Effect of Vitamin D Deficiency and Replacement on Endothelial Function in Asymptomatic Subjects

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Context: Vitamin D receptors are present in many tissues. Hypovitaminosis D is considered to be a risk factor for atherosclerosis.

Objective: This study explores the effects of vitamin D replacement on insulin sensitivity, endothelial function, inflammation, oxidative stress, and leptin in vitamin D-deficient subjects.

Design, Setting, and Patients: Twenty-three asymptomatic vitamin D-deficient subjects with 25-hydroxyvitamin D [25(OH)D] levels below 25 nmol/liter were compared with a control group that had a mean 25(OH)D level of 75 nmol/liter. The vitamin D-deficient group received 300,000 IU im monthly for 3 months. The following parameters were evaluated before and after treatment: vitamin D metabolites, leptin, endothelial function by brachial artery flow mediated dilatation (FMD), insulin sensitivity index based on oral glucose tolerance test, and lipid peroxidation as measures of thiobarbituric acid reactive substances (TBARS).

Results: FMD measurements were significantly lower in 25(OH)D-deficient subjects than controls ($P = 0.001$) and improved after replacement therapy ($P = 0.002$). Posttreatment values of TBARS were significantly lower than pretreatment levels ($P < 0.001$). A positive correlation between FMD and 25(OH)D ($r = 0.45$; $P = 0.001$) and a negative correlation between FMD and TBARS ($r = -0.28$; $P < 0.05$) were observed. There was a significant increase in leptin levels after therapy, and the leptin levels were positively correlated with 25(OH)D levels ($r = 0.45$; $P < 0.05$).

Conclusions: This study shows that 25(OH)D deficiency is associated with endothelial dysfunction and increased lipid peroxidation. Replacement of vitamin D has favorable effects on endothelial function. Vitamin D deficiency can be seen as an independent risk factor of atherosclerosis. Hypovitaminosis D-associated endothelial dysfunction may predispose to higher rates of cardiovascular disease in the winter. **(*J Clin Endocrinol Metab* 94: 4023–4030, 2009)**

Vitamin D deficiency is a common problem worldwide. Vitamin D levels are affected by seasonal fluctuations, dietary intake, and clothing habits. Vitamin D deficiency has been considered to be a risk factor for hypertension, various cancer types, and autoimmune diseases, and it may also play a role in the pathogenesis of type 2 diabetes (1). Vitamin D deficiency has been reported to have negative effects on insulin release and β-cell function in type 2 diabetic patients (2–4). A study of patients having vitamin D deficiency and osteomalacia revealed that vitamin D replacement positively affects insulin release (5). Borissova *et al.* (6) have reported that in addition to af-

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Abbreviations: BMI, Body mass index; CVD, cardiovascular disease; FMD, flow-mediated dilatation; HDL, high-density lipoprotein; HOMA, homeostasis model of assessment; hsCRP, highly sensitive C-reactive protein; iPTH, immunoreactive PTH; ISI, insulin sensitivity index; LDL, low-density lipoprotein; MDA, malondialdehyde; OGTT, oral glucose tolerance test; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; PON, paraoxonase; TBARS, thiobarbituric acid reactive substances.

fecting insulin release, hypovitaminosis D also increases insulin resistance and facilitates the development of metabolic syndrome. Previous studies have shown that insulin resistance is associated with endothelial dysfunction, an early marker of atherosclerosis (7–9). Thus, it is possible to argue that hypovitaminosis D may trigger insulin resistance and endothelial dysfunction, which both play a role in the development of atherosclerosis. The effects of vitamin D on the endothelium might be due to the changes in PTH, inflammation, oxidative stress, adipokines or energy metabolism due to weight gain.

This study evaluated the effects of hypovitaminosis D and vitamin D replacement on endothelial function, insulin resistance, oxidative stress markers, and leptin as a predictor of energy metabolism in asymptomatic 25-hydroxyvitamin D [25(OH)D]-deficient subjects.

Subjects and Methods

Subject selection

Seventy-three volunteers (48 females and 25 males) were screened for vitamin D deficiency between January and May 2006. Plasma 25(OH)D levels below 25 nmol/liter were considered low (10). Twenty-three of 27 subjects with 25(OH)D deficiency were included in the study after exclusion of subjects with any of systemic diseases or using medication. Young healthy volunteers were selected because they are a homogeneous group with no comorbidities or chronic diseases that could affect endothelial function. Twenty-three age- and sex-matched subjects with normal 25(OH)D levels were included as the control group (10).

