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LABORATORY STUDY

Carboxylated and intact osteocalcin predict adiponectin concentration in hemodialyzed patients

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ABSTRACT

Purpose Disrupted bone metabolism in patients with chronic kidney disease (CKD) is associated with elevated concentrations of biochemical bone markers. Recently, animal studies show the role of osteocalcin in energy metabolism, which is partially confirmed in humans. The aim of our study was to evaluate the relationships between serum concentrations of bone markers and indices of energy metabolism in CKD patients on maintenance hemodialysis; in particular, the relationship between various forms of osteocalcin and adiponectin. **Patients and methods** The cross-sectional study included 155 hemodialyzed stage 5 CKD patients. Serum concentrations of glucose, insulin, adiponectin, bone alkaline phosphatase (bALP), tartrate resistant acid phosphatase (TRAP), carboxylated (cOC), undercarboxylated (ucOC), and intact osteocalcin (OC) were determined. **Results** In total cohort, bALP, TRAP, cOC, and ucOC negatively correlated with BMI. All analyzed bone markers positively correlated with adiponectin in total cohort and in men. In multiple linear regression analysis including all patients, log(cOC) and log(intact OC) were the only bone markers that predicted log(adiponectin) (beta = 0.22; $p = 0.016$ and beta = 0.26; $p = 0.010$) independently of sex, dialysis vintage, CRP, insulin, iPTH concentrations, BMI, and age. **Conclusions** Our data confirm the positive association between cOC, intact OC, and adiponectin concentrations in CKD patients on maintenance hemodialysis.

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Introduction

Renal osteodystrophy associated with chronic kidney disease (CKD) is characterized by deterioration of bone metabolism resulting in dysregulation of bone resorption and bone formation.[1] The consequence of metabolic bone disease is elevation of serum bone marker concentrations including bone alkaline phosphatase (bALP) (which reflects bone forming activity) and tartrate resistant acid phosphatase (TRAP) (considered as a marker of bone resorption). Osteocalcin (OC), also called bone Gla protein, is regarded as a biochemical marker of bone metabolism, and correlates with osteoblast activity.[2,3] Like other Gla proteins, OC undergoes γ-carboxylation in a vitamin K dependent manner. It carries three carboxylated glutamic acid (Gla) residues at positions 17, 21, and 24, known to mediate strong binding of OC to hydroxyapatite.[2,4] Therefore, for a long time OC was believed to regulate and enable mineralization of extracellular bone matrix.[2,3]

However, as recently shown in animal experiments, OC does not influence neither bone turnover rate nor bone mineralization, but may act directly as a circulating hormone involved in regulation of glucose metabolism and adipose tissue mass.[5,6] Moreover, in mice, OC increases pancreatic β-cell proliferation and insulin secretion, as well as expression and release of adiponectin from adipocytes, increasing insulin sensitivity, and energy utilization.[7] These findings were supported by the beneficial effect of OC on the progression of metabolic disease, obesity, and type 2 diabetes mellitus (T2DM) in wild mice. In animal studies, the undercarboxylated form of OC (ucOC), but not the fully carboxylated one (cOC), is an active hormone. Biological activity of OC is diminished with higher extent of γ-carboxylation.[7]

Adiponectin is strongly expressed in visceral adipose tissue and bone marrow, and its concentration is decreased in obesity, T2DM, cardiovascular disease (CVD), and dyslipidemia.[8] Animal and clinical data

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Table 1. Demographic, clinical, and biochemical characteristics of patients.

