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Osteocalcin and vitamin D status are inversely associated with homeostatic model assessment of insulin resistance in Canadian Aboriginal and white women: the First Nations Bone Health Study☆,☆☆

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Abstract

Objective: Osteocalcin, a protein synthesized by osteoblasts, and vitamin D status have independently been implicated in energy metabolism and glucose regulation. This study was conducted to simultaneously explore the relationships among osteocalcin, vitamin D status and indicators of glucose metabolism and adiposity in a mixed-ethnicity cohort of adult women.
Design: Cross-sectional.
Methods: Aboriginal and white women (n=368) over 25 years of age (45.3±13.6 years) were studied for measures of osteocalcin and 25-hydroxy vitamin D [25(OH)D] plus glucose metabolism including glucose, insulin, C-peptide, hemoglobin A1c (HbA1c) and homeostatic model assessment of insulin resistance (HOMA-IR). Measures of adiposity included body mass index (BMI) plus total body fat and trunk fat from dual-energy X-ray absorptiometry.
Results: Aboriginal women had higher BMI, fat and markers of dysglycemia. Osteocalcin was not different between groups, but 25(OH)D was lower in Aboriginal women. Osteocalcin was inversely related to all five parameters of glucose metabolism, whereas 25(OH)D was inversely related to insulin, C-peptide and HOMA-IR. After accounting for age, ethnicity or adiposity using regression analyses, glucose, HbA1c and HOMA-IR were inversely related to both osteocalcin and 25(OH)D. However, only 25(OH)D was inversely related to C-peptide, and neither osteocalcin nor 25(OH)D was related to insulin.
Conclusions: These data from a unique mixed Aboriginal and white population suggest that both vitamin D and osteocalcin are involved in glucose control.

Keywords: Osteocalcin; Vitamin D; Glucose; Ethnicity

1. Introduction

The relationship between osteocalcin and glucose homeostasis and insulin sensitivity in both animals [1] and humans [2] is relatively new. Osteocalcin is specifically secreted by osteoblasts [1], but also endothelial cells [3] and embryonic stem cells [4]. Mice lacking osteocalcin have reduced insulin secretion, insulin sensitivity and glucose tolerance, with beta-cell proliferation accompanied by increased visceral fat [1]. In experimental studies, the uncarboxylated form of osteocalcin stimulates insulin expression in beta-cells, enhances insulin sensitivity in adipocytes and improves glucose intolerance [1]. In middle-aged men and after adjustment for age and body mass index (BMI), uncarboxylated osteocalcin associates with enhanced beta-cell function, but carboxylated osteocalcin associates with improved insulin sensitivity [5,6]. An inverse association between carboxylated osteocalcin and glucose metabolism exists in men [7–9] and women [10]. In a prospective analysis, osteocalcin inversely related to glucose and homeostatic model assessment of insulin resistance (HOMA-IR), and those with higher osteocalcin had a lower rise in glucose 3 years later [11]. Further proof of principle originates from research in adults receiving treatment for diabetes mellitus type 2; osteocalcin was higher with better response to treatment [12]. Additionally, glucose tolerance and insulin sensitivity partially improved in obese mice given daily injections of osteocalcin [13].

Regardless of whether carboxylation of osteocalcin is critical in the relationship to insulin resistance, the question that remains

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unanswered is what accounts for low osteocalcin in those with insulin resistance. Osteocalcin, but not other biomarkers of osteoblast activity, associates with oral glucose tolerance test in patients with diabetes mellitus type 2 [14,15]. Osteocalcin synthesis is suppressed by cortisol [16], and patients with insulin resistance have elevated cortisol [17,18]. Therefore, is osteocalcin a key metabolic signal explaining how elevated cortisol leads to insulin resistance? Elevated cortisol is a response to higher amounts of 11beta-hydroysteroid dehydrogenase type-1, in turn stimulated by tissue calcitriol [1,25(OH)$_2$D] [19]. Obese humans [20] and mice [21] have elevated calcitriol and low vitamin D status as indicated by serum 25-hydroxy vitamin D [25(OH)D]. This may explain the incomplete improvement in insulin sensitivity with osteocalcin injection in obese mice [13] since 25(OH)D is expected to be low.