The study was approved by the local ethics committee of Marmara University School of Medicine (reference no. MARYC-2005-223) and was carried out in accordance with the Declaration of Helsinki. All subjects gave informed consent before participation.

Study protocol

Height, weight, waist/hip ratio, body mass index (BMI), and blood pressure of subjects were noted. Blood samples were collected for measuring biochemical parameters, and a 75-g oral glucose tolerance test (OGTT) was performed. Endothelial function was determined as flow-mediated dilatation (FMD). Vitamin D_3 treatment with 300,000 IU (Nobel Ilac A.S., Istanbul, Turkey) was administered im to the 25(OH)D-deficient group once a month for 3 months. At the end of the third month of treatment, the subjects were reevaluated for the same parameters.

Biochemical parameters

Plasma 25(OH)D levels were measured with HPLC (Spectra System, GmbH, Munich, Germany). 25(OH)D HPLC assay effectively distinguishes $25(OH)D_2$ from $25(OH)D_3$ (11). Intraassay and interassay coefficients of variation were 1.5–2.6% and 3.6–4%, respectively. According to the reference range of the test kit, deficiency was defined as 25(OH)D level below 25 nmol/liter and insufficiency as below 50 nmol/liter, and the recommended level of 25(OH)D was above 100 nmol/liter. Levels of 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] were measured with a RIA (Biosource, Brussels, Belgium). This method does not distinguish between $1,25(OH)D_2$ and $1,25(OH)D_3$. Intraassay and interassay coefficients of variation were between 6.9 and 12%, respectively. Normal ranges were between 19 and 65 pg/ml. Insulin and intact PTH levels were measured with chemiluminescence immunometric method (Diagnostic Products Corp., Los Angeles, CA). The intraassay coefficients of variation and the total sensitivity were 3.3–5.5% and 4.1–7.3%, respectively, for the insulin kit, and they were 4.2–5.7% and 6.3–8.8%, respectively, for the immunoreactive PTH (iPTH) kit. Normal ranges were between 0.7 and 9 μIU/ml for insulin and between 10 and 65 pg/ml for iPTH. Highly sensitive C-reactive protein (hsCRP) levels were measured with an immunoturbidometric assay (Roche Diagnostics GmbH, Indianapolis, IN). Intraassay and interassay coefficients of variation of the kit were 0.28–1.34% and 2.51–5.7%, respectively. The normal level of hsCRP was below 0.5 mg/dl. Fibrinogen levels were measured by a clotting method based on the presence of excessive thrombin in the plasma (STA; Diagnostica Stago, Paris, France). Intraassay and interassay coefficients of variation of the kit were 2.5–2.9% and 2.6–2.7%, respectively. Normal ranges of fibrinogen were between 2 and 4 g/liter. Serum leptin levels were measured with the ELISA. Intraassay and interassay coefficients of variation of the leptin kit (Biosource) were 4.6 and 3.6%, respectively. Calcium, phosphorus, and glucose concentrations were measured with an enzymatic colorimetric assay method (Roche Diagnostics GmbH). Intraassay and interassay coefficients of variation of the glucose kit were 0.7–0.9% and 6.3–8.8%, respectively. Intraassay and interassay coefficients of variation of the calcium kit were 0.4–0.9% and 0.8–1.6% and of the phosphorus kit were 0.8–0.9% and 1.4–1.8%, respectively.

The measurement of lipid peroxidation [thiobarbituric acid reactive substances (TBARS)] was performed with the method described by Yagi (12). The serum samples were boiled with thiobarbituric acid after an extraction procedure with butanol, and the absorbance of the pink color at the upper phase of the liquid was measured at 532 nm. The results were defined using a standard solution of 1, 1, 3, 3-tetraethoxypropane and were expressed as nanomoles of malondialdehyde (MDA) per milliliter serum.

Paraoxonase (PON) enzyme activity was determined by the hydrolysis of p-nitrophenol (13). The milieu of the reaction was prepared to include 1.2 mmol/liter paraoxon, 1.32 mmol/liter $CaCl_2$, 132 mmol/liter Tris-base, and 2.63 mmol/liter NaCl (pH 8.5) and observed 405 nm at 25 C for 3 min. The results were multiplied by the coefficient of molar consumption ($\varepsilon = 18.05 \times 10^{-3}$) and expressed as the unit (U/liter).

