Relationship between total vitamin D metabolites and complications in patients with type 2 diabetes

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Abstract. In our previous study, it was shown that endogenous vitamin D_3 and its metabolites are associated with diabetic microvascular complications and cardiovascular risk factors. The aim of the present study was to determine if the relationship between total vitamin D (vitamin D_2 supplements plus endogenous vitamin D_3) was a better predictor of complications in type 2 diabetes (T2DM). A total of 460 patients with T2DM participated in the present cross-sectional study. Plasma levels of total vitamin D and its metabolites (1,25-dihydroxyvitamin D (1,25(OH)D), 25-hydroxyvitamin D (25(OH)D) and 24,25-dihydroxyvitamin D (24,25(OH)D) were measured by isotope-dilution liquid chromatography tandem mass spectrometry analysis. 1,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 were associated with diabetic retinopathy and coronary artery disease, but total 1,25-dihydroxyvitamin D and total 25-hydroxyvitamin D levels were not statistically associated with any complications. Total 1,25-dihydroxyvitamin D showed the same positive association as 1,25-dihydroxyvitamin D_3 for hypertension and dyslipidemia, and total 25-hydroxyvitamin D showed the same positive association as 25-hydroxyvitamin D_3 for dyslipidemia. Total 24,25-dihydroxyvitamin D showed the same positive association only with dyslipidemia as did 24,25-dihydroxyvitamin D_3. However, total 25-hydroxyvitamin D was associated with hypertension, whereas 25-hydroxyvitamin D_3 was not. Vitamin D_3 metabolites were associated with diabetic retinopathy, whereas total vitamin D levels were not, suggesting that endogenous vitamin D_3 metabolites are a better measure of diabetic microvascular complications. However, both total vitamin D and vitamin D_3 metabolites were associated with cardiovascular risk factors in patients with type 2 diabetes.

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Introduction

Vitamin D deficiency may increase the risk of development of type 2 diabetes (T2DM) (1-3), given its inverse relationship with diabetes onset (4). Both insulin resistance and b cell dysfunction are associated with vitamin D deficiency (5), and obesity can exacerbate vitamin D deficiency through sequestering of vitamin D into adipose tissue (6). Vitamin D deficiency in T2DM has also been associated with microvascular complications, although causality remains unclear (7).

Vitamin D_3 (also known as cholecalciferol) is produced endogenously in the body, whereas vitamin D_2 is ingested in the diet as ergosterol, with mushrooms and fungi being the primary sources, and is then converted to ergocalciferol by ultraviolet light; multiple 25-hydroxylases then convert the ergosterol and cholecalciferol to vitamin D_2 ($25(OH)D_2$) and vitamin D_3 ($25(OH)D_3$), respectively (8,9). In the kidney, vitamin D is converted to the active $1,25(OH)_2D$ form by 1a-hydroxylase, or to $24,25(OH)_2D$ (10) (Fig. 1); $1,25(OH)_2D$ is produced in extrarenal tissues and may, and act locally (10).

Vitamin D_2 is readily available as an oral supplement to counter vitamin D deficiency, and is often preferentially used over vitamin D_3 as the latter is more costly; therefore, the vitamin D levels and its metabolites assayed and reported may be a composite of vitamin D_2 and vitamin D_3, as assays may not distinguish between them (11).

In our previously study, the association between of vitamin D_3 deficiency and microvascular or cardiovascular risk complications in patients with T2DM was reported (12). Therefore, the aim of the present study was to determine if total vitamin D was a better predictor of complications in T2DM than vitamin D_3 alone.

Patients and methods

Study population. A total of 460 patients with T2DM (median age 55.2 years; age range, 30-90 years; 227 male and 233 female) were recruited from patients attending the Hamad

General Hospital diabetes clinic, Qatar, between July 2013 and July 2015 (Table I). The criteria for inclusion in the study were: Qatari ethnicity and ≥30 years old. T2DM was diagnosed based on the World Health Organization guidelines (13). For inclusion in the T2DM cohort, one or more of the following criteria had to be met: Fasting plasma glucose of >7 mmol/l, HbA1c >6.5% or a diagnostic glucose tolerance test. All diabetic patients had an estimated glomerular filtration rate of >60 ml/min/kg to ensure that vitamin D levels were not confounded by renal dysfunction. A total of 290 control subjects were included in the present study (median age 46.1 years; age range, 30-85 years; 151 male and 139 female). To be included in the nondiabetic control group, a normal glucose tolerance test was required. Criteria for exclusion were type 1 diabetes, gestational diabetes or secondary diabetes due to steroid treatment. The T2DM subjects underwent retinal photography, foot examination and measurement of blood pressure.

