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ORIGINAL ARTICLE

Serum 25-hydroxyvitamin D3 concentrations and carotid artery intima-media thickness among type 2 diabetic patients

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Summary

Objective To estimate the prevalence of hypovitaminosis D among type 2 diabetic adults and to assess the relationship between hypovitaminosis D and intimal medial thickening (IMT) of the common carotid artery, a marker of preclinical atherosclerosis.

Design, patients and measurements We compared winter serum 25-hydroxyvitamin D3 [25(OH)D] concentrations in 390 consecutive type 2 diabetic patients and 390 nondiabetic controls who were comparable for age and sex. Common carotid IMT was measured with ultrasonography only in diabetic patients by a single trained operator blinded to subjects' details.

Results The prevalence of hypovitaminosis D (i.e. 25(OH)D ≤ 37·5 nmol/l) was higher in diabetic patients (34·0 *vs* 16·4%, $P < 0·001$) than in controls. Among diabetic patients, those with hypovitaminosis D ($n = 130$) had a marked increase in common carotid IMT (1·10 ± 0·15 *vs* 0·87 ± 0·14 mm, $P < 0·001$) when compared with their vitamin D-sufficient counterparts ($n = 260$). These patients also had significantly higher haemoglobin A1c, fibrinogen and C-reactive protein (hs-CRP) concentrations. In multivariate regression analysis, low 25(OH)D concentrations independently predicted carotid IMT ($P < 0·001$) in people with type 2 diabetes after adjustment for classical risk factors, diabetes duration, HbA1c, calcium, renal function tests, inflammatory markers, use of medications, and presence of the metabolic syndrome (as defined by the Adult Treatment Panel III criteria).

Conclusions Hypovitaminosis D is highly prevalent in type 2 diabetic adults and is strongly and independently associated with increased carotid IMT. Further investigation into whether vitamin D may play a role in the prevention of atherosclerosis appears to be warranted.

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Introduction

The importance of hypovitaminosis D is related primarily to bone integrity. Vitamin D3 is essential not only for bone development and growth in children and maintenance of bone in adults, but also for the prevention of osteoporosis and fractures in the elderly.[1,2]

In addition to its traditional calcium-related effects on the skeleton, hypovitaminosis D has been now recognized to exert nonskeletal adverse effects on several other organ systems. There is accumulating experimental and clinical evidence suggesting that serum concentrations of 25-hydroxyvitamin D3 [25(OH)D] may be inversely associated with some cancers,[1,2] type 2 diabetes,[3–5] metabolic syndrome[6] and cardiovascular disease (CVD).[7,8]

Much remains to be learned, however, about the associations between vitamin D status, metabolic syndrome, and atherosclerosis. Furthermore, the available data in humans arguing that inadequate vitamin D status may be a novel risk factor for CVD remain conflicting.[8]

Because currently there is a lack of information on associations between vitamin D status and carotid artery intima-media thickness (IMT) (a reliable marker of preclinical, generalized atherosclerosis)[9] among type 2 diabetic individuals, people in whom the incidence rates of CVD are very high, we sought to estimate the prevalence of hypovitaminosis D and to assess the relationship between serum 25(OH)D concentrations and carotid IMT in a representative sample of type 2 diabetic outpatients. Clarification of these relationships may help to suggest possible underlying mechanisms, and may be of clinical importance in planning preventative and therapeutic strategies.

Patients and methods

We studied 390 type 2 diabetic outpatients who consecutively attended our clinic during the winter months (November–March) after exclusion of those with recent history of acute illness or advanced chronic liver or renal disease, and those who were taking any medications known to affect vitamin D metabolism, including vitamin/mineral supplements. The control group consisted of 390 (M/F = 253/137; age = 58 ± 7 years) nondiabetic volunteers, who were well matched for age and sex.

The local Ethics Committee approved the protocol. Written informed consent was obtained from all participants.

Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Waist circumference was measured with a standardized tape, in a standing position, at the level of the umbilicus. Blood pressure was measured in triplicate with a standard mercury manometer. Information on smoking history and other lifestyle characteristics was obtained from all participants by questionnaire.