The endocrine pancreas expresses vitamin D receptors and is a site for extrarenal production of 1,25(OH)$_2$D [22]. Vitamin D status is strongly related to insulin sensitivity even after correcting for age, gender and BMI [23,24]. In a secondary analysis of vitamin D supplementation [11], adults with impaired fasting glucose and receiving vitamin D supplementation (700 IU plus 500 mg calcium) over 3 years had less elevation in fasting plasma glucose compared with those who received placebo [11]. In a short-term randomized controlled trial using 1332 IU vitamin D/day over 1 month in adults with type 2 diabetes, the initial secretion of insulin following an intravenous glucose challenge was significantly elevated by 34% accompanied by nonsignificant reductions (24%) in insulin resistance [25]. Even though 25(OH)D concentrations increased [25], longer durations are required in adults to arrive at concentrations of greater than 75 nmol/L which are thought to enhance glucose homeostasis [26]. Only one study has included measures of vitamin D status and osteocalcin in relation to insulin resistance and observed no interrelationships [5]; however, vitamin D status was not reported. Recently, we reported that compared to white women, Canadian Aboriginal women have lower vitamin D status, higher parathyroid hormone and a trend for lower osteocalcin values [27]. Aboriginal women also have higher indices of obesity and central adiposity [28] that may explain the predisposition to insulin resistance and diabetes [5]. This study explored the relationships among osteocalcin and vitamin D status with indicators of glucose metabolism and adiposity in Aboriginal and white adult women.

2. Methods and materials

2.1. Study population

The study population consisted of 368 urban Aboriginal and white females stratified by age (25–39 years, 40–59 years, 60–75 years) (Table 1). Study recruitment was conducted from June 2002 through March 2004. A small subgroup of rural Aboriginal women (N=42) did not undergo total body measurements since no equipment was available in the rural communities and are excluded from this report. Likewise, this data set only includes women with a complete data set for all biochemistry and measures of adiposity. The University of Manitoba Research Ethics Board approved this study.

Women for the urban cohort (operationally defined as current residence within 50 km of the provincial capital, Winnipeg, Manitoba) were selected randomly from the Manitoba Health population registry. Aboriginal ethnicity was determined from the Canadian government's 1994–1999 Status Verification System maintained by First Nations and Inuit Health Branch and Indian and Northern Affairs Canada, with the presence of a treaty status code in the provincial health registry file providing a secondary indicator of Aboriginal ethnicity as described in detail in Leslie et al. [29].

2.2. Measurements

After enrolment, consenting subjects completed detailed baseline measurements that included an interviewer-administered subject questionnaire, and fasting biochemical markers of bone and glucose metabolism. Weight was measured without shoes to the nearest 0.5 lb with a portable digital scale (Tanita TBF-612) and subsequently converted to kilogram. Height was measured to the nearest 0.1 cm with a Harpenden pocket stadiometer (Holtain Ltd, Crosswell, United Kingdom). BMI (kg/m^2) was calculated as weight (kg) divided by height (m) squared.

Table 1
Characteristics of white and Aboriginal women

Variable	All (n=368)	White (n=181)	Aboriginal (n=187)
Age (years)	45.3±13.6	47.2±14.7	43.5±12.2 **
Height (m)	162.9±6.2	163.5±6.1	162.3±6.2
Weight (kg)	77.8±17.7	76.1±18.0	79.5±17.3
BMI (kg/m^2)	29.3±6.5	28.5±6.8	30.1±0.5 **
DXA			
Total fat (kg)	29.6±10.9	28.3±11.4	30.8±10.3 *
Trunk fat (kg)	15.3±6.4	13.8±6.3	16.7±6.2 **
Fat (%)	36.9±6.4	35.9±7.0	37.9±5.7 **
Plasma			
Osteocalcin (mmol/L)	144.7±64.4	151.0±68.2	138.7±60.2
25(OH)D (nmol/L)	62.6±29.3	70.5±31.0	55.0±25.4 **
Glucose (mmol/L)	5.6±1.9	5.3±1.2	5.9±2.3 **
Insulin (μU/ml)	10.4±9.4	8.9±8.0	11.8±10.4 **
C-Peptide (ng/ml)	2.0±1.0	1.9±0.9	2.2±1.1 **
HbA1c (%)	6.1±1.4	5.9±1.0	6.2±1.7 *
HOMA-IR	2.9±4.9	2.4±4.3	3.4±5.4 *

Data are mean±S.D.
* P<.05.
** P<.01 using t tests for white vs. Aboriginal.