Insulin sensitivity index (ISI)

Whole body insulin sensitivity during OGTT was calculated by the formula reported previously (14). After an overnight fast, blood samples were drawn just before and 30, 60, 90, and 120 min after the administration of 75 g glucose for the measurements of serum glucose and insulin concentrations.

Endothelial function assessment

Endothelial function was evaluated by a high-resolution Doppler ultrasonography examination of the right brachial artery, measuring FMD as described before (15). All evaluations

TABLE 1. Demographic data of patients

	Vitamin D-deficient group (n = 23)		Control group (n = 23)	P
	Before treatment	After treatment		
Age (yr)	23.3 ± 3.0		22.5 ± 2.2	
Sex (M/F)	15/8		15/8	
BMI (kg/m^2)	22.9 ± 3.6	25 ± 4.7	21.4 ± 2.7	0.054
Hip/waist	0.77 ± 0.06	0,78 ± 0.09	0.75 ± 0.07	0.449
Systolic BP (mm Hg)	116.7 ± 15.4	109.4 ± 11.8	111.5 ± 11.7	0.419
Diastolic BP (mm Hg)	75.6 ± 8.5	68.8 ± 4.9	72.1 ± 8.3	0.06

M, Males; F, females; BP, blood pressure.

were performed after a fasting period of 8 h, at a room temperature of 20–25 C, by a single experienced vascular sonographer who was blind to the diagnosis and clinical records. Endothelial function of the female subjects was assessed during the follicular phase of menstrual cycle. Intraobserver variation of the sonographer was less than 2%.

A linear array transducer with a frequency of 10 MHz (GE Vingmed, System Five; GE Healthcare, Horten, Norway) was used to acquire images. Brachial artery evaluation was performed 2 cm above the elbow. The electrocardiogram was monitored continuously. After measuring the basal diameter of the brachial artery, the cuff of a sphygmomanometer was placed on the forearm. Arterial occlusion was created by cuff inflation to 250–300 mm Hg for 5 min. After the cuff was deflated, lumen diameter was noted 1 min later to assess FMD. FMD was expressed as the percentage change in vessel diameter during reactive hyperemia.

After 10 min of rest following reactive hyperemia, 5 mg nitroglycerine was administered sublingually to determine endothelium-independent vasodilatation. Lumen diameter was measured 4–5 min after nitroglycerine administration. Endothelium-independent vasodilatation was expressed as the maximum percentage increase above baseline lumen diameter. For each measurement, the mean of the three highest measurements from the five consecutive cardiac cycles was taken.

Statistical analyses

All statistical calculations were performed with the SPSS (Statistical Package for Social Sciences) for Windows 15.0 software (SPSS, Chicago, IL). Comparisons between the groups were made with the paired *t* test and ANOVA where appropriate. The Pearson correlation with two-tailed probability values was used to estimate the strength of association between variables. A stepwise multiple regression analysis was performed to define the predictors of FMD. The level of statistical significance was set at $P < 0.05$. All results were expressed as mean ± SD.

Results

The demographic data of the groups are presented in Table 1. The two groups were similar with respect to age, BMI, waist/hip ratio, and blood pressure.

Baseline plasma 25(OH)D levels were lower in the vitamin D-deficient group compared with the control group (20.4± 6.8 *vs.* 75.1 ± 15.3 nmol/liter; $P < 0.01$) and significantly increased after treatment (116.9 ± 45.5 nmol/liter; $P < 0.05$) (Fig. 1). Most of the subjects were responsive to vitamin D replacement (Fig. 2). A significant increase in plasma 1,25(OH)$_2$D levels was observed after treatment (75.4 ± 48.4 pg/ml; $P < 0.001$).

FMD measurements for the deficient group were significantly lower compared with the controls (7.0 ± 3.2 *vs.* 11.2 ± 5.2%; $P = 0.001$). There was a significant increase in FMD values (10.4 ± 3.3%) after treatment (Fig. 1), the variances of which are shown in Fig. 2. The pretreatment endothelium-independent vasodilatation measures with