All study subjects had received vitamin D_2 supplements, 50,000 units weekly, prescribed for at least the preceding 4 months. Diabetes subjects were all treated with at least 2 antidiabetic medications that included metformin, and whilst patients were prescribed insulin, compliance could not be confirmed.

This study was approved by Weill Cornell Institutional Review Board (approval no. IRB# 13-00063); all study subjects provided written informed consent. Trial conduct was undertaken in accordance with the International Conference on Harmonisation-Good Clinical Practice (ICH GCP) and the Declaration of Helsinki (14).

Study design. The study design has been previously described (15). Briefly, patients were fasted overnight, and subsequently, blood samples were collected, as well as the baseline weight and blood pressure. Fasting venous blood was collected in fluoride oxalate and serum gel tubes (BD Diagnostics; Becton, Dickinson and Company). The samples were separated by centrifugation at 2,000 x g for 15 min at 4˚C, and within 1 h of collection, the aliquots were stored at -80˚C. Overnight urine samples were also collected, aliquoted and stored at -80˚C, and analyzed in batches. Blood pressure was measured using an automated device (NPB-3900; Nellcor Puritan Bennett) during each visit. Blood pressure measurements were performed after the subjects had been seated quietly for at least 5 min, using the right arm which was supported at heart level. For each measurement, three readings were taken, at least 2 min intervals, and the mean of the three readings was recorded (15).

Diabetic retinopathy was diagnosed using fundoscopy and diabetic neuropathy was diagnosed based on the vibration perception threshold (Neurothesiometer NU-1, Horwell-UK) of the great toe being >25 V (16).

Coronary artery disease (CAD) was defined as a history of myocardial infarction or angina, confirmed by coronary angiography (17). Peripheral arterial disease (PAD) was defined as a history of claudication or pain at rest with evidence of artery stenosis on ultrasound or lower limb angiography (18). Stroke was defined as a sudden onset neurological deficit lasting >24 h (19).

Serum vitamin D levels were measured using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (12).

Statistical analysis. Statistical analysis was performed as described previously (20). The sample size used in the present study was based on a previous study, which found that 51% of diabetics without microvascular complications and 80% with retinopathy exhibited vitamin D deficiency (7). Using the 49% of patients without retinopathy as the comparison group, a sample size of 274 diabetic patients was selected, which provides 80% statistical power to detect a 68% prevalence of vitamin D deficiency in the retinopathy group. When examining the mean differences in vitamin D, the 460 patients, assuming 40% (n=184) have retinopathy, this would yield an harmonic mean of the sample size of ~132. Using this sample size provided 95% power for a difference in vitamin D means of 0.35 deviations using a t-test, which was considered a moderate-sized effect.

Data trends were visually and statistically evaluated for normality. Non-parametric tests (Mann Whitney U) were used on data that violated the assumptions of normality when tested using a Kolmogorov-Smirnov Test (20). Statistical analysis was performed using SPSS version 24.0. Values are reported as the median (inter-quartile range).

Results

Baseline characteristics. The baseline characteristics for the T2DM and the control cohorts are shown in Table I. The diabetic patients were significantly older (P<0.001) and had a higher BMI (P<0.001) compared to the control subjects. The diabetic patients also had elevated HbA1c (P<0.001) and fasting glucose levels compared with the control group (P<0.001).

Vitamin D measurements. The levels of total 25(OH)D were significantly higher compared with the $25(OH)D_3$ (P<0.001). The levels of total $1,25(OH)_2D$ and total $24,25(OH)_2D$ did not differ compared with the levels of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ (P>0.05).

The lower active total $1,25(OH)_2D$ levels were associated with hypertension and dyslipidemia in diabetic patients (P=0.03) in comparison with the lower $1,25(OH)_2D_3$ levels, which were associated with diabetic retinopathy (P=0.006) hypertension and dyslipidemia (both P=0.01) as well as CAD (P=0.012). There was no association between either total $1,25(OH)_2D$ or $1,25(OH)_2D_3$ levels with diabetic neuropathy, PAD or CAD. Total 25(OH)D levels were associated with both hypertension and dyslipidemia (P<0.001) in comparison with $25(OH)D_3$ levels, which were associated with diabetic retinopathy (P=0.03) and dyslipidemia (P=0.04). There was no association between either total 25(OH)D or $25(OH)D_3$ levels with diabetic neuropathy, dyslipidemia, PAD, CAD or stroke. Total $24,25(OH)_2D$ levels were associated with dyslipidemia (P=0.03) in accordance with $24,25(OH)_2D_3$ levels (P<0.02). There was no association between either total $24,25(OH)_2D$ or $24,25(OH)_2D_3$ with diabetic neuropathy, diabetic retinopathy, hypertension, PAD, CAD or stroke. None of the vitamin D metabolites were associated with diabetic neuropathy or stroke (Table II).