All urban subjects underwent total body scanning with a dual-energy X-ray absorptiometry (DXA) device (Hologic QDR-4500W; Waltham, MA, USA). All results were reviewed by a single study investigator with extensive experience in clinical and research DXA (W.D.L.). Scans with major artifacts (e.g., surgical implants) or scanning errors (e.g., mispositioning) were excluded. The total body scan provides a rapid and precise noninvasive measure of total body and regional body composition as the following three components: bone mineral mass, fat mass and lean tissue mass [12]. For this report, total body fat (kg and %) and trunk fat (kg) values were analyzed. Although DXA measures of trunk fat mass reflect fatty elements in soft tissue as well as adipose tissue in subcutaneous and visceral depots, DXA-derived measures account for 80% of the variation in intraabdominal fat as measured by computed tomography (CT) [13] and explain 79% of the variance in insulin sensitivity [14]. Mean values of total abdominal fat do not significantly differ from those obtained with CT [15].

2.3. Biochemistry

A morning blood sample was taken for the purpose of assessing biomarkers of bone and glucose metabolism, and serum was stored at −70°C until analyzed. It is accepted that the best indicator of vitamin D status is serum or plasma 25(OH)D [30]. Serum 25(OH)D was measured using a radioimmunoassay that equally detects D_2 and D_3 isomers (Diasorin Inc., Stillwater, MN, USA) with interassay precision coefficient of variation of 6%–13 %. Serum glucose, hemoglobin A1c (HbA1c) and osteocalcin [1–43] were measured using a Roche Modular system (Roche Diagnostics, Montreal, Canada). Serum insulin and C-peptide were measured on an Immulite analyzer. HOMA-IR was calculated as the product of the fasting insulin (μU/ml) and plasma glucose concentration (mmol/L) divided by 22.5 [31].

2.4. Statistical analysis

Normality of the data was tested using the method of Kolmogorov and Smirnov. With the exception of glucose and HbA1c, all other biochemical values were nonnormally distributed and were log transformed prior to analysis. Differences in measures of adiposity and glucose metabolism were compared between ethnic groups using t test for continuous data. Based on plasma osteocalcin and 25(OH)D quartiles, data for indices of glucose metabolism were analyzed using mixed-model analysis of variance (ANOVA) with fixed effects of quartiles, ethnicity and random effects of age in the model followed by post hoc Bonferroni multiple comparisons testing. Pearson correlation was followed by multiple regression analyses to explore if both osteocalcin and vitamin D were related to the parameters of glucose metabolism while accounting for age, adiposity and ethnicity. Assumptions of the regression models were tested for random residuals and normality using quantile–quantile plots; all models were improved when the dependent variable was log transformed. Interactions among the independent variables were explored for each parameter of glucose metabolism. Data are presented as mean (S.D.) values unless otherwise stated, and significance was accepted at P≤.05.

3. Results

The study population consisted of 187 urban Aboriginal and 181 urban white women. Aboriginal women were slightly younger with significantly greater BMI and measures of body fat than white women (Table 1). Osteocalcin values were not different between groups

(*P*=.067), while 25(OH)D was significantly lower in Aboriginal women. All measures of glucose metabolism were higher in Aboriginal women.

Using osteocalcin quartiles, there were main effects of ethnicity for all five glucose parameters with higher values in Aboriginal women (Fig. 1). However, for glucose and HbA1c, no differences were observed among quartiles within white women, whereas quartile 1 of the Aboriginal women had the highest values compared to all other groups. For HOMA-IR, higher values were observed in quartile 1 compared to quartiles 3 and 4 for white women, but no differences were observed among quartiles for Aboriginal women. Both C-peptide and insulin were not different among quartiles for white and Aboriginal women. When quartiles were constructed using plasma 25(OH)D, there were main effects of ethnicity for all five glucose parameters, with higher values in Aboriginal women (Fig. 2). Main effects of quartile were observed for HOMA-IR, with the lowest values observed in quartile 3 of white women, and similarly, for C-peptide, the lowest values were observed for quartiles 3 and 4. No effect of plasma 25(OH)D quartile was observed for HbA1c. An interaction effect between quartile and ethnicity for insulin showed that, within white women, quartiles 3 and 4 had lower values compared to quartiles 1 and 2, but this was not observed within Aboriginal women.