Venous blood was drawn in the morning after an overnight fast. Plasma glucose, lipids, creatinine and other biochemical blood measurements were determined by an automatic colourimetric method (DAX 96, Bayer Diagnostics, Milan, Italy). LDL cholesterol was calculated by the Friedewald's equation, except when triglycerides exceeded 4·5 mmol/l (eight patients). Haemoglobin A1c was measured by an automated HPLC analyser (Menarini-Arkray HA-8140, Florence, Italy); the upper limit of normal for the laboratory was 5·9%. Plasma fibrinogen and C-reactive protein (hs-CRP) concentrations were determined with the Clauss method (IL Test PT-Fibrinogen HS, Instrumentation Laboratory, Milan, Italy) and with a highly sensitive immuno-turbidimetric assay (Roche Diagnostics, Milan, Italy), respectively. It is known that vitamin D3 circulates in the blood stream largely as 25(OH)D.[1,2] Although unhydroxylated vitamin D3 and 1,25-dihydroxyvitamin D3 can be measured in the circulation, the best estimates of vitamin D status are provided by measurement of 25(OH)D; this is due to its long serum half-life (~3 weeks) and because the 25-hydroxylation step is unregulated, thus reflecting substrate availability.[1,2] Serum 25(OH)D concentrations were measured using an automated chemiluminescence immuno-assay (DiaSorin Liaison, Stillwater, MN, USA); intra- and inter-assay coefficients of variation were below 4% and 9%; analytical sensitivity was of 12·5 nmol/l. To avoid seasonal variations, all 25(OH)D samples were collected during the winter months in both control and diabetic individuals. Reference ranges for 25(OH)D concentrations in our laboratory during the winter months (a period in which sunlight exposure is poor) were 25–160 nmol/l. Urinary albumin excretion rate was measured as the albumin-to-creatinine ratio (ACR) by an immuno-nephelometric method; micro- and macro-albuminuria were defined as an ACR > 2·5 and > 25 mg/mmol, respectively. Estimated glomerular filtration rate (e-GFR) was calculated with the simplified equation proposed by the Modification of Diet in Renal Disease (MDRD) Study Group.[10]

Metabolic syndrome (MetS) was defined according to the Adult Treatment Panel (ATP) III criteria;[11] in accordance with this definition, a person with type 2 diabetes was classified as having MetS if he/she had at least two of the following risk determinants:

- waist circumference > 102 cm in males or > 88 cm in females;
- triglycerides = 1·7 mmol/l;
- HDL < 1·0 mmol/l in males and < 1·29 mmol/l in females or treatment;
- blood pressure = 130/85 mmHg or receiving treatment for hypertension.

Carotid IMT was measured with ultrasonography by a single trained operator who was blind to the clinical characteristics of the participants. Carotid IMT measurements were performed only in diabetic patients and not in control subjects. IMT measurements were made bilaterally at the level of the common carotid artery far wall and always in stenotic-free segments, as previously reported.[12,13] For each subject, three measurements on both sides were performed, on the anterior, lateral and posterior projection of the near and far wall. All readings were then averaged. Repeated measurements on the same subjects (that were done in a subgroup of 100 subjects) gave coefficients of variation below 9%. A carotid plaque was defined as a focal thickening of > 1·2 mm at the level of common carotid artery.[9]

Data are presented as means ± SD or percentages. Because of skewness and kurtosis of the distributions, carotid IMT, serum triglyceride, hs-CRP and 25(OH)D concentrations were logarithmically transformed for statistical analyses and then back-transformed to their natural units for presentation in text and tables. Statistical analyses included unpaired-*t*-test, χ^2-test with Yates' correction for continuity (for categorical variables), univariate linear correlations and analysis of covariance. The independence of the association of carotid IMT with low 25(OH)D concentrations was assessed by multivariate linear (when carotid IMT, the dependent variable, was considered as a continuous variable) or logistic (when carotid IMT was modelled as categorical variable stratifying the study population in tertiles) regression analysis. In the fully adjusted regression model, together with 25(OH)D, sex, age, BMI, smoking history, diabetes duration, HbA1c, LDL cholesterol, calcium, renal function tests, markers of inflammation (fibrinogen or hs-CRP), use of medications (lipid-lowering, antihypertensive, antiplatelet or hypoglycaemic drugs), and presence of ATP III-defined MetS were included as covariates. Separate regression models were also tested with individual components of the MetS simultaneously included (as continuous or categorical variables) in the same equation instead of the MetS a single entity. Hypovitaminosis D was defined as a serum 25(OH)D concentration ≤ 37·5 nmol/l.[2,14] *P*-values < 0·05 were considered statistically significant.

