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Carboxylated and undercarboxylated osteocalcin in metabolic complications of human obesity and prediabetes

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Abstract

Background Carboxylated osteocalcin (Gla-OC) participates in bone remodeling, whereas the undercarboxylated form (Glu-OC) takes part in energy metabolism. This study was undertaken to compare the blood levels of Glu-OC and Gla-OC in nonobese, healthy obese, and prediabetic volunteers and correlate it with the metabolic markers of insulin resistance and early markers of inflammation.

Methods Nonobese (body mass index [BMI] <30 kg/m^2; n = 34) and obese subjects (30 <BMI <40 kg/m^2; n = 98), both sexes, aged 25 to 65 years, were divided into healthy control, normal weight subjects, healthy obese, and obese with biochemical markers of prediabetes. The subgroups with obesity and low or high Gla-OC or Glu-OC were also considered for statistical analysis. After 2 weeks of diet standardization, venous blood was sampled for the determination of Gla-OC, Glu-OC, lipid profile, parameters of inflammation (hsCRP, interleukin 6, sE-selectin, sPECAM-1, and monocyte chemoattractant protein 1), and adipokines (leptin, adiponectin, visfatin, and resistin).

Results Gla-OC in obese patients was significantly lower compared to nonobese ones (11.36 ± 0.39 vs 12.69 ± 0.90 ng/mL, P = .048) and weakly correlated with hsCRP (r = −0.18, P = .042), visfatin concentration (r = −0.19, P = .033), and BMI (r = −0.17, P = .047). Glu-OC was negatively associated with fasting insulin levels (r = −0.18, P = .049) and reduced in prediabetic individuals compared with healthy obese volunteers (3.04 ± 0.28 vs 4.48 ± 0.57, P = .025).

Conclusions Decreased blood concentration of Glu-OC may be a selective early symptom of insulin resistance in obesity, whereas the decreased level of Gla-OC seems to be associated with the appearance of early markers of low grade inflammation accompanying obesity.

KEYWORDS

carboxylated osteocalcin, obesity, prediabetes, undercarboxylated osteocalcin

1 | INTRODUCTION

Osteocalcin (OC or bone Gla protein, BGP) and matrix Gla protein (MGP) were the first members of the growing family of vitamin K-dependent carboxylated proteins synthesized outside the liver and found not to be involved in coagulation.[1] Osteocalcin is released into

the circulation when new bone is formed and is considered a marker of bone turnover.[2] It was later identified as being secreted under normal, nonpathological conditions. The mature OC protein is small (49 amino acids in humans) and contains 3 glutamate residues, which once γ-carboxylated are responsible for its binding to calcium and hydroxyapatite. In turn 2 conserved cysteine residues of this protein form the