To explore relationships between osteocalcin and 25(OH)D with measures of adiposity and glucose metabolism, Pearson correlation analyses were conducted (Table 2). Osteocalcin was inversely associated with body weight, BMI, body fat measures, glucose, insulin, C-peptide, HbA1c and HOMA-IR. Plasma 25(OH)D was also inversely associated with weight and all measures of adiposity but was only related to insulin, C-peptide and HOMA-IR. Osteocalcin and 25(OH)D were not correlated. To examine if both osteocalcin and 25(OH)D

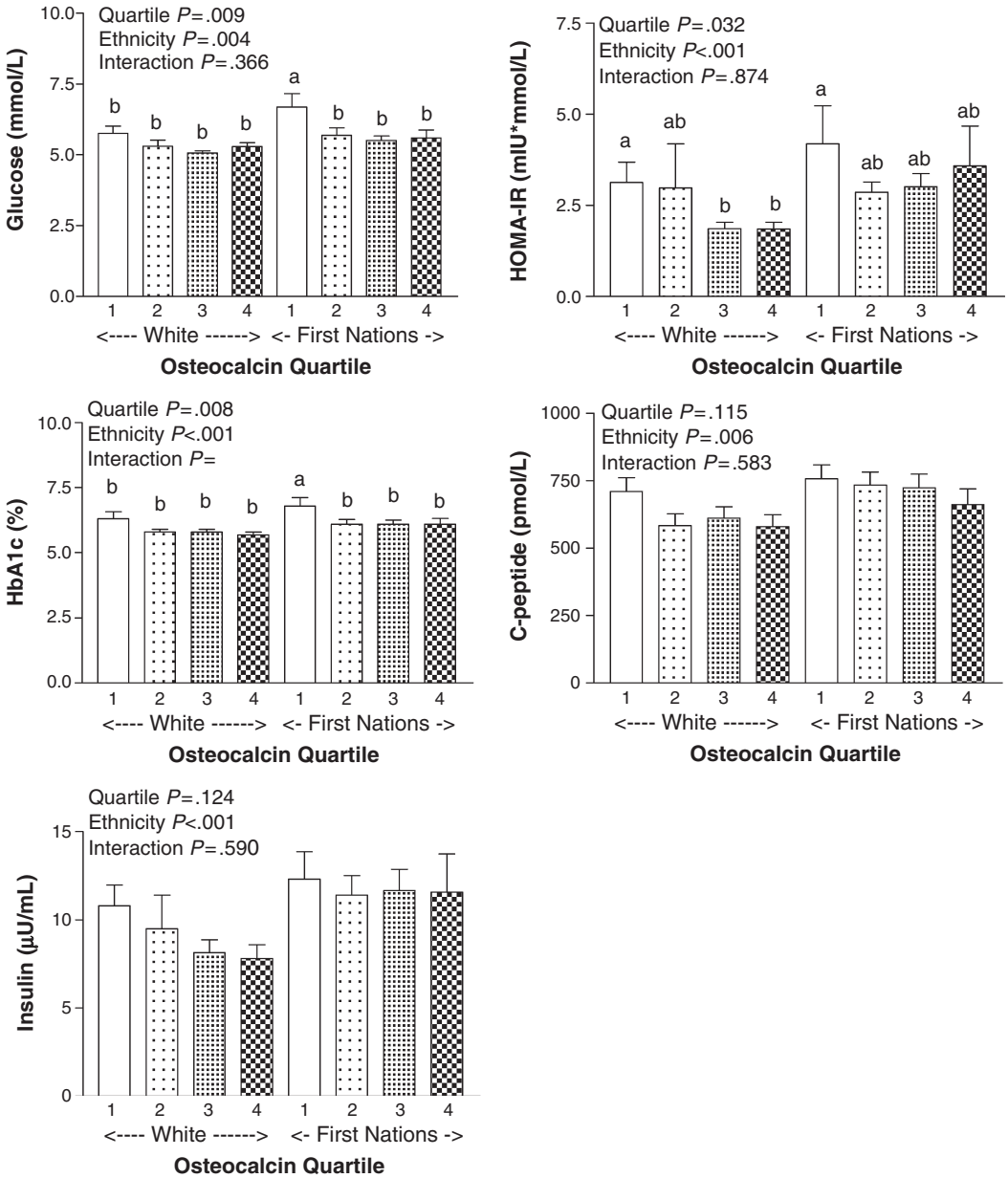

Fig. 1. Parameters of glucose metabolism according to osteocalcin quartile. Quartile 1: 31 to 105, quartile 2: 105 to 133, quartile 3: 133 to 171, quartile 4: 171 to 528 mmol/L of osteocalcin. Values are mean±S.E.M., and different superscript letters indicate significant differences; $P<.05$ using mixed-model ANOVA and random effects of age; $n=92$ per quartile. Data were log transformed prior to analysis.