Results

The mean (± SD) concentration of 25(OH)D was 62·7 ± 23 nmol/l (median: 54 nmol/l; range: 12·5–140 nmol/l) among nondiabetic controls and 48·2 ± 26 nmol/l (median: 40 nmol/l; 12·5–168 nmol/l) among those with type 2 diabetes. The age- and sex-adjusted prevalence of hypovitaminosis D was higher in diabetic patients than in control subjects (34 *vs* 16·4%, $P < 0·001$).

The baseline characteristics of type 2 diabetic patients grouped according to vitamin D status are shown in Table 1. Those with hypovitaminosis D were slightly older and had higher concentrations of HbA1c, hs-CRP and fibrinogen than their vitamin D-sufficient diabetic counterparts. The proportion using aspirin was greater among those with hypovitaminosis D, whereas the proportion using lipid-lowering, antihypertensive or hypoglycaemic drugs was essentially the same in both groups. Sex, BMI, waist circumference, diabetes duration, smoking history, blood pressure, lipids, calcium, estimated GFR and albuminuria were not statistically different between the groups.

Notably, patients with hypovitaminosis D had a markedly greater carotid IMT than their vitamin D-sufficient counterparts, with no significant differences between sexes (not shown). These patients also had a greater prevalence of carotid atherosclerotic plaques (74·6% *vs* 38·9%, $P < 0·001$), defined as a focal thickening > 1·2 mm at the level of common carotid artery.

Table 1. Baseline characteristics of type 2 diabetic patients ($n = 390$), grouped according to their vitamin D status

Variable	Hypovitaminosis		
	With*	Without†	*P*-value
n	130	260	–
Sex (M/F)	79/51	174/86	NS
Age (years)	59 ± 6	57 ± 7	0·053
BMI (kg/m^2)	28·4 ± 5	28·5 ± 5	NS
Waist circumference (cm)	105 ± 11	105 ± 12	NS
Diabetes duration (years)	12 ± 8	11 ± 7	NS
Diet only (%)	13	17	NS
Oral hypoglycaemic agents only (%)	58	60	NS
Insulin treatment (%)	29	23	NS
Current smokers (%)	23	17	NS
Statin users (%)	29	26	NS
Aspirin users (%)	40	22	0·001
Anti-hypertensive users (%)	71	69	NS
Blood pressure (mmHg)			
Systolic	146 ± 18	144 ± 17	NS
Diastolic	80 ± 8	80 ± 7	NS
HbA1c (%)	7·5 ± 1·3	7·2 ± 1·4	0·05
Triglycerides (mmol/l)	2·01 ± 1·1	1·82 ± 1·0	NS
HDL cholesterol (mmol/l)	1·35 ± 0·3	1·35 ± 0·4	NS
LDL cholesterol (mmol/l)	3·37 ± 0·8	3·35 ± 0·7	NS
e-GFR (ml/min/1·73 m^2)	91 ± 21	94 ± 18	NS
Calcium (mmol/l)	2·34 ± 0·1	2·36 ± 0·2	NS
Fibrinogen (g/l)	4·69 ± 0·8	4·21 ± 0·9	0·001
hs-CRP (mg/l)	4·98 ± 6·5	4·30 ± 6·1	0·001
Micro- or Macro-albuminuria (%)	27	23	NS
ATP III – Metabolic syndrome (%)	80	76	NS
Common carotid artery IMT (mm)	1·10 ± 0·15	0·87 ± 0·14	0·001

*D3 [25(OH)D: 28 ± 7 nmol/l]; †D3 [25(OH)D: 60 ± 23 nmol/l].
Data are the means ± SD, unless otherwise indicated. Differences were assessed by the unpaired *t*-test (for continuous variables) and by the χ^2-test (for categorical variables).
e-GFR, glomerular filtration rate as estimated by the MDRD equation.