intramolecular disulfide bond, which contributes to stabilizing its three-dimensional structure upon binding of its γ-carboxylated glutamate (Gla) residues to calcium.[3] The undercarboxylated OC (Glu-OC) does not appear to bind calcium but could be involved in interactions with its recently identified G protein-coupled receptor family C group 6 member A (GPRC6A).[4] It has been demonstrated that it is expressed in many tissues including: liver, skeletal muscle, brain, testis, bone and pancreatic β-cells.[5–7] $Gprc6a^{-/-}$ mice have more white fat compared with wild-type (WT) animals. They are glucose intolerant, insulin resistant, develop hepatic steatosis and have reduced testosterone levels.[8] All these observations suggested that GPRC6A mediates metabolic function of osteocalcin. Recent studies have shown that carboxylated osteocalcin (Gla-OC) interacts with hydroxyapatite crystals and modulates its growth, whereas the Glu-OC has a hormone-like function in energy metabolism, fertility and brain development.[4,9–11] The administration of exogenous Glu-OC into WT mice decreased their fat mass and serum triglyceride (TG) levels. In white adipose tissue, Glu-OC inhibited the expression of lipolysis mediating genes: triglyceride lipase (*Tgl*) and perilipin, but activated the expression of adiponectin and its target genes.[12] Osteocalcin affected the expression of *Pgc1a*, *Nrf1*, and *Mcad* - genes implicated in energy consumption and mitochondrial biogenesis in muscles,[13] whereas in the brown adipose tissue expression of genes involved in thermogenesis: *Ucp1* and *Pgc1a* was increased.[12] Osteocalcin was demonstrated to affect glucose uptake in skeletal and vascular muscle cells.[14] It has also been reported that osteocalcin can directly modulate glucose transport in adipocytes, suppress the secretion of proinflammatory cytokines and induce the secretion of anti-inflammatory cytokines as adiponectin.[15] Oury et al[10] have recently demonstrated that disrupting osteocalcin signaling leads to glucose intolerance in both humans and mice, providing evidence that there is some similarity between mouse and human OC in mediated pathways. Several available papers present osteocalcin studies in humans.[16–19] Patients with type 2 diabetes have been reported to have a lower concentration of serum osteocalcin compared to healthy counterparts.[17] Semenkovich and Teitelbaum[18] suggested existing a cycle in which the metabolic events of diabetes downregulate osteoblast function, which in turn leads to less secretion of osteocalcin and a greater aggravation of insulin resistance. However, results from human studies have been inconsistent and often have implicated total osteocalcin association with insulin sensitivity adipokines and inflammatory markers.[19]

This study was undertaken to compare the blood level of Glu-OC and Gla-OC in nonobese, obese (prediabetic) and healthy obese (without biochemical parameters' pathology) volunteers and correlate them with the metabolic markers of insulin resistance and early markers of inflammation.

2 | MATERIALS AND METHODS

2.1 | Subjects and anthropometry

The study protocol and the entire study were approved by the Bioethics Committee of the Jagiellonian University in Cracow (written consent, opinion no. KBET/82/B/2009) functioning according to the third edition of the *Guidelines on the Practice of Ethical Committees in Medical Research* issued by the Royal College of Physicians of London. All participants gave written informed consent prior to participation in the study. Consent was obtained from each subject after full explanation of the purpose and nature of all used procedures. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Obese (30 <body mass index [BMI] <40 kg/m^2, n = 98) and nonobese (BMI <30 kg/m^2, n = 34) women and men, aged 25 to 65 years were included into the study. The exclusion criteria were conditions that might affect the metabolic parameters and response to diet such as: chronic diseases (cardiovascular diseases, cancer, chronic inflammation), diabetes mellitus and other metabolic disorders, severe kidney or liver failure, dietary restrictions or supplementation with: vitamins, β-carotene or n-3 polyunsaturated fatty acids, therapy with: hormones, anti-inflammatory drugs or other drugs known to affect lipid or glucose metabolism, smoking or excessive use of alcohol, pregnancy or lactation. All patients enrolled into this study were asked to follow an isocaloric diet with a low amount of antioxidative vitamins, polyunsaturated fatty acids and alcohol for 2 weeks before and during the study. The diet instructions were presented to each patient and diet compliance was controlled every 2 weeks by a dietitian.

Body weight was measured to the nearest 0.1 kg with a digital scale, and height was measured to the nearest 0.5 cm using a wall-monitored stadiometer. Waist and hip circumferences were obtained with a tape measure. Body composition was estimated with the bioelectrical impedance method using Segmental Body Composition Analyser TANITA BC 418 MA (Tanita, Japan). Blood pressure was measured in the supine position after 10 minutes of rest with an automatically inflating cuff.

2.2 | Sample collection and analysis

After 2 weeks of diet standardization, venous blood samples were drawn after 12 hours overnight fasting for measurements of basal plasma Gla-OC, Glu-OC, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, TGs, hsCRP, interleukin 6 (IL-6) and soluble adhesion molecules: E-selectin (sE-selectin), monocyte chemoattractant protein 1 (MCP-1), soluble platelet/endothelial cell adhesion molecule 1 (sPECAM-1/CD31), vascular cell adhesion protein 1 (sVCAM-1) and adipokines (leptin, adiponectin, visfatin, and resistin). To control kidney function, estimated glomerular filtration rate (eGFR) was calculated using the MDRD equation.