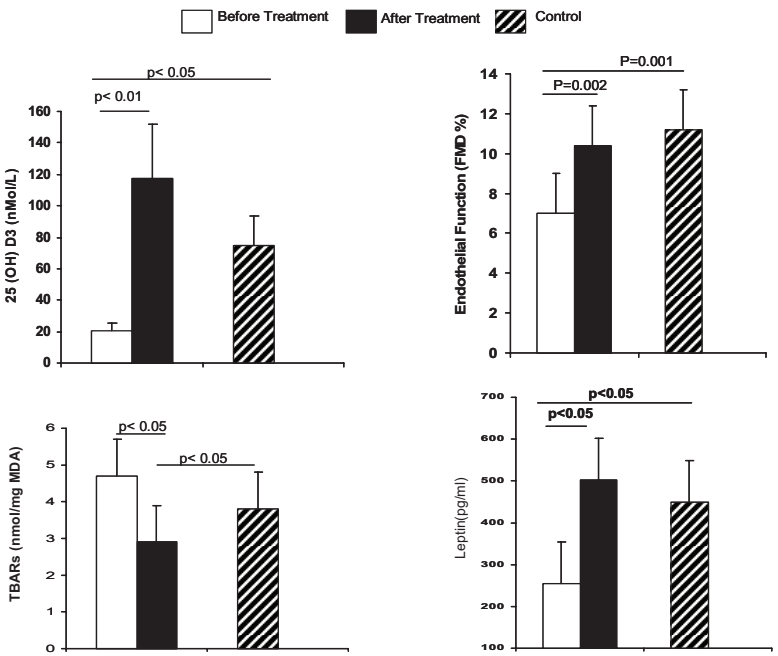

FIG. 1. Plasma 25(OH)D$_3$ levels and FMD values were significantly increased after treatment compared with the baseline and the control group, whereas levels for TBARS were significantly decreased after vitamin D treatment compared with the baseline and the control group. Leptin levels were low before treatment compared with the control group and significantly increased after vitamin D therapy.

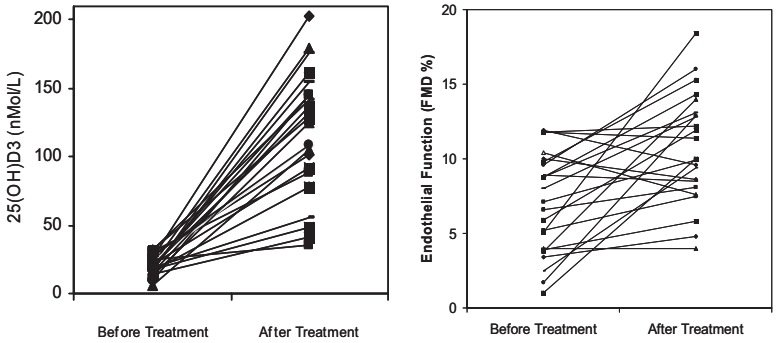

FIG. 2. Individual changes in FMD percentages and vitamin D levels were seen after treatment.

nitroglycerine were similar between the groups (21.6 ± 6.1 *vs.* 24.6 ± 10.5% in the vitamin D-deficient group and controls) and did not change after the treatment (20.8 ± 7.1%; $P > 0.05$).

Both baseline and posttreatment calcium and phosphorus levels were similar between the groups (Table 2), whereas higher PTH levels significantly decreased after treatment in the vitamin D-deficient group. The levels of inflammatory markers (hsCRP, leukocyte, and fibrinogen), total cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol or low-density lipoprotein (LDL)-cholesterol levels were similar between the groups (Table 2). The areas under the curves for serum glucose and insulin levels obtained during OGTT were not different between the groups.

Although basal TBARS levels of the deficient group (4.7 ± 1.7 ng/mg MDA) and the control group (3.8 ± 0.8 ng/mg MDA) were not different, TBARS levels decreased significantly after treatment (2.9 ± 0.7 ng/mg MDA; $P < 0.0001$) (Fig. 1). Serum PON activity did not show any difference between the groups before and after treatment. Baseline serum leptin levels (254.6 ± 108.3 pg/ml) in the deficient group were lower than the controls (449 ± 148 pg/ml; $P < 0.001$), whereas leptin levels increased significantly (502 ± 240.7 pg/ml; $P < 0.001$) after treatment (Fig. 1).

Correlation and regression analyses

According to the correlation data of the 25(OH)D-deficient group and controls, the 25(OH)D level was correlated positively with the $1,25(OH)_2D$ level ($r = 0.57$; $P < 0.001$) and leptin ($r = 0.50$; $P < 0.001$), whereas they were negatively associated with TBARS ($r = -0.26$; $P < 0.05$) and PTH ($r = -0.33$; $P = 0.02$). Although FMD was correlated positively with 25(OH)D level ($r = 0.45$; $P = 0.001$), a negative correlation was observed between FMD and TBARS ($r = -0.28$; $P < 0.05$) (Fig. 3).