	Entire group ($n = 155$)	Women ($n = 66$)	Men ($n = 89$)	p-Value
Age, years	58 ± 14	59 ± 16	58 ± 13	0.7
Dialysis vintage, months	60 (31-111)	48 (25-96)	67 (36-115)	0.1
BMI, kg/m^2	23.7 (21.0-25.8)	21.9 (19.8-25.3)	24.8 (22.6-26.8)	<0.001
Premenopausal women, *n* (%)	5 (3)	5 (8)	-	-
Hypertension, *n* (%)	114 (74)	47 (71)	67 (75)	0.6
Diabetes mellitus type 2, *n* (%)	20 (13)	8 (12)	12 (13)	0.8
Ischemic heart disease, *n* (%)	75 (48)	35 (53)	40 (45)	0.3
Current smoking, *n* (%)	49 (32)	13 (20)	36 (40)	0.006
Hemoglobin, g/dL	11.5 ± 2.5	11.2 ± 1.8	11.7 ± 2.6	0.2
Albumin, g/L	37.9 ± 3.5	37.6 ± 3.5	38.2 ± 3.5	0.3
C-reactive protein, mg/L	4.34 (1.86-10.70)	4.49 (1.58-10.70)	4.16 (2.08-10.70)	0.7
Total cholesterol, mmol/L	4.78 ± 1.20	5.12 ± 1.37	4.52 ± 0.99	0.002
LDL-cholesterol, mmol/L	2.72 ± 0.90	2.91 ± 1.00	2.57 ± 0.79	0.1
HDL-cholesterol, mmol/L	1.15 ± 0.34	1.28 ± 0.36	1.05 ± 0.29	0.004
Triglycerides, mmol/L	1.88 (1.22-2.86)	1.91 (1.21-2.77)	1.86 (1.36-2.87)	0.8
Fasting glucose, mmol/L	4.63 (4.23-5.30)	4.56 (4.23-5.16)	4.76 (4.23-5.30)	0.8
Insulin, μIU/mL	11.63 (7.53-14.19)	9.55 (6.78-13.76)	10.94 (7.76-17.20)	0.1
HOMA IR	2.13 (1.42-3.06)	1.99 (1.28-2.79)	2.25 (1.56-3.43)	0.2
Adiponectin, μg/mL	13.40 (9.26-19.16)	17.36 (10.75-21.49)	11.88 (8.34-16.91)	0.002
iPTH, pg/mL	408 (135-844)	514 (216-1156)	350 (132-687)	0.027
cOC, ng/mL	105.2 (65.7-191.2)	113.0 (69.0-222.9)	102.6 (65.2-168.3)	0.2
ucOC, ng/mL	32.5 (12.1-89.6)	44.4 (17.0-119.1)	23.25 (8.97-70.70)	0.003
Intact OC, ng/mL	72.4 (44.8-142.7)	95.5 (54.4-155.9)	67.3 (33.9-118.7)	0.043
bALP, U/L	54.1 (36.7-89.8)	64.6 (41.8-135.7)	44.5 (33.7-78.2)	0.002
TRAP, U/L	10.22 (7.30-14.83)	13.35 (8.31-16.46)	8.95 (6.98-13.42)	0.001

Notes: BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; iPTH, intact parathormone; HOMA IR, homeostasis model assessment-insulin resistance; cOC, carboxylated osteocalcin; ucOC, undercarboxylated osteocalcin; bALP, bone alkaline phosphatase; TRAP, tartrate resistant acid phosphatase.

revealed that adiponectin regulates energy homeostasis by suppressing hepatic gluconeogenesis, stimulating fatty acid oxidation, and lowering triglyceride concentration in liver and skeletal muscles and by enhancing glucose uptake in skeletal muscles.[9,10]

The relationship between bone osteocalcin release and energy metabolism has been well documented in both *in vitro* and *in vivo* animal experiments, but has not been fully confirmed in humans. Numerous clinical observations acknowledged the role of OC as a hormone regulating energy metabolism; however, most of them concerned patients with altered glucose metabolism. Serum OC was significantly reduced in T2DM patients compared to non-diabetic controls and negatively correlated with body mass index (BMI), adipose tissue mass, glucose, homeostasis model assessment index of insulin resistance (HOMA IR), and glycated hemoglobin (HbA_1C), while positively correlated with adiponectin.[11–18]

The aim of our study was to evaluate the relationships between concentrations of bone markers (intact OC, cOC, ucOC, bALP, TRAP), and indices of energy metabolism (fasting glucose, insulin and adiponectin concentrations, calculated HOMA IR, and BMI) in stage 5 CKD patients treated with maintenance hemodialysis. Also, we attempted to explore whether OC (as a bone hormone or as a bone metabolism marker) correlates with adiponectin. Finally, we analyzed whether sex has an impact on these relationships.