On 2 other different days, subjects underwent a 2-hour oral glucose tolerance test (OGTT) and an 8-hour oral lipid tolerance test (OLTT). Fasting and postprandial blood samples were drawn for OGTT and OLTT and analyzed for concentrations of glucose, insulin, free fatty acids (FFAs), TGs and glucose-dependent insulinotropic polypeptide (GIP). Blood was sampled without stasis through an indwelling catheter into syringes. Within 30 minutes, blood samples for all estimated parameters (except FFAs) were centrifuged at 1000x g for 10 minutes at 4°C, and the supernatants were immediately stored at −80°C until analysis.

OGTT was conducted according to the World Health Organization (WHO) and International Diabetes Federation (IDF) guidelines. Tests were performed during morning hours (8:00–11:00 AM) after a 10-hour overnight fasting. Blood samples were collected at the following 5 time points: baseline (fasting), and then 30, 60, 90 and 120 minutes after the ingestion of 75 g glucose dissolved in 250 mL water.

OLTT was performed as described before.[20] All patients were asked not to drink alcohol or drinks containing caffeine for 3 days before the test. The day before the OLTT, a last low-fat meal was eaten by the participants before 6 PM (2 slices of bread without any fatty products and unsweetened tea). Only water was allowed to drink thereafter. Test breakfasts were given at 7:30 AM, and postprandial studies were performed from 7:30 AM to 5:30 PM. Test breakfast meal (caloric content 1018 kcal, consisting of: 73% of fat, 16% of protein and 11% of carbohydrates) contained: light bread – 50 g, butter – 20 g, cream cheese – 60 g, pork loin roast – 100 g and mayonnaise – 40 g. Venous blood samples were taken 5 times before the meal (fasting sample, 0 hours) and postprandial at time points: 2, 4, 6 and 8 hours after breakfast.

2.3 | Blood analyses

Plasma Gla-OC and Glu-OC were determined by ELISA (Takara, Japan). Intra- and interassay coefficients of variation were: <4.8% and <2.4% (Gla-OC), <6.66%, and <9.87% (Glu-OC), respectively. Total osteocalcin level was calculated as the sum of Gla-OC and Glu-OC. Plasma glucose, total cholesterol, HDL cholesterol, and TGs were assayed by automated, enzymatic colorimetric methods (Allmed, Poland) using the MaxMat Analyzer. The intra- and interassay variability coefficients were as follows: 2.3% and 3.5% (glucose), 1.4% and 3.4% (TGs), 1.4% and 3.8% (total cholesterol), 2.1% and 2.8% (HDL cholesterol), respectively. LDL cholesterol was calculated from measured values of total cholesterol, TGs, and HDL cholesterol according to the Friedewald formula.

FFAs concentration was measured immediately in non frozen plasma by enzymatic quantitative colorimetric method (Roche Diagnostics GmbH, Germany).

The determination of serum insulin was performed by immunoradiometric method (DIAsource, ImmunoAssays, Belgium) and read using the gamma counter (LKB Instruments). Within- and between-run imprecision CVs were 2.1% and 6.5%, respectively. Basal insulin resistance was estimated using homeostasis model of assessment (HOMA-IR).[21] Insulin sensitivity post oral glucose load was determined using an oral glucose insulin sensitivity index (OGIS) proposed by Mari et al,[22] which can be downloaded as a calculator for Excel spread sheets from the web page http://webmet.pd.cnr.it/ogis.