Total 25(OH)D levels were significantly higher compared with $25(OH)D_3$ (P<0.001), but those of $1,25(OH)_2D$ and $24,25(OH)_2D$ did not differ between total and D_3 metabolites. When the subjects with 25(OH)D deficiency (≤20 ng/ml) were compared to those who were replete (≥30 ng/ml), there was no difference in hypertension, dyslipidemia, retinopathy

Table I. Clinicopathological characteristics, vitamin D levels and metabolite levels in the Control and Type 2 Diabetic cohorts.

Parameter	Control[c]	Diabetes[c]	P-value
Age, years	46 (30.0-85)	55 (30-90)	<0.001[b]
BMI, kg/m^2	30.1 (21.7-53.5)	32.4 (17.0-61.0)	<0.001[b]
HbA1c, %	5.6 (4.4-9.0)	7.9 (4.9-15.9)	<0.001[b]
Glucose, mmol/l	5.2 (2.9-11.1)	8.6 (2.3-29.0)	<0.001[b]
Total 1,25(OH)$_2$D, ng/dl	0.044 (0.000-2.087)	0.02 (0.000-0.189)	<0.001[b]
Total 25(OH)D, ng/dl	19.58 (4.41-63.73)	26.46 (0.00-61.21)	<0.001[b]
Total 24,25(OH)D, ng/dl	0.387 (0.000-4.486)	0.290 (0.000-7.772)	<0.001[b]
Total 3epi25(OH)D , ng/dl	0.206 (0.000-7.564)	0.326 (0.000-4.001)	0.005[a]

[a]P<0.01, [b]P<0.001. [c]Median (range).

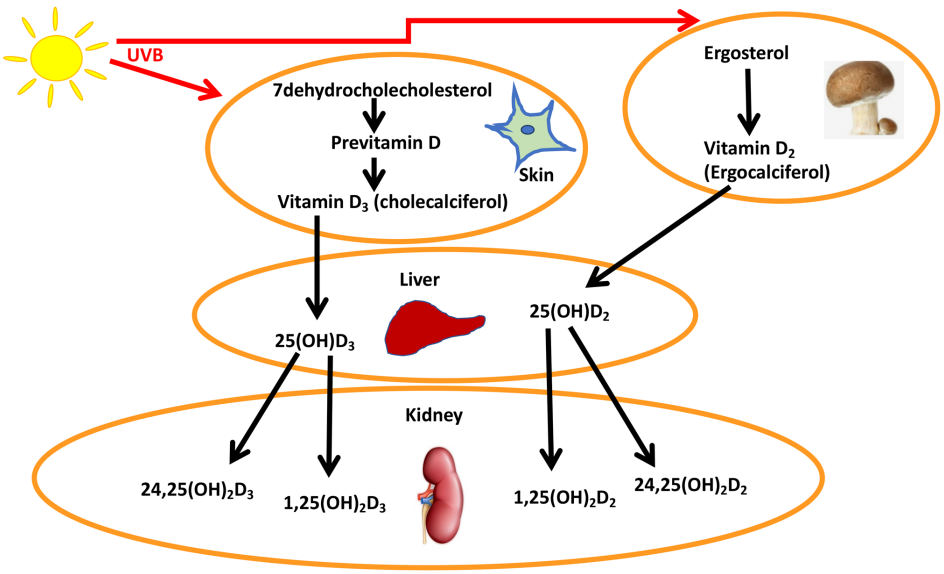

Figure 1. Vitamin D pathways. In the skin, 7-dehydrocholesterol is converted to pre-vitamin D3 and then to vitamin D_3. This is transported to the liver via DBP, which also transports 25(OH)D_3 to the kidney. In the kidney, 25(OH)D_3 is taken up by tubular cells and undergoes conversion to the active 1,25 $(OH)_2D_3$ and 24,25$(OH)_2D_3$. Vitamin D_2 comes from the diet (yeasts and fungi) and is converted to 25(OH)D_2 in the liver and to 1,25$(OH)_2D_2$ and 24,25$(OH)_2D_2$ in the kidney. DBP, vitamin D binding protein 25(OH)D_3, 25 hydroxy vitamin D.

or neuropathy (data not shown). There was no correlation between the estimated glomerular filtration rate and any of the vitamin D metabolites (data not shown).