Subjects and methods

Patients and blood samples

We recruited 155 CKD patients, undergoing maintenance hemodialysis, at the age of 58 ± 14 years (66 women, 89 men); median hemodialysis vintage was 59 (51-68) months. The demographic and clinical data are shown in Table 1. Data on co-morbidities (hypertension, diabetes, and ischemic heart disease) were collected based on patients' records. Data on menopausal status and smoking status were based on the interview.

Blood samples for the measurement of blood hemoglobin and serum concentrations of albumin, glucose, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol, triglycerides, C-reactive protein (CRP), and intact parathyroid hormone (iPTH) were obtained at the initiation of the mid-week hemodialysis session, in the morning hours (between 6:00 am and 9:00 am), after overnight fast. These measurements were performed using standard laboratory methods. PTH was determined using second-generation iPTH assay. Separate fasting blood samples for the measurement of serum concentrations of insulin, adiponectin, bALP, TRAP, cOC, ucOC, and intact OC were

obtained simultaneously (between 6:00 am and 9:00 am). Samples were centrifuged at 3000 × *g*, after which serum was separated and kept in aliquots at −75 °C until assayed (not longer than 6 months).

The results of laboratory tests in hemodialyzed patients were compared with the reference values provided by the laboratory for the routine tests and by the manufacturers of the immunochemical tests described below. Serum cOC and ucOC concentrations were also measured in samples from 36 healthy individuals (mean age: 58 ± 8; 16 women and 20 men) in order to evaluate the reference values for the parameters.

The study was approved by the Jagiellonian University Bioethics Committee and all patients provided informed consent for participation.

Immunochemical methods

Serum concentration of insulin was measured using the enzyme linked immunosorbent assay (Insulin, Dako Cytomation Ltd, Ely, UK). The detection limit for this assay was 0.5 μU/mL with the intra- and inter-assay precision (coefficients of variation) 7.5 and 9.3%, respectively. Adiponectin was measured using quantitative sandwich enzyme immunoassay technique (Human Total Adiponectin Quantikine ELISA, R&D Systems, Abingdon, UK). The detection limit for this assay was 0.246 ng/mL with the intra- and inter-assay precision 5.0 and 7.9%, respectively. bALP activity was measured with the enzyme immunoassay (METRA^{TM} BAP Kit, Quidel Corporation, San Diego, CA). The detection limit for this assay was 0.7 U/L with the intra- and inter-assay precision 5.8 and 7.6%, respectively. Serum activity of TRAP was measured by immunocapture enzyme-activity assay (Human TRAP 5 Assay, BioVendor Laboratory Medicine Inc., Brno, Czech Republic). The detection limit for this assay was 0.1 U/L with the intra- and inter-assay precision 3.7 and 7.1%, respectively.

Fully carboxylated (Gla-type) osteocalcin (cOC) serum concentrations were measured using an enzyme immunoassay kit (Gla-type Osteocalcin EIA, Takara Bio Inc., Otsu, Japan). The detection limit was 0.5 ng/mL with the intra- and inter-assay precision 2.4 and 4.8%, respectively. Undercarboxylated (Glu-type) osteocalcin (ucOC) serum concentrations were measured using an enzyme immunoassay kit (Glu-type Osteocalcin EIA, Takara Bio Inc., Otsu, Japan). The detection limit for this assay was 0.25 ng/mL with the intra- and inter-assay precision 6.7 and 9.9%, respectively. Intact osteocalcin was measured using an enzyme immunoassay kit (MicroVue Osteocalcin EIA, QUIDEL Corporation, San Diego, CA). The detection limit was 0.45 ng/mL with the intra- and inter-assay precision 10.0 and 8.8%, respectively.

Calculations

HOMA IR was calculated according to the following formula: HOMA IR = fasting glucose concentration (mmol/L) × fasting insulin concentration (μU/mL)/22.5. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$.