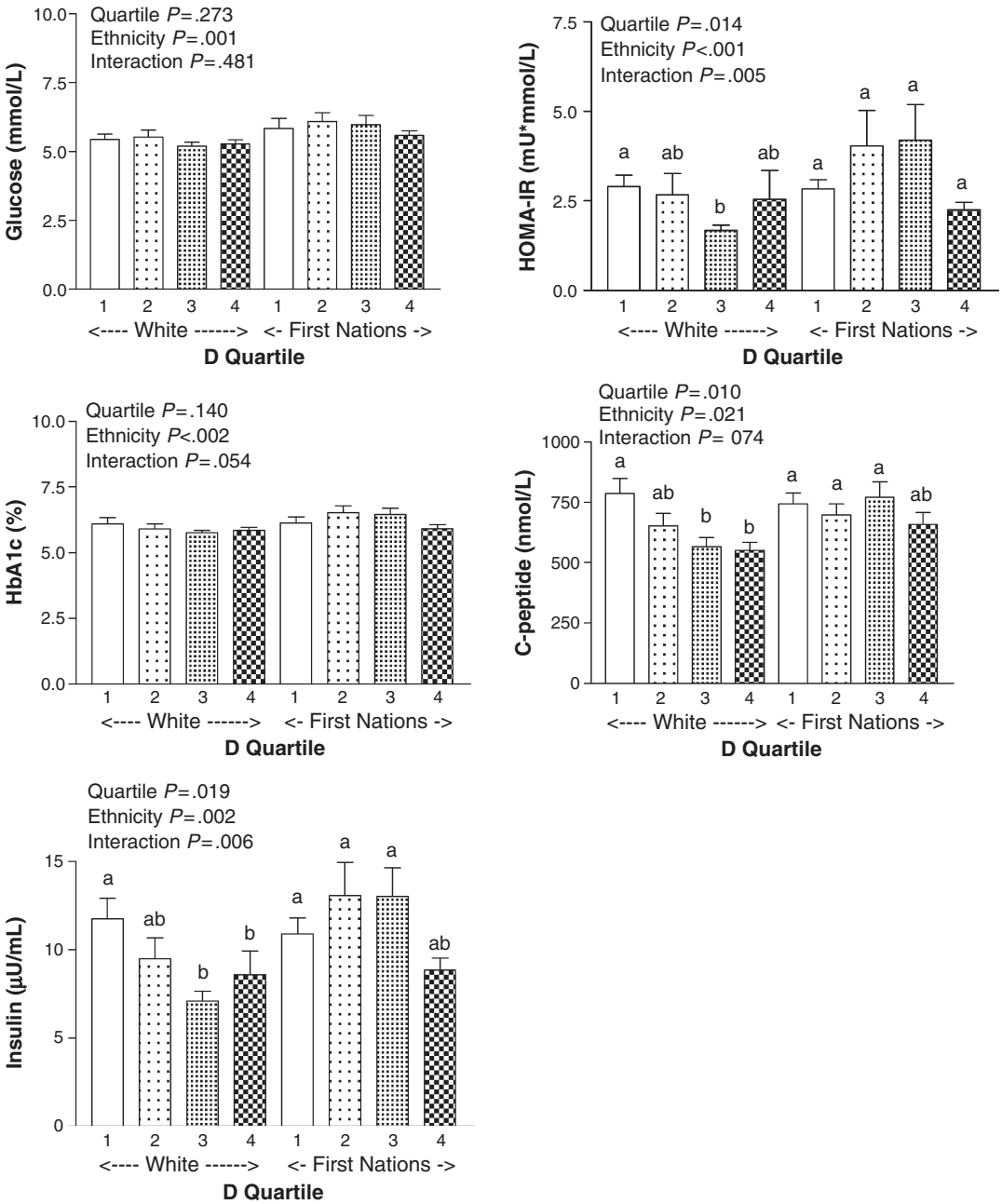

Fig. 2. Parameters of glucose metabolism according to 25(OH)D quartile. Quartile 1: 15 to 40, quartile 2: 40 to 57, quartile 3: 57 to 80, quartile 4: 80 to 179 nmol/L of 25(OH)D. Values are mean±S.E.M., and different superscript letters indicate significant differences; $P<.05$ using mixed-model ANOVA and random effects of age; $n=92$ per quartile. Data were log transformed prior to analysis.

were significantly related to the parameters of glucose metabolism, multiple linear regression (Table 3) was conducted. After accounting for other variables, osteocalcin was significantly related to glucose, HbA1c and HOMA-IR, whereas 25(OH)D was significantly related to glucose, C-peptide, HbA1c and HOMA-IR. Aside from HOMA-IR, substituting DXA measures of adiposity for BMI did not improve model fit. Neither osteocalcin nor 25(OH)D remained in the model for insulin. No significant interactions were observed.

4. Discussion

This report confirms that carboxylated osteocalcin and 25(OH)D are related to glucose metabolism in a group of mixed-ethnicity adult women, even after accounting for age and measures of adiposity. Previously, such a relationship has been observed for osteocalcin in white men and women [2,8] and, more recently, in Asians [5,32]. Values for HOMA-IR and glucose according to our highest and lowest quartiles of osteocalcin were both lower than those similarly reported in men [5], suggesting that osteocalcin has a relationship across a wide range of glucose