To measure plasma concentrations of incretin—glucose dependent insulinotropic polypeptide (GIP), human GIP ELISA (Human Total GIP; EMD Millipore, St Charles, MO) was used. The inter- and intra-assay coefficients of variation were: 1.8% to 6.1% and 3.0% to 8.8%, respectively.

Plasma leptin, adiponectin (adipocyte complement-related protein of 30 kDa, Acrp 30), resistin, IL-6, sE-Selectin, MCP-1, and sVCAM-1 were determined using ELISA (R&D Systems Europe, Ltd, Abingdon, United Kingdom). Within- and between-run imprecision CVs were

3% and 4% (leptin), 4% and 6% (adiponectin), 5.3% and 8.2% (resistin), 6% and 7% (IL-6), 6% and 8% (sE-Selectin), 5% and 6% (MCP-1), and 3.5% and 7.7% (sVCAM-1), respectively. Visfatin (Nampt/PBEF) and sPECAM-1/CD31 were measured by ELISA (BioVendor, Czech Republic). The inter- and intra-assay CVs were 6% and 7% (visfatin), 1.7% and 7.4% (sPECAM-1), respectively. CRP was determined by the highly sensitive immunoturbidimetric method (APTEC Diagnostics nv, Belgium). Within- and between-run imprecision CVs were 1.66% and 2.08%, respectively.

2.4 | Statistical analyses

The Shapiro-Wilk test was used to test data for a Gaussian distribution. Normally distributed data are presented as mean ± SEM otherwise as median and interquartile range (IQR). Differences between the main studied groups (obese vs nonobese) and the formed subgroups were analyzed by unpaired t test, the Mann-Whitney U test, or the Kruskal-Wallis test and the Dunn test (comparison of results between multiple groups) for nonnormally distributed data. Continuous variables were log transformed if required. The Spearman rank correlation was used to find association between variables. All analyses were performed with Statistica software (StatSoft). The $P < .05$ was considered statistically significant. Areas under curves (AUC) during OGTT or OLTT were calculated by the trapezoidal method.[23]

3 | RESULTS

The characteristics of the study population of nonobese and obese participants is presented in Tables 1 and 2. Obese volunteers (n = 98) differ from nonobese ones (n = 34) in regard to anthropometric measurements: BMI (34.0 vs 28.4 kg/m^2, $P < .001$), adipose tissue mass (40.8% vs 35.0%, $P < .001$), and blood pressure (Table 1). Patients with higher BMI also showed increased plasma leptin levels and fasting insulin. Area under the OGTT and OLTT insulin concentration-time curve and basal insulin resistance index HOMA-IR were also increased in obese patients (Table 2). Subjects in both groups participating in the study did not differ in: plasma fasting and postprandial lipid levels (total cholesterol, LDL cholesterol, HDL cholesterol, TGs, and FFAs) as well as GIP levels.

Serum hsCRP and IL-6 were higher in obese subjects. Plasma levels of ALT, AST, urea, and uric acid were also elevated in this group of patients (Tables 1 and 2). In the whole group of obese patients in comparison to nonobese subjects, the level of Gla-OC was significantly lower, whereas there were no significant differences in total osteocalcin as well as Glu-OC content (Table 2). For further analysis, the whole group of study participants (n = 132) was divided into 2 groups (Table 3) or 4 groups (Table 4) in accordance to the higher Gla-OC (>11.2 ng/mL) and the lower Gla-OC (<11.2 ng/mL) level based on the median value. We observed that the group with lower Gla-OC level (n = 66) demonstrated a lower amount of Glu-OC, and Gla/Glu ratio, which was accompanied by higher blood concentrations of inflammatory cytokines: hsCRP and visfatin. In this group of patients, lower levels of total and LDL cholesterol, sPECAM-1, urea, creatinine and higher eGFR were observed in

TABLE 1 Characteristics of subjects participating in the study (anthropometric, biochemical, and lipid measurements)