To determine the factors influencing endothelial function after treatment, the results for the same subjects were evaluated before and after treatment. FMD was positively correlated with 25(OH)D levels ($r = 0.36$; $P = 0.007$) and $1,25(OH)_2D$ levels ($r = 0.35$; $P = 0.001$) and also with PON ($r = 0.26$; $P = 0.03$), whereas they were negatively correlated with TBARS ($r = -0.42$; $P = 0.002$) (Fig. 4). $1,25(OH)_2D$ levels were positively correlated with leptin ($r = 0.39$; $P = 0.01$) but were negatively correlated with iPTH ($r = -0.33$; $P = 0.02$) and TBARS ($r = -0.46$; $P = 0.001$) after treatment. We found a negative correlation between leptin and TBARS ($r = -0.26$; $P < 0.05$), PON and PTH ($r = -0.34$; $P < 0.05$), PTH and CRP ($r = -0.27$; $P < 0.05$). There were positive correlations between homeostasis model of assessment (HOMA) and lep-

TABLE 2. Laboratory results and glycemic data of the study groups

	Vitamin D-deficient group (n = 23)		Control group (n = 23)	*P*
	Before treatment	After treatment		
$25(OH)D_3$ (nmol/liter)	20.4 ± 6.8	116.9 ± 45.5	75.1 ± 15.3	<0.001
$1,25(OH)_2D$ (pg/ml)	28.9 ± 12.6	79.4 ± 54.1	45.0 ± 12.6	<0.001
iPTH (pg/ml)	58.9 ± 29.4	40.6 ± 22.0	44.6 ± 13.4	<0.05
Ca (mg/dl)	9.6 ± 0.4	9.6 ± 0.4	9.7 ± 0.6	0.183
P (mg/dl)	3.9 ± 0.6	3.9 ± 0.6	3.9 ± 0.5	0.928
Total cholesterol (mg/dl)	155.6 ± 25	156.2 ± 27	158.2 ± 35	0.914
Triglycerides (mg/dl)	71.4 ± 36	67.7 ± 29	60 ± 22	0.420
HDL (mg/dl)	58.2 ± 14	58 ± 13	60.5 ± 14	0.743
LDL (mg/dl)	83 ± 21.6	84.7 ± 25.1	85.8 ± 27.2	0.949
Fasting BG (mg/dl)	86.9 ± 7.4	85.7 ± 6	84 ± 6.4	0.558
Fasting insulin (mIU/ml)	6 ± 1.6	7.3 ± 2.5	6 ± 3	0.122
HOMA	1.3 ± 0.3	1.5 ± 0.5	1.2 ± 0.6	0.183
ISI	1.7 ± 0.5	1.6 ± 0.7	2 ± 1	0.458
AUC glucose (mg/h/ml)	54.4 ± 46.6	53.6 ± 48.2	51.1 ± 43.9	0.916
AUC insulin (mU/h/ml)	78.3 ± 38.4	100 ± 78.8	89.1 ± 43.3	0.623

BG, Blood glucose; AUC, area under the curve.