Statistical analysis

The number of patients and percentage was reported for categorical variables and mean ± SD or median (lower – upper quartile) for continuous variables with normal or non-normal distribution, respectively. The Shapiro-Wilk test was used to assess normality. Contingency tables were analyzed with the Pearson chi-squared test. The differences between groups were tested with *t*-test or Mann-Whitney test, as appropriate. Pearson coefficient was calculated to assess the correlations, after log-transformation of right-skewed variables. The multiple regression models were constructed using independent variables that significantly correlated with the dependent variable in simple analysis and the pre-specified confounders, i.e. log(iPTH) and age. The results were considered significant at $p \leq 0.05$. The computations were performed using Statistica10.0 software package (StatSoft, Tulsa, OK).

Results

Study population's characteristics and the evaluation of studied parameters in regard to sex

Table 1 summarizes the demographic, clinical, and biochemical characteristics of the participants. Despite the fact that patients were normoglycemic and the majority had normal insulin concentrations (i.e., 2.0–25.0 μIU/mL), we noted elevated HOMA IR values. The concentrations of adiponectin in the majority of hemodialyzed patients were within the reference interval (i.e., 0.865–21.42 μg/mL). However, the mean adiponectin concentration in hemodialyzed patients (14.92 μg/mL) was more than two times higher than the mean reported by the manufacturer of the test for healthy subjects (i.e., 6.64 μg/mL). Bone turnover markers bALP and TRAP were slightly elevated (reference values: 11.0–41.0 U/L and 2.67–6.47 U/L, respectively), while cOC, ucOC, and intact OC concentrations were several times higher than the manufacturers' reference values of 11.9–27.1 ng/mL,1.2–10.5 ng/mL, and 3.7–10.0 ng/mL, respectively (Table 1). Also, cOC and ucOC in hemodialyzed patients were significantly higher than the concentrations observed by us in 36 healthy subjects, i.e., 18.4 (16.7–20.5) and 4.49 (2.79–6.59) ng/mL, respectively

Table 2. Simple correlations between log(adiponectin) and the markers of bone metabolism.

	Entire group (n=155)		Women (n=66)		Men (n=89)	
	r	p-Value	r	p-Value	r	p-Value
log(cOC)	0.29	<0.001	0.26	0.034	0.30	0.004
log(ucOC)	0.23	0.004	0.11	0.4	0.24	0.024
log(intact OC)	0.31	<0.001	0.20	0.1	0.34	0.001
log(bALP)	0.28	<0.001	0.11	0.4	0.37	<0.001
log(TRAP)	0.30	<0.001	0.17	0.2	0.33	0.002

Note: log, natural logarithm; see Table 1.

(p<0.001 for both variables). Sex-specific differences included lower BMI values, higher total and HDL-cholesterol, lower quantity of active smokers, higher adiponectin, iPTH, and bone markers: ucOC, intact OC, bALP, and TRAP concentrations in women (Table 1).

Relationships between the concentrations of bone markers and metabolism indices

We found no correlations between the concentration of glucose or insulin as well as HOMA-IR values and the studied markers of bone turnover (cOC, ucOC, intact OC, bALP, TRAP). BMI negatively correlated with cOC, ucOC, bALP, and TRAP (r=−0.20, p=0.012; r=−0.17, p=0.039; r=−0.21, p=0.013, and r=−0.32, p<0.001, respectively, for log-transformed variables), but not with intact OC (r=−0.16, p=0.052). Moreover, cOC, ucOC, intact OC, bALP, and TRAP concentrations were positively correlated with adiponectin (Table 2).

Interestingly, all the bone markers significantly correlated with adiponectin in men, but only cOC correlated with adiponectin in women (Table 2).

Relationships between bone markers and adiponectin after adjustment for other predictors of adiponectin concentrations

Except for the associations mentioned above, the variables significantly correlated with log(adiponectin) in the studied group included log(BMI) (r=−0.44, p<0.001), log(dialysis vintage) (r=0.17, p=0.041), log(CRP) (r=−0.16, p=0.046), log(insulin) (r=−0.32, p=0.001), log(fasting glucose) (r=−0.19, p=0.031) and log(HOMA IR) (r=−0.29, p=0.002).