homeostasis in both men and women. These relationships are also observed in healthy men with low HOMA-IR values ~1.5 [6]. In addition to our complementary observations for osteocalcin, to our knowledge, our study is the first to report in the same cohort significant relationships between vitamin D status and HOMA-IR plus indices of insulin metabolism. By comparing ethnic groups in our study, we were able to confirm that all markers of

Table 2
Correlation of plasma osteocalcin and 25(OH)D with adiposity and glucose metabolism

Variable	Osteocalcin (log mmol/L)		25(OH)D (log nmol/L)	
	r	*P* value	*r*	*P* value
Weight (kg)	−0.25	<.0001	−0.21	<.0001
BMI (kg/m^2)	−0.26	<.0001	−0.22	<.0001
DXA				
Total fat (kg)	−0.24	<.0001	−0.22	<.0001
Trunk fat (kg)	−0.25	<.0001	−0.26	<.0001
Fat (%)	−0.19	0.0002	−0.18	<.0001
Plasma				
osteocalcin (log mmol/L)	–	–	−0.05	.36
25(OH)D (log nmol/L)	−0.05	0.36	–	–
Glucose (mmol/L)	−0.20	.0001	−0.08	.12
Insulin (log μU/ml)	−0.17	.0009	−0.19	.0002
C-Peptide (log ng/ml)	−0.15	.0042	−0.21	<.0001
HbA1c (%)	−0.23	<.0001	−0.08	.11
HOMA-IR (log)	−0.21	<.0001	−0.19	.0003

Data are Pearson correlation coefficients. All biochemistry data were log transformed prior to analysis except for glucose and HbA1c; n=368.

glucose metabolism were higher in Aboriginal than white women, consistent with the higher risk for development of diabetes [33]. However, the relationships between osteocalcin and vitamin D with glucose metabolism were evident in both ethnic groups as shown in the regression analyses.