	Nonobese (n = 34)	Obese (n = 98)	P*
Age, y	48.05 ± 1.94	46.74 ± 1.15	n.s.
Sex, female (n)	27	72,00	n.s.
BMI, kg/m^2	28.4 (2.2)[†]	34 (12.8)	.000
WHR	0.87 (0.53)	0.89 (0.26)	.537
Adipose tissue mass, %	35.0 (6.4)	40.8 (11.3)	.000
Systolic BP, mm Hg	120 (24)	130 (52)	.020
Diastolic BP, mm Hg)	80 (28)	85 (23)	.010
APTT, s	29 (6.4)	29.0 (15.3)	.908
Urea, mmol/L	4.4 (4.1)	5.0 (3.2)	.030
Uric acid, mmol/L	240 (227.5)	302 (191.9)	.020
ALT, U/L	17 (39)	24 (60)	.001
AST, U/L	18 (12)	20 (45)	.030
Total bilirubin, mmol/L	9.80 (21.5)	8.6 (12.4)	.195
Creatinine, mmol/L	61.4 (36.5)	65.3 (31.1)	.235
MDRD	91.8 (26.5)	91.4 (43.8)	.621
Total cholesterol, mmol/L	5.38 ± 0.15[‡]	5.54 ± 0.11	.986
HDL cholesterol, mmol/L	1.30 ± 0.03	1.31 ± 0.02	.997
LDL cholesterol, mmol/L	3.49 ± 0.14	3.55 ± 0.09	.765
Fasting NEFA, mmol/L	0.69 ± 0.03	0.76 ± 0.02	.141
AUC OGTT NEFA, mmol·L^{-1}·min^{-1}	155.24 ± 10.03	174.76 ± 8.56	.152
AUC OLTT NEFA, mmol·L^{-1}·min^{-1}	1546.02 ± 88.92	1554.73 ± 50.68	.429
Fasting triglycerides, mmol/L	1.31 ± 0.11	1.51 ± 0.07	.201
AUC OGTT triglycerides, mmol·L^{-1}·min^{-1}	615.98 ± 61.81	682.58 ± 33.11	.508
AUC OLTT triglycerides, mmol·L^{-1}·min^{-1}	3597.52 ± 374.66	3924.56 ± 193.02	.424

Selection of 2 subgroups: nonobese and obese participants. ALT indicates alanine transaminase; APTT, activated partial thromboplastin time; ASP, aspartate transaminase; AUC, area under the curve; BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDRD, an estimated Glomerular Filtration Rate (eGFR) calculated from serum creatinine using the Modification of Diet in Renal Disease; NEFA, nonesterified fatty acids; OGTT, oral glucose tolerance test; OLTT, oral lipid tolerance test; WHR, waist-to-hip ratio.

*Significant difference between nonobese and obese group (unpaired t test or Mann-Whitney U test for nonnormally distributed variables), P < .05.

[†]Median (interquartile range); all such values.

[‡]Mean ± SEM; all such values.

comparison with subjects presenting Gla-OC higher than 11.2 ng/mL (Table 3). A weak negative correlation of Gla-OC with: hsCRP ($r = -0.18$, $P = .042$), visfatin concentration ($r = -0.19$, $P = .033$), BMI ($r = -0.17$, $P = .047$) and eGFR ($r = -0.31$, $P = .000$) was found (Table 5). No correlation of Gla-OC with insulin resistance parameters (fasting Insulin, HOMA-IR or glucose levels) was found (Table 5). On the contrary, Glu-OC inversely correlated with fasting insulin level and HOMA IR index (Table 5). Comparison of nonobese and obese subjects regarding Gla-OC levels (Table 4) showed that obese patients with Gla-OC >11.2 ng/mL presented higher Glu-OC, total and LDL cholesterol as well as sPECAM-1 level than obese ones with Gla-OC <11.2 ng/mL. Such results were not observed in the group of nonobese subjects (Table 4). Obese subjects with a Gla-OC level less than 11.2 ng/mL demonstrated higher plasma hsCRP level than nonobese ones. Therefore, for further analysis, the group of obese subjects was divided in accordance to metabolic disturbances. We selected healthy obese subjects (without any metabolic disturbances) (n = 29) and obese patients presenting symptoms of prediabetes with or without atherogenic dyslipidemia (n = 32). The group with prediabetes included subjects whose fasting plasma glucose was ≥5.6 and <7.0 mmol/L or the level of glucose at the 2 hours of OGTT was