Discussion

Total 25(OH)D levels were significantly higher compared with those of 25(OH)D_3, and this reflects the vitamin D_2 supplementation that these patients were taking. However, the levels of total 1,25$(OH)_2$D and total 24,25$(OH)_2$D did not differ to those of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$. This result is in agreement with a study on high dose vitamin D_2 supplementation, which showed that vitamin D_2 was less efficacious at raising serum 25(OH)D levels than vitamin D_3, and that vitamin D_2 did not increase the 1,25$(OH)_2$D levels to the same degree that vitamin D_3 supplementation did (21). This also supports the notion that vitamin D_3 is better than vitamin D_2 for treating vitamin D deficiency (21), and that vitamin D_2 supplements may not sufficiently increase the levels of active 1,25$(OH)_2$D.

Both 25(OH)D_3 and 1,25$(OH)_2D_3$ were associated with diabetic retinopathy, whereas neither total 25(OH)D nor total 1,25$(OH)_2$D levels were associated with diabetic retinopathy. It has been reported that in type 2 diabetes, vitamin D deficiency is associated with development of microvascular complications (22) and a recent meta-analysis highlighted an association between vitamin D deficiency and retinopathy (23); however, these outcomes were not specifically correlated to either total vitamin D or the vitamin D_3 forms. These data may also suggest why vitamin D and diabetes studies reported in the literature are conflicting on the relationship of complications and benefits of vitamin D supplementation if the effects of total vitamin D differs from that of vitamin D_3 (24).

Total vitamin D, in comparison with vitamin D_3 metabolites, showed very similar associations with the other cardiovascular parameters, including dyslipidemia, hypertension, PAD and stroke. The only additional significant result was the association between 1,25$(OH)_2D_3$ and CAD, that was not seen with total 1,25$(OH)_2$D.

Table II. Total vitamin D and vitamin D_3 level in patients with diabetes based microvascular diabetic complications and cardiovascular complications.

Complication	Total 1,25 (OH)D, ng/dl[d]	P-value	Total 25(OH) D, ng/dl[d]	P-value	Total 24,25 (OH)D, ng/dl[d]	P-value	1,25(OH) 2D3, ng/dl[d]	P-value	25(OH) D3, ng/dl[d]	P-value	24,25(OH) 2D3, ng/dl[d]	P-value
Diabetic retinopathy		0.1		0.1		0.87		0.006[b]		0.03[a]		0.97
No	0.018 (0.000-0.043)		25.42 (18.23-35.90)		0.288 (0.187-0.604)		0.028 (0.010-0.047)		5.24 (3.07-12.02)		0.278 (0.185-0.587)	
Yes	0.025 (0.010-0.047)		30.82 (20.91-37.73)		0.302 (0.200-0.472)		0.015 (0.006-0.035)		7.50 (3.97-15.66)		0.302 (0.197-0.472)	
Diabetic neuropathy		0.8		0.3		0.73		0.48		0.63		0.77
No	0.020 (0.000-0.044)		26.06 (18.05-36.25)		0.288 (0.189-0.506)		0.015 (0.000-0.040)		6.14 (3.34-14.00)		0.283 (0.188-0.506)	
Yes	0.020 (0.000-0.042)		28.56 (22.27-36.31)		0.310 (0.622)		0.013 (0.000-0.033)		6.18 (3.73-11.53)		0.271 (0.185-0.622)	
Hypertension		0.03[a]		<0.001[c]		0.29		0.009[b]		0.46		0.23
No	0.030 (0.011-0.047)		22.65 (15.46-33.24)		0.297 (0.193-0.587)		0.021 (0.006-0.043)		6.39 (3.44-13.66)		0.295 (0.193-0.587)	
Yes	0.015 (0.000-0.042)		29.43 (20.77-38.82)		0.278 (0.187-0.518)		0.012 (0.000-0.032)		5.87 (3.31-13.88)		0.260 (0.187-0.518)	
Dyslipidemia		0.003[b]		0.009[b]		0.03[a]		0.003[b]		0.04[a]		0.02[a]
No	0.033 (0.014-0.054)		22.81 (16.00-31.29)		0.330 (0.221-0.638)		0.024 (0.009-0.045)		6.86 (3.84-14.47)		0.330 (0.221-0.638)	
Yes	0.016 (0.000-0.041)		28.29 (19.82-37.60)		0.267 (0.185-0.472)		0.013 (0.000-0.033)		5.30 (3.02-12.02)		0.260 (0.183-0.471)	
Peripheral artery disease		0.19		0.23		0.7		0.22		0.55		0.65
No	0.019 (0.000-0.044)		26.48 (18.74-36.35)		0.289 (0.191-0.521)		0.014 (0.000-0.038)		6.06 (3.38-13.88)		0.283 (0.189-0.521)	
Yes	0.025 (0.017-0.050)		21.27 (14.07-32.78)		0.315 (0.144-0.622)		0.025 (0.012-0.050)		10.38 (4.06-15.40)		0.260 (0.144-0.622)	
Coronary artery disease		0.99		0.28		0.44		0.01[b]		0.75		0.45
No	0.021 (0.000-0.045)		25.98 (18.36-36.14)		0.295 (0.190-0.515)		0.028 (0.011-0.041)		6.28 (3.38-14.00)		0.289 (0.190-0.515)	
Yes	0.018 (0.005-0.039)		29.73 (21.65-36.97)		0.241 (0.189-0.604)		0.015 (0.005-0.027)		5.43 (3.21-11.69)		0.241 (0.187-0.604)	

