-placental transport.[39]

Lower VDBP concentrations were predictive of PE at the third trimester, and the ratio of 1,25(OH)$_2$D/VDBP

was predictive at V2 and V3. A larger study is needed to determine whether this ratio is a better predictor than 1,25$(OH)_2$D alone. VDBP, like many plasma proteins, significantly increased as pregnancy advanced, independent of diabetes or PE status.

Strengths and Limitations: In this study, we measure not only 25(OH)D, but also active 1,25$(OH)_2$D and VDBP in a longitudinal study of T1DM women with and without (late-onset) PE. We include estimates of bioavailable and free concentrations. Our cohort, although small, was rigorously phenotyped, and was free of proteinuria and hypertension at enrolment. Gestational time-points were well-defined, and all study visits were prior to PE onset. A non-diabetic control group provided reference values for normal pregnancy.

Limitations include reliance on affinity constants to estimate free forms of vitamin D: these constants may vary between individuals, and may be altered by pregnancy.[38] Our cohort was predominantly Caucasian, and there are ethnic differences in vitamin D metabolism and function, including variation of VDBP allelic forms according to race.[40, 41] Sunshine exposure affects total 25(OH)D levels, and vitamin D deficiency may have seasonal and geographical variations. In our subset, 51% of the women were from Norway, 29% from Australia, and 20% from USA. Small numbers precluded stratification by season or location, but most participants, regardless of origin, were vitamin D insufficient or deficient. The absence of vitamin D-sufficient women is a limitation.

Interpretation: Vitamin D is converted from pro-hormone, 25(OH)D, to active hormone by 25(OH)D-1α-hydroxylase, predominantly in the kidney, but also in macrophages and, during pregnancy, in the placenta.[42] Higher levels of 'active vitamin D' in diabetic women with subsequent PE could reflect early subclinical renal or placental dysfunction. The kidney is the major site of its formation, and furthermore, VDBP is filtered through the glomerulus and is reabsorbed by the proximal tubules.[2, 36] We may hypothesise that early renal dysfunction, prior to PE onset, perturbs both 1,25$(OH)_2$D and VDBP.[43, 44] Women were excluded from our study if they had microalbuminuria or more severe albuminuria at V1. Nevertheless, other evidence from the MAMPED cohort supports the concept that subtle, early renal abnormalities confer PE risk: specifically increased first trimester *urinary neutrophil-gelatinase associated lipocalin* (creatinine corrected) (uNGALcc) and elevated 'estimated glomerular filtration rates' (eGFR).[29] Relating current data to these prior findings, we observed, specifically in DM+PE+ women at first trimester, that total, bioavailable and free 25(OH)D were negatively correlated with eGFR (all $p<0.05$), while total and free 1,25$(OH)_2$D at V2 were positively correlated with uNGALcc ($p<0.05$). Regarding VDBP, any renal insult during or even before pregnancy could alter glomerular and tubular processing. Overall, these lines of evidence support the notion that subtle subclinical renal dysfunction, preceding microalbuminuria, is associated with PE.

An alternative possibility is that the association between active vitamin D and subsequent PE is 'defensive', a response to early stresses initiating disease. Active vitamin D has protective functions for the fetoplacental unit, inhibiting inflammatory cytokines[45] and inducing anti-microbial peptide synthesis.[46] In a rat model (reduced utero-placental perfusion, RUPP), treatment of animals with 1,25$(OH)_2$D early in gestation ameliorated PE, apparently by reducing oxidative and ER stresses;[47] and in a cross-sectional study in non-diabetic humans, plasma 1,25$(OH)_2$D was lower in those with than without PE,[48] perhaps reflecting defeat of protective responses.

Associations between vitamin D and PE may differ between 'mild, late onset' PE and 'early-onset severe' form of the disease, and Bodnar*et al.* have suggested that the association between vitamin D deficiency and PE is limited to the latter.[12] In the present prospective study, an overwhelming majority (˜90%) of PE cases in T1DM women were 'mild, late onset'. A prospective study of early-onset, severe disease was beyond the scope of MAMPED.

Whether any of the associations we have identified reflect a causal relationship, and how they might be affected by vitamin D supplementation is unknown. Currently, during pregnancy, all women are recommended to take 600 IU vitamin D daily,[6] but a recent study shows that 4,000 IU daily is more effective at maintaining sufficiency without toxicity.[3] Whether supplementation reduces PE risk for women with diabetes is unknown: a recent multicentre study to assess efficacy in preventing GDM showed a marginal reduction

in fasting glucose, but was underpowered to address PE.[49] In that study, and in contrast to our T1DM patients, women were largely vitamin D sufficient at study entry.[50] Studies of effects of vitamin D on human hypertension have yielded generally negative results.[49]

Conclusion: This is the first longitudinal, observational study to investigate associations of vitamin D metabolites and VDBP with PE in women with T1DM. In the late second trimester, $1,25(OH)_2D$ and $1,25(OH)_2D$/VDBP ratio were good predictors of PE. Further studies should address the value of these biomarkers, the significance of differential changes of 25(OH)D and $1,25(OH)_2D$ during pregnancy, mechanistic implications, and whether optimising vitamin D status during pregnancy is effective in reducing the high prevalence of PE in T1DM women.

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Data Availability: The data that support the findings of this study are available from the corresponding author on reasonable request.

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Author Contributions:

Timothy J. Lyons had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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