We performed multiple regression analysis in order to check whether the associations between adiponectin and the bone markers studied are independent of sex, dialysis vintage, CRP, BMI, glucose metabolism, iPTH, and age. However, all the bone markers were strongly correlated with each other [correlation coefficients ranging from r=0.51 for log(cOC) and log(TRAP) to as high as r=0.95 for log(cOC) and log(intact OC)].

Table 3. Multiple linear regression models to predict log(adiponectin) in hemodialyzed patients (n=155).

Independent variable	beta ± SE	p-Value	beta ± SE	p-Value
Female sex	0.14 ± 0.09	0.1	0.12 ± 0.10	0.2
Age	0.03 ± 0.09	0.7	0.08 ± 0.10	0.4
log(BMI)	−0.26 ± 0.10	0.010	−0.26 ± 0.10	0.013
log(dialysis vintage)	0.07 ± 0.09	0.5	0.05 ± 0.10	0.6
log(CRP)	−0.06 ± 0.09	0.5	0.05 ± 0.10	0.6
log(insulin)	−0.21 ± 0.09	0.023	−0.23 ± 0.10	0.021
log(iPTH)	−0.05 ± 0.11	0.7	−0.18 ± 0.14	0.2
log(cOC)	0.27 ± 0.11	0.026	–	–
log(intact OC)	–	–	0.43 ± 0.14	0.007
Whole model	R^2=0.28; p<0.001		R^2=0.28; p<0.001	

Note: See Tables 1 and 2.

Therefore, we decided to include them in the multiple regression models separately, in order to avoid redundancy of predictors. Log-transformed cOC and intact OC appeared to be the only bone markers that significantly predicted adiponectin concentrations independently of age and sex, log(dialysis vintage), log(BMI), log(insulin concentrations), log(CRP), and log(iPTH) (Table 3).

Discussion

Bone metabolism markers in hemodialyzed patients

The CKD group was characterized by variably accelerated bone metabolism, as reflected by elevated bone markers. The concentrations of bALP and TRAP were less elevated than cOC, ucOC, and intact OC. Our observations may support the complex mechanism of OC release from bone into blood, which underlay OC as a marker of bone turnover (involving both bone formation and bone resorption).[19–21] The elevation of ucOC concentrations may also reflect vitamin K status, which is significantly reduced in hemodialyzed patients.[22,23] Finally, diminished glomerular filtration rate can cause the increase in blood OC concentrations in CKD patients as a result of accumulation of this protein due to renal failure.[2,24]

Bone markers and energy metabolism

Obesity or elevated BMI are supposed to reduce bone fracture risk and protect from osteoporosis.[25] Moreover, it was demonstrated that T2DM is associated with higher bone mineral density.[26,27] Additionally, some studies have shown lower bone turnover rates in subjects with higher BMI.[28] In the present study, we showed the inverse association between BMI and serum concentrations of bone metabolism markers (bALP, TRAP, cOC, and ucOC) in stage 5 CKD patients. Thus, our data confirmed the negative associations between BMI and

OC reported earlier in clinical observations regarding the populations with preserved renal function.[14,16,28]