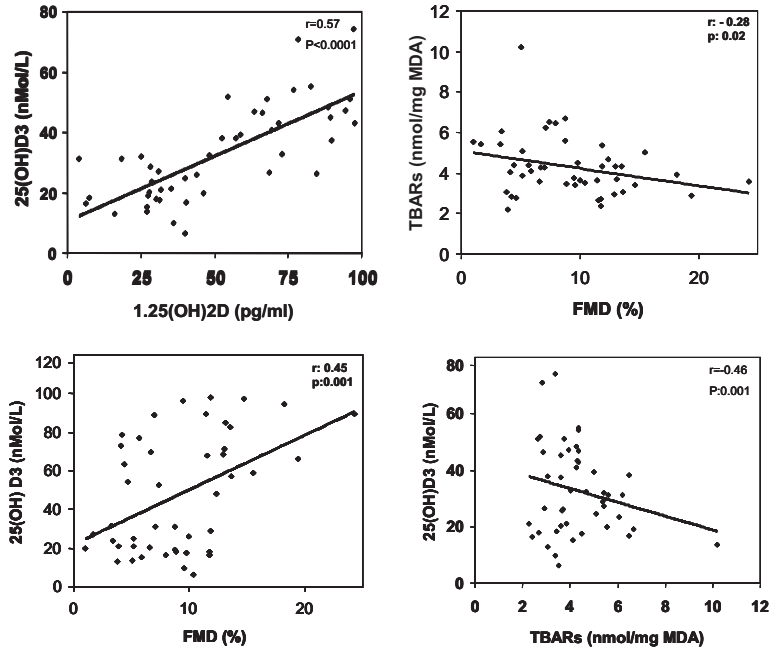

FIG. 3. The correlation data of the vitamin D-deficient subjects and the control group showed a positive association between 25(OH)D_3 and FMD values (r = 0.45; $P = 0.001$), also between 25(OH)D_3 and 1,25(OH)$_2$D levels (r = 0.57; $P < 0.0001$). There was a negative correlation between FMD and TBARS (r = −0.28; $P < 0.05$), also between 25(OH)D_3 levels and TBARS (r = −0.46; $P = 0.001$).

tin (r = 0.45; $P < 0.05$) and also between HOMA and CRP (r = 0.31; $P < 0.05$).

Linear regression analysis was performed to determine the predictors of endothelial function. Among 25(OH)D, 1,25(OH)$_2$D, PTH, hsCRP, HOMA, ISI, TBARS, PON, and leptin that were included in the linear regression model, 25(OH)D, 1,25(OH)$_2$D, PTH, TBARS, and PON were shown to be significantly and independently affecting endothelial function (r^2 of the model = 0.31; $P = 0.01$).

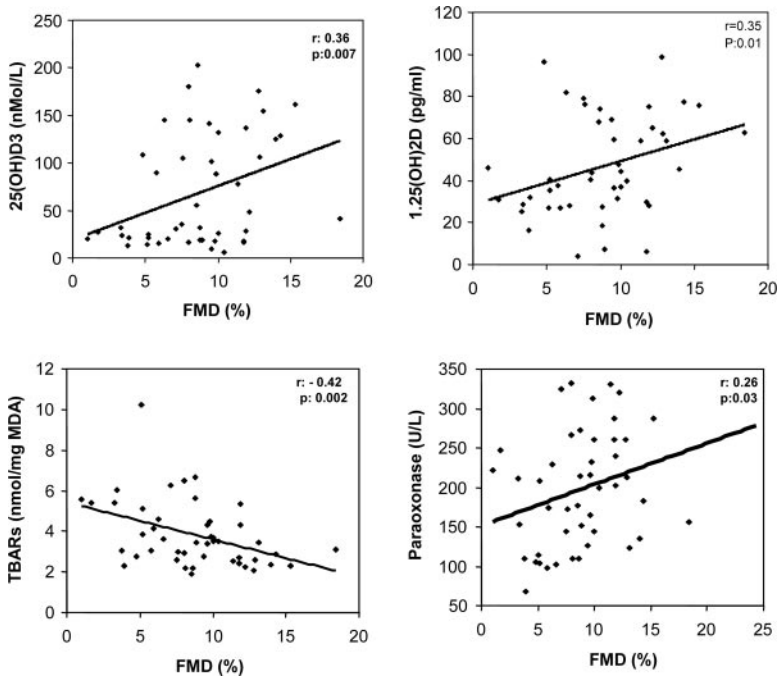

FIG. 4. The correlation data of the vitamin D-deficient group before and after treatment showed a positive association between FMD values and 25(OH)D_3 (r = 0.36; $P = 0.007$), between FMD and 1,25(OH)$_2$D (r = 0.35; $P = 0.01$), and also PON (r = 0.26; $P < 0.05$). A significant negative correlation was observed between FMD and TBARS (r = −0.42; $P < 0.005$).

Discussion

Our study, which aimed to evaluate the effects of hypovitaminosis D and vitamin D replacement on endothelial function, insulin resistance, oxidative stress markers, and leptin as a predictor of energy metabolism in asymptomatic vitamin D-deficient subjects, demonstrated a relationship between vitamin D levels and endothelial function in asymptomatic vitamin D-deficient people, and it confirms earlier studies that suggest a possible relationship between hypovitaminosis D and atherosclerosis.

Previous studies have shown an inverse relationship between vitamin D levels and coronary calcification (16, 17). Additionally, vitamin D deficiency was reported to be more common in people diagnosed with acute coronary syndrome compared with healthy controls. Furthermore, it has been suggested that the higher incidence of cardiovascular diseases (CVDs) in winter might be explained by lower vitamin D levels observed in this season (18). However, no relation between 1,25(OH)$_2$D, PTH levels, and coronary calcifications as determined by electron beam tomography has been reported by others (19).