Clinical criteria for normal glucose metabolism includes blood glucose <5.5 mmol/L and, for those with established diabetes, glucose 4 to 7 mmol/L and HbA1c <7% [34]. The American Diabetes Association now endorses HbA1c as one of four options for the diagnosis of diabetes with a recommended cut point above 6.5% [35]. The majority of our participants had fasting glucose (73% <5.5 mmol/L) and HbA1c (88.3% <7%) within these targets. Although the quartile of lowest osteocalcin had mean HbA1c of <7%, mean glucose values were mildly hyperglycemic, with lower values observed across quartiles in the Aboriginal women. The mechanism by which osteocalcin enhances glucose disposal is proposed as a feedback loop [36]. Indeed, a number of studies report low serum osteocalcin concentration in patients with type 2 diabetes [37–41] and increases with improvement of glycemic status [42,43]. Osteocalcin concentration was lower in postmenopausal women with type 2 diabetes compared to women with impaired fasting glucose or normal glucose [10]. In the latter study, there was an inverse correlation between osteocalcin concentration and fasting glucose (r=−0.20), fasting insulin (r=−0.13), HbA1c (r=−0.22), insulin resistance (HOMA-IR, r=−0.016) and BMI (r=−0.11) and positive association with age (r=0.15). The magnitude of these associations closely parallels that observed in our study.

Similarly, in a longitudinal study in men and women without diabetes, serum osteocalcin was positively linked to insulin sensitivity (r=0.23), and this association was stronger among lean subjects. After adjustment for age and BMI, serum osteocalcin had the strongest relationship to insulin sensitivity among lean subjects [2]. In a cross-sectional study on nondiabetic lean men, there was a negative association between serum osteocalcin and insulin secretion (r=−0.41). Serum osteocalcin was negatively associated with insulin resistance (HOMA-IR) (r=−0.43). However, there was no association observed between change in serum osteocalcin and insulin resistance during weight loss, but baseline serum osteocalcin levels were associated with insulin sensitivity and secretion [2]. In Chinese men and women, osteocalcin is also inversely associated with diabetes and glucose metabolism [32], and in Swiss men, osteocalcin is inversely associated with glucose and fat mass [8]. Another report in postmenopausal women also observed negative associations between osteocalcin and glucose, HbA1c, HOMA-IR and BMI [10]. Osteocalcin was related to glucose and HbA1c after adjustment for age and BMI in multivariate analyses, although the HbA1c values were lower than ours. Since these reports were of people without diabetes and the relationships were similar to our study, the possibility that some of the women could have undiagnosed diabetes at the time of the study was not an underlying confounder responsible for the linkages between osteocalcin and markers of glucose metabolism.

In contrast to osteocalcin, vitamin D status more strongly associated with measures of insulin metabolism than glycemia. This is consistent with previous work that demonstrated that short-term treatment with 1,25(OH)$_2$D in adults with diabetes and managed without insulin improved insulin and C-peptide [44]. Similarly, *in vitro*, 1,25(OH)$_2$D stimulates insulin secretion in rat beta-cells [45]. The presence of vitamin D receptors in pancreas and the production of 1,25(OH)$_2$D [22,46] imply a fundamental role for vitamin D in the endocrine pancreas. Few studies have focused on mechanisms, but one using a vitamin D analogue (low calcemia) in culture demonstrated greater intracellular calcium concentration and insulin release [47]. In response to glucose, beta-cells cultured with 1,25(OH)$_2$D increase calcium concentration through opening of calcium channels

Table 3
Significantly related variables in multiple linear regression of parameters of glucose metabolism in adult white and Aboriginal women