The associations between serum OC and glucose concentrations have been well established in clinical studies.[12–14,16] It was shown that serum OC was negatively associated with BMI, fat mass, fasting glucose concentration, fasting insulin, and HOMA IR.[12,14,16] All these observations confirm the postulated role of OC in energy metabolism in mice i.e., maintenance of normoglycemia, the decline in BMI, and adipose tissue mass and the elevation of serum adiponectin concentration even under conditions of overfeeding.[6,7] In the animal model, only ucOC has hormonal activity [6]. However, data from clinical observational studies are conflicting, mainly in regard to the type of OC determined (total OC, cOC, or ucOC). Some authors observed the correction of glucose tolerance and improvement of β-cell function in concurrence with elevated ucOC levels,[15,29] while others ascribe the main role in the improvement of insulin sensitivity to cOC and total OC.[13,17,18] In our population of hemodialyzed patients, none of the bone markers correlated with glucose or insulin concentrations, or HOMA IR. In hemodialyzed patients, chronic inflammation and uremic toxicity may induce glucose intolerance, insulin resistance, and influence serum glucose concentrations as well as serum adiponectin levels.[30,31] Still, the recent study of Okuno et al.[29] shows the negative correlations between ucOC and glucose, HbA_1C and glycated albumin in patients on maintenance hemodialysis. However, our patients differ from the group of Okuno; in particular, the median iPTH concentrations in our group were more than two times higher (408 vs. 120 pg/mL), and the percentage of diabetic patients were significantly lower (13% vs. 51%). The demand for fasting blood sampling lead to exclusion of many diabetic HD patients from our group. Importantly, Okuno et al.[29] show much lower ucOC among diabetic than non-diabetic patients. We hypothesize that high bone turnover in our patients, as reflected by increased iPTH and bone markers, may interfere with or mask the relationships between OC and glucose metabolism. Additionally, we suppose that the discrepancy between our results and Okuno et al. may be in part attributed to the discrepancies between the immunoassays used for ucOC determination.

Bone markers and adiponectin concentrations

We observed positive correlations between adiponectin serum concentrations and the bone markers (bALP, TRAP, cOC, ucOC, and intact OC) in hemodialyzed patients. The correlations of adiponectin with all the bone markers were significant in men, but they were not significant in women, except for cOC.

It has been shown in the experimental murine models that the biological activity of OC depends on its blood concentration. In low concentrations, OC affects insulin synthesis rate and its release from β-cells. However, higher concentrations of OC stimulate adiponectin release from adipose tissue, without stimulating effect on the pancreas.[7] This observation is consistent with our results, showing positive correlation of highly elevated cOC and ucOC concentrations with adiponectin, but not glucose, insulin or HOMA IR. We also observed sex-related differences in those relationships. This is consistent with the report of Kanazawa et al.[16] In women and men, there are different factors involved in bone metabolism regulation (e.g., estrogen levels, menopause, or hormonal replacement therapy in women).[32,33] Other determinants of OC concentration, i.e., age and physical activity, smoking habits, seasonal variability, and diet differences (intake of green vegetables) or obesity may also influence the results.[34–36]

Several clinical studies have shown positive associations between the concentrations of adiponectin and biochemical bone markers.[37,38] Bacchetta et al.[39] for the first time have shown the inverse relationship between OC and bone mineral density (BMD) and positive between OC and adiponectin concentrations in patients with CKD stage 2-4. The authors additionally have underlined that the complex interrelations between adipocytes and bone tissue in CKD may have significant impact on cardiovascular risk stratification. Our data support Bacchetta's results, especially in men.

Multiple linear regression analysis showed that in the entire group of hemodialyzed CKD patients only cOC and intact OC as a bone-related markers correlated with adiponectin independently of age, sex, dialysis maintenance, BMI, CRP, insulin, and iPTH. This result suggests that cOC may act as a bone hormone, associated with higher adiponectin concentrations, although studies performed on animal models suggested that only ucOC is the hormonally active form of OC that regulates glucose metabolism.[6,7] Clinical studies do not always support observations performed in animals, and in contrast to rodents, in humans cOC rather than ucOC may be the endocrine regulator of energy metabolism.[17,36]

The selection of the study population (i.e., hemodialyzed CKD patients) to evaluate the relationships between bone and energy metabolism has both advantages and disadvantages. In stage 5 CKD patients, we observed highly elevated serum OC concentrations, rarely seen in other diseases. In hemodialyzed patients, uremic toxemia and chronic inflammation influence

both bone and energy metabolism.[31,40] Despite these complex interrelations, our data confirm the positive associations between two forms of osteocalcin, namely cOC and intact OC, and adiponectin in CKD patients treated with maintenance hemodialysis.

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Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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