A very recent study clearly revealed the increased risk of CVD in vitamin D-deficient (20) patients recruited from 1739 Framingham Offspring Study participants without prior CVD. There was a graded increase in cardiovascular risk across plasma levels of 25(OH)D, with multivariable-adjusted hazard ratios of 1.53 for levels between 10 and 15 ng/ml and 1.80 for levels below 10 ng/ml (95% confidence intervals, 1.00 to 2.36 and 1.05 to 3.08, respectively; P for linear trend = 0.01), and it was con-

cluded that 25(OH)D deficiency was associated with an increased incidence of CVD.

In our study, the endothelial function of 25(OH)D-deficient subjects was significantly disturbed in comparison to controls, whereas a significant improvement was observed after replacement, and a positive correlation between 25(OH)D levels and FMD was considered to be consistent with this relationship. The effect of vitamin D on endothelial function may be through either direct or indirect mechanisms. Although vitamin D receptors are present on endothelial cells, a direct effect of vitamin D on endothelial cells has not been documented (21). On the other hand, data regarding the indirect effects of vitamin D and evidence for its protective effect against oxidative stress seems to be more abundant (22–26). We observed that FMD measurements decreased in three subjects and did not change in two subjects, despite increased 25(OH)D and decreased TBARS levels. We could not find any significant difference in terms of biochemical or clinical parameters between nonresponders compared with responders. This lack of difference could be related to methods used for FMD determination, which is a highly operator-dependent procedure.

Wiseman (22) reported in 1993 that the hydrophobic parts of cholecalciferol, $1,25(OH)_2D_3$, ergocalciferol, and 7-hydroxy D_3 interfered with fatty acid residues that impair the viscosity of cell membrane and thus protected the cell membrane from lipid peroxidation and the harmful effects of free radicals *in vitro* in a pioneer study investigating the antioxidant effects of vitamin D_3. Later, Kuzmenko *et al.* (23) investigated the effects of vitamin D on oxidative stress and lipid peroxidation in animals by determining the changes in lipid peroxidation before and after replacement in vitamin D-deficient animals. They demonstrated that high levels of TBARS in vitamin D-deficient animals were decreased significantly after vitamin D_3 replacement but still remained higher than the control group (24, 25). Sardar *et al.* (26) suggested that vitamin D was an antioxidant due to an increase in hepatic glutathione levels in rats that received cholecalciferol. In another study, it was proposed that the antioxidant effect of vitamin D_3 was much more than that of vitamin E, melatonin, or β-estradiol (27). Although it was first presumed that the effects of vitamin D on oxidative stress were mostly due to its genomic and nongenomic functions, novel data indicated that vitamin D was effective through improving antioxidant mechanisms.

In our study, $1,25(OH)_2D$ levels increased after vitamin D supplementation and were positively correlated with 25(OH)D in all study groups. Also FMD results were significantly correlated with $1,25(OH)_2D$ levels after treatment. These results indicate that vitamin D has an endocrine effect on the endothelium that is important for endothelial function rather than for the local synthesis of $1,25(OH)_2D$.

We have shown that increased oxidative stress in 25(OH)D-deficient people decreases with replacement. High levels for TBARS, which indicate lipid peroxidation, decreased significantly after replacement in deficient individuals. Because there is a positive association between FMD and 25(OH)D levels and because increases in 25(OH)D levels with replacement decreased TBARS levels significantly, the positive effects of vitamin D replacement on endothelial function can be explained by a decrease in oxidative stress.

PON enzyme, an indicator of oxidative stress, inhibits LDL oxidation *in vitro* and protects against CVD (28). The activity of this enzyme is decreased by increased oxidative stress (29). In our study, although there was no significant increase in PON levels after treatment, the regression analysis revealed that PON activity was one of the main determinants of endothelial function. An increase in PON activity after treatment was not expected because subjects included in the study were asymptomatic without any underlying disease and had normal lipid levels. The positive correlation between PON activity and endothelial function also suggests that vitamin D reduces oxidative stress.