As expected,[1,2,5,6] serum concentrations of 25(OH)D were inversely correlated to age, sex, HbA1c, fibrinogen, hs-CRP and presence of the ATP-III defined MetS (not shown). Moreover, there was a graded, positive, relationship between e-GFR tertiles and 25(OH)D concentrations (1°→3° tertile: 17 ± 25 *vs* 48 ± 25 *vs* 79 ± 26 nmol/l, respectively; $P < 0·005$).

The marked differences in carotid artery wall thickness that were observed between the two groups (see Table 1) were only slightly weakened after adjustment for sex, age, BMI, smoking history, diabetes duration, HbA1c, LDL cholesterol, calcium, e-GFR, inflammatory markers (fibrinogen or hs-CRP), use of medications, and presence of the MetS (by analysis of covariance; $P < 0·001$). Identical results were found after excluding patients who were taking lipid-lowering drugs (multiple-adjusted carotid IMT values between the groups: 1·07 ± 0·15 *vs* 0·84 ± 0·14 mm, respectively; $P < 0·001$ by analysis of covariance).

Table 2. Multiple linear regression analysis: independent predictors of carotid artery intima-media thickness among 390 type 2 diabetic adults

Dependent variable	Independent variables	Standardized beta coefficients	*P*-value
Carotid IMT	Age	0·41	0·001
	Sex	0·38	0·001
	Smoking	0·23	0·001
	Fibrinogen	0·18	0·005
	25(OH)D	–0·40	0·001
	R^2 model	0·64	

In this model BMI, diabetes duration, HbA1c, LDL cholesterol, calcium, e-GFR, use of medications (lipid-lowering, antihypertensive, antiplatelet or hypoglycaemic drugs), and ATP III-defined MetS were also included as covariates, but they were not independently associated with carotid IMT.

In univariate regression analysis, carotid IMT correlated positively to age, BMI, cigarette smoking, HbA1c, fibrinogen, hs-CRP, LDL cholesterol (*r*-values ranging from 0·61 to 0·14; $P < 0·01$ or less), and negatively to 25(OH)D ($r = –0·56$, $P < 0·001$). Moreover, carotid IMT was significantly greater in males, in patients having the MetS, and in those taking antiplatelet, lipid-lowering or antihypertensive drugs ($P < 0·001$ for all).

As shown in Table 2, in the fully adjusted regression model male sex, age, smoking, fibrinogen and 25(OH)D were independently associated with carotid IMT, whereas BMI, diabetes duration, HbA1c, LDL cholesterol, calcium, e-GFR, use of medications and the MetS were not. This regression model accounted for 64% ($R^2 = 0·64$) of total variance in carotid IMT; the relative contribution of 25(OH)D in the explanation of this variance was about 20%. Almost identical results were obtained in regression models that also adjusted for the individual components of the MetS (included as continuous variables), or in models in which 25(OH)D was included as a categorical variable [i.e. 25(OH)D ≤ 37·5 nmol/l]. Finally, the results remained essentially unchanged after excluding patients who were taking lipid-lowering drugs ($n = 105$).

Very similar results were obtained even when the independence of the association between 25(OH)D and carotid IMT (3° tertile *vs* tertiles 1–2°) was tested by multiple logistic regression analysis. Also in this case, low concentrations of 25(OH)D independently predicted carotid IMT (multiple-adjusted odds ratio 1·97; 95%C.I. 1·3–3·5; $P < 0·001$) after adjustment for multiple potential confounders.