Parameter	R^2	Variable	Estimate	95%CI	*P* value
Glucose (log mmol/L)	0.18	Constant	0.930	0.782, 1.079	<.0001
		Age (year)	0.002	0.002, 0.003	<.0001
		Ethnicity (0=white)	0.033	0.014, 0.052	.0008
		Osteocalcin (log mmol/L)	−0.108	−0.161, −0.055	<.0001
		25(OH)D (log nmol/L)	−0.051	−0.098, −0.004	.032
Insulin (log μU/ml)	0.37	Constant	0.157	0.051, 0.263	.004
		Ethnicity (0=white)	0.066	0.020, 0.112	.005
		BMI (kg/m^2)	0.025	0.021, 0.028	<.0001
C-peptide (log pmol/L)	0.31	Constant	2.457	2.258, 2.655	<.0001
		BMI (kg/m^2)	0.017	0.014, 0.020	<.0001
		25(OH)D (log nmol/L)	−0.105	−0.196, −0.013	.026
HbA1c (log %)	0.20	Constant	0.961	0.844, 1.078	<.0001
		Age (y)	0.002	0.001, 0.003	<.0001
		Ethnicity (0=white)	0.024	0.009, 0.039	.002
		Osteocalcin (log mmol/L)	−0.101	−0.143, −0.059	<.0001
		25(OH)D (log nmol/L)	−0.041	−0.078, −0.004	.028
HOMA-IR (log)	0.28	Constant	0.171	−0.373, 0.715	<.0001
		Age (y)	0.002	−0.001, 0.004	.132
		Ethnicity (0=white)	0.078	0.017, 0.138	.013
		Fat (%)	0.021	0.016, 0.026	<.0001
		Osteocalcin (log mmol/L)	−0.229	−0.399, −0.058	.009
		25(OH)D (log nmol/L)	−0.158	−0.310, −0.006	.041

[48]. This is somewhat consistent with our regression observations using C-peptide since values were lower with better vitamin D status. Thus, there is growing evidence to suggest that vitamin D has a direct role in the endocrine pancreas.

Vitamin D status and osteocalcin were however significantly associated with all indices of adiposity, and adiposity in turn was positively associated with adverse glucose metabolism. These observations suggest an indirect relationship of vitamin D with glucose metabolism that may simply be a reflection of how increasing fat mass sequesters vitamin D, resulting in lower circulating 25(OH)D [49]. Many of our study participants were obese (44% had BMI ≥30 kg/m^2), and of these, 80% had plasma 25(OH)D below the suggested optimal target of 75 nmol/L. Therefore, we conducted multiple linear regression analysis and demonstrated that, after accounting for age, ethnicity and/or adiposity, both osteocalcin and 25(OH)D were inversely related to glucose, HbA1c and HOMA-IR, whereas only 25(OH)D was related to C-peptide. Based on the regression equations, for each 50-nmol/L increase in 25(OH)D, glucose will decline by 0.6 mmol/L, HbA1c will decline by 0.9%, and HOMA-IR will decline by 0.6 unit. Similarly, for each 50-mmol/L increase in osteocalcin, glucose declines by 0.5 mmol/L, HbA1c declines by 0.5% and HOMA-IR declines by 0.3 unit. Additionally, for each 50-nmol/L increase in 25(OH)D, C-peptide would increase by ~100 pmol/L. Further research is required to establish ideal targets of 25(OH)D to improve or maintain glucose metabolism in both healthy and obese people. Such intervention is now under way in adults with diabetes [50].

There are limitations to our study, including the fact that diabetes is underdiagnosed in the general population and that Aboriginal women are at particularly high risk [33]. Vitamin D intakes in our study (<15 μg/day) [51] are also below suggested targets (25 μg/day) needed to achieve a recommended level of 75 nmol/L 25(OH)D [52]. With our cross-sectional analyses, it is unclear if improving vitamin D status might confer benefit to glucose metabolism. Controlled studies are warranted to establish this relationship. Additionally, our measure of osteocalcin was confined to the carboxylated isoform, and we did not conduct measures of insulin resistance such as oral glucose tolerance testing. Finally, the mechanism(s) and feedback loops by which both osteocalcin and vitamin D status influence glucose homeostasis remain unclear. Future studies should incorporate biochemical measures of cortisol and 1,25(OH)$_2$D to help guide research regarding mechanisms of action.

In summary, in a group of adult Canadian women of white and Aboriginal ancestry, osteocalcin and vitamin D status are related to biomarkers of glucose metabolism after accounting for age, ethnicity and adiposity. One randomized controlled trial designed to establish the benefits of vitamin D and calcium supplementation on bone mass also suggests that glucose disposal is improved in white men and women [11]. Therefore, future studies of vitamin D supplementation in Aboriginal women to improve vitamin D status should include measures of osteocalcin and glucose tolerance.

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