Table II. Continued.

Complication	Total 1,25 (OH)D, ng/dl[d]	P-value	Total 25(OH) D, ng/dl[d]	P-value	Total 24,25 (OH)D, ng/dl[d]	P-value	1,25(OH) 2D3, ng/dl[d]	P-value	25(OH) D3, ng/dl[d]	P-value	24,25(OH) 2D3, ng/dl[d]	P-value
Stroke		0.75		0.28		0.23		0.05[a]		0.41		0.24
No	0.020 (0.000-0.044)		26.08 (18.42-36.27)		0.290 (0.192-0.541)		0.015 (0.000-0.040)		6.18 (3.38-14.00)		0.285 (0.191-0.541)	
Yes	0.023 (0.000-0.040)		30.13 (25.49-36.10)		0.218 (0.151-0.431)		0.011 (0.000-0.024)		4.92 (2.32-11.20)		0.218 (0.151-0.431)	

[a]P<0.05, [b]P<0.01, [c]P<0.001. [d]Median (interquartile range).

When the deficient and replete 25(OH)D populations were compared, there was no difference between them, suggesting that the serum 25(OH)D levels were not related to the development of hypertension or dyslipidemia. These changes were not a result of altered renal function, as there was no correlation with estimated glomerular filtration rate.

There is increasing evidence showing that vitamin D deficiency serves a role in the pathogenesis of type 2 diabetes (25-27) with evidence from epidemiological studies linking vitamin D deficiency and insulin resistance (28,29). In adults at high risk of developing type 2 diabetes, supplementation with cholecalciferol has been shown to improve b cell function (30) and $1,25(OH)_2D$ may also improve insulin sensitivity by activating peroxisome proliferator-activated receptor δ (31). Conversely, long-term studies have found that vitamin D and calcium supplementation do not offer protection against the risk of diabetes development (22), and giving supplements to vitamin D replete patients with T2DM had no effect on insulin resistance or glycemic control (32); however, differing meta-analyses have shown an improvement in HbA1c in response to supplementation with vitamin D in some studies (33,34), but not in others (35).

Both the total $24,25(OH)_2D$ and $24,25(OH)_2D_3$ levels were significantly associated with dyslipidemia; $24,25(OH)_2D$ may not be an inactive metabolite, as it has been shown to suppress Apo A-1 in hep G cells (36), and it may exhibit a physiological role in growth plate formation (8); therefore, a direct effect on lipid metabolism cannot be excluded.

The strength of the present cross-sectional study was the homogeneous Qatari population studied and the number of participants assessed using state-of-the-art measurements of 25(OH)D and metabolites, and these results may be generalizable to other Qatari populations. However, this study was limited by its cross-sectional design and that, whilst all subjects were prescribed vitamin D_2 supplements, it was not possible to ascertain compliance. Additionally, the results may not be generalizable to other ethnicities, for which a multi-center approach with participation from institutes in several different countries is required.

In conclusion, vitamin D_3 metabolites were associated with diabetic retinopathy, whereas total vitamin D levels were not, suggesting that endogenous vitamin D_3 metabolites are the better measure of diabetic microvascular complications. However, both total vitamin D and vitamin D_3 metabolites were associated with cardiovascular risk factors in patients with type 2 diabetes.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AEB analyzed and interpretated the data as well as wrote the manuscript. LHMA analyzed and interpretated the data. SRD performed the statistical analysis. AL performed the vitamin D measurements. EAA and AH participated in data analysis and interpretation, as well as prepared the manuscript. SLA designed the study and contributed to the discussion. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Weill Cornell Institutional Review Board (approval no. IRB# 13-00063). All study subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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