Because vitamin D is the major regulator of calcium metabolism, the relationship between calcium metabolism and endothelial function should be investigated. In a cross-sectional study, 3212 patients were evaluated by questionnaires for their nutritional habits and heart disease. The prevalence of heart disease was found to be higher in people with elevated PTH levels (30). In our study, PTH levels, which were higher in 25(OH)D-deficient subjects, decreased to levels similar to controls after treatment, whereas no differences were observed in calcium and phosphorus levels. A negative correlation between 25(OH)D and PTH levels was found, and this was consistent with the literature (31). Because no relationship between PTH, calcium, and phosphorus levels and endothelial function was observed, it would not be appropriate to attribute the positive effects of vitamin D replacement on endothelial function to the change in PTH levels.

In our study, no relationship was shown between endothelial function and hsCRP, fibrinogen, or leukocyte counts, which are indicators of low-grade inflammation. It has been reported that hsCRP levels are correlated negatively with $1,25(OH)_2D$ levels in healthy individuals, but vitamin D replacement for 12 wk does not change hsCRP or cytokine levels (32, 33). The levels of hsCRP are increased in almost all chronic inflammatory diseases (34). In a patient with chronic inflammatory disease, hsCRP

levels decreased significantly after a 1-yr treatment with vitamin D, and it was consequently suggested that vitamin D deficiency could play a role in tissue injury due to inflammation (35).

Considering that vitamin D deficiency is accompanied by impairment of insulin release and insulin resistance (3), it is possible to expect a tendency toward endothelial dysfunction and atherosclerotic disease in vitamin D-deficient people. However, in our study we did not observe any difference in insulin release or resistance in the 25(OH)D-deficient group compared with the control group, and there was no difference in insulin sensitivity parameters although BMI was increased after vitamin D treatment.

Based on the data in the literature, it might be concluded that vitamin D has a prominent effect on insulin release, but evidence for its effect on insulin resistance is not powerful. It has been shown that there are vitamin D receptors on the β-cells of the pancreas and that vitamin D acts on insulin release (5, 36). In recent years, studies were performed to explore the effects of vitamin D on insulin sensitivity and metabolic syndromes. Borissova *et al.* (6) reported that whereas insulin release improved after 1332 IU/d cholecalciferol treatment for 1 month, insulin resistance also improved in 21.4% of type 2 diabetic women whose $25(OH)D_3$ levels were low. Later Chiu *et al.* (4) reported the presence of vitamin D deficiency in 37% of 126 healthy volunteers, and a positive correlation between ISI and 25(OH)D levels was documented by the hyperglycemic clamp. However, other studies performed in different patient groups have demonstrated no relationship between vitamin D deficiency and insulin resistance or insulin sensitivity (37). Although it is known that vitamin D level is related to sunlight exposure and seasonal changes, individual factors should also be taken into consideration in the evaluation of insulin resistance (38).

Increased BMI has been reported after vitamin D replacement therapy (39). The mechanism of the increase in fat mass with vitamin D therapy is not clear. In this study, we measured leptin to evaluate vitamin D action on adipokines and energy metabolism. Interestingly, we found a significant increase in leptin levels after replacement, and leptin levels were positively correlated with 25(OH)D. It is not easy to explain the exact mechanism of vitamin D action on leptin metabolism. There are a few studies in the literature that report inhibitory effects of leptin on vitamin D. Matsunuma *et al.* (40) suggested that leptin administration clearly decreased the elevated $1,25(OH)_2D$ concentrations in leptin-deficient *ob/ob* mice. In another study, direct inhibitory effect of $25(OH)D_3$ on leptin secretion was reported in human adipose tissue culture, which was in contrast with our findings (41). These results are far from unraveling the relationship between leptin and vitamin D. Further studies are certainly needed to clarify the interactions of adipokines and vitamin D.

Although we observed that supraphysiological 25(OH)D levels have a favorable effect on endothelial function, there are experimental data that indicate atherogenic effects of pharmacological doses of 25(OH)D (42). We couldn't observe any difference between the subjects with increased and unchanged FMD in terms of 25(OH)D and $1,25(OH)_2D$ levels. All subjects were followed between 2006 and 2009, and none had hypercalciuria or any other side effects of vitamin D.

In conclusion, 25(OH)D deficiency is associated with endothelial dysfunction. When vitamin D is administered to people with 25(OH)D deficiency, endothelial function improves. We suggest that this improvement in endothelial function is likely to be due to the effects of vitamin D replacement on oxidative stress and lipid peroxidation. The reason for the increase in the prevalence of CVDs during the winter may be endothelial dysfunction due to 25(OH)D deficiency (18). Vitamin D administration to people with chronic inflammatory diseases or those at high risk for atherosclerotic diseases during the winter months is a subject worth investigating.

Acknowledgments

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