Discussion

In a representative sample of type 2 diabetic outpatients, we found a relatively high prevalence (~35%) of hypovitaminosis D [defined as a serum 25(OH)D concentration ≤ 37·5 nmol/l][2,14] and, for the first time, a strong inverse association between serum 25(OH)D concentrations and carotid artery IMT, a marker of preclinical atherosclerosis.[9] Notably, this association was independent of a broad spectrum of potential confounders, including classical risk factors, glycaemic control, MetS components, renal function tests, markers

of inflammation and use of medications. In particular, the exclusion of patients taking statins, drugs that are known to reduce carotid IMT and that could prevent bone loss and fractures, did not substantially modify our results.

These findings confirm some previous evidence demonstrating that hypovitaminosis D is highly prevalent in people with type 2 diabetes,[4,5] and suggest that hypovitaminosis D might be an underestimated, novel, risk factor for CVD among type 2 diabetic adults.

Clearly, we must be cautious in making any causal inference, given the cross-sectional design of this study. Further follow-up and interventional studies are necessary to determine whether hypovitaminosis D predicts incident CVD in people with type 2 diabetes, and to determine whether vitamin D supplementation would be protective against CVD and what mechanisms might account for such protection.

The most likely explanation for these data would be that low concentrations of 25(OH)D reflect the presence of preclinical atherosclerosis and/or underlying cardio-metabolic risk factors, which have been found to relate to the severity of hypovitaminosis D,[1–5] or are merely a marker of an 'unhealthy' lifestyle (poor sunlight exposure or inadequate diet), which itself could promote the development of atherosclerosis. However, since in this study 25(OH)D was inversely associated with carotid IMT independent of multiple potential confounders, it could also be hypothesized that inadequate vitamin D status might directly contribute to the pathogenesis of atherosclerosis. Although the published studies in humans arguing that hypovitamininosis D is a novel risk factor for CVD remain still conflicting,[15–17] there is now accumulating experimental and clinical evidence suggesting both associative relationships and mechanisms for biological plausibility.[8,18–26] It has been reported that serum 25(OH)D concentrations are significantly lower in nondiabetic patients with acute myocardial infarction[7] or congestive heart failure[21] than in healthy controls. A significant, inverse, relationship between serum 25(OH)D concentrations and coronary artery calcifications has been also reported.[22,23] Additionally, type 2 diabetic individuals with clinically relevant CVD have lower 25(OH)D concentrations than their vitamin D-sufficient diabetic counterparts without CVD.[24] Finally, some interventional studies documented that long-term vitamin D supplementation in nondiabetic, vitamin D-deficient individuals significantly reduced plasma levels of CRP, tissue matrix-metalloproteinases (MMP 1 and 9) and its inhibitors (TIMP-1),[25] and had beneficial effects on the elastic properties of the common carotid artery in postmenopausal women.[26]

The following four potential, biological, mechanisms might be important for the protective effects of vitamin D3 against atherosclerosis: vitamin D3 can inhibit various aspects of inflammation,[1,8,19,20] which have been established as a key pathogenic mechanism in atherosclerosis; vitamin D3 can exert an antiproliferative effect on vascular smooth muscle cells and myocardial cell hypertrophy and proliferation,[8,21,27] which underlies the pathogenesis of congestive heart failure; vitamin D3 can improve insulin secretion/resistance,[3,6] which is thought to play a causal role in atherosclerosis; and vitamin D3 can act as a negative endocrine regulator for the renin-angiotensin system,[28,29] which itself plays an important independent role in hypertension and cardiovascular health.

This study has some limitations that should be noted. Because our study was a cross-sectional one, the causative nature of the associations reported cannot be established. Follow-up studies will be required to sort out the time sequence of events. Further, we were unable to evaluate any possible effects of parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3, because measurements of these variables were not performed in this study. Indeed, it is known that low concentrations of vitamin D often result in mild elevations of PTH (secondary hyperparathyroidism), which has been linked to hypertension, insulin resistance and significant increases in the serum levels of many acute-phase proteins.[30]

In conclusion, our results show that type 2 diabetic adults have significant reductions in serum 25(OH)D concentrations (*vs* matched controls) that predict preclinical atherosclerosis, independent of classical risk factors, renal function tests, inflammatory markers, use of medications and presence of the metabolic syndrome. These findings suggest the need for ongoing evaluation of the possible protective role of vitamin D3 supplementation in the development of atherosclerosis.

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