between 7.8 and 11 mmol/L. Lipid disturbances were considered when serum fasting TG level was ≥1.7 mmol/L or the HDL cholesterol concentration in men was <1.03 mmol/L and in women <1.29 mmol/L (Table 6).

Healthy obese volunteers presented higher Glu-OC level than those from the obese group with prediabetic disturbances (Table 6). Insulin resistance symptoms (glucose, insulin, and HOMA-IR) as well as GIP levels were significantly higher in volunteers with prediabetes (Table 6). The segregation of all obese subjects (n = 98) into 2 subgroups based on Glu-OC level (Glu-OC <2.97 ng/mL and Glu-OC >2.97 ng/mL) revealed that subjects with lower levels of undercarboxylated osteocalcin (n = 49) demonstrated increased fasting insulin levels and insulin resistance index HOMA IR (Table 7), pointing to decreased insulin sensitivity. On the contrary, no elevation of the investigated biochemical markers of inflammation was found in this group (Table 7).

Thus, the lower level of Glu-OC in obese subjects characterized the subjects with insulin resistance expressed by the increased fasting plasma insulin and HOMA-IR values, whereas the Gla-OC level correlated with the markers of inflammatory status indicated in our study by the elevation of blood hsCRP and visfatin level.

Gla-OC/Glu-OC ratio had a tendency to distinguish obese healthy individuals from prediabetic subjects (Table 6). The Gla-OC/Glu-OC

TABLE 2 Characteristics of subjects participating in the study (insulin sensitivity, inflammatory, and osteocalcin measurements)

	Nonobese (n = 34)	Obese (n = 98)	P*
AUC OGTT Glucose, mmol·L^{-1}·min^{-1}	3378 (2564.5)†	3456 (1957)	.250
AUC OLTT Glucose, mmol·L^{-1}·min^{-1}	9252 (2265)	9660 (2341.2)	.140
AUC OGTT Insulin, mIU·mL^{-1}·min^{-1}	36609.93 ± 6199.96‡	48561.10 ± 2745.49	.004
AUC OLTT Insulin, mIU·mL^{-1}·min^{-1}	31008 (122556)	42528 (110166)	.001
Fasting GIP, pg/mL	23.3 (43.4)	26.1 (106.3)	.199
AUC OGTT GIP, pg·mL^{-1}·min^{-1}	70796.84 ± 6411.65	71573.00 ± 2859.29	.480
AUC OLTT GIP, pg·mL^{-1}·min^{-1}	394982.14 ± 34515.85	365472.83 ± 14362.21	.350
Fasting glucose, mmol/L	5.22 ± 0.08	5.24 ± 0.05	.488
Fasting insulin, mIU/mL	12.49 ± 1.48	16.72 ± 0.78	.000
HOMA-IR	2.06 (8.06)	3.45 (7.28)	.000
OGIS, mL·min^{-1}·m^{-2}	387.11 ± 15.12	370.79 ± 7.65	.056
sE-Selectin, pg/mL	32.95 (46.23)	37.23 (47.45)	.073
MCP-1, pg/mL	357.31 ± 16.85	366.58 ± 11.61	.310
sVCAM-1, ng/mL	588.56 ± 22.37	590.81 ± 16.54	.548
sPECAM-1, ng/mL	67.46 ± 2.91	72.43 ± 1.69	.634
hs CRP, mg/L	0.80 (5.75)	2.22 (8.41)	.001
IL-6, pg/mL	1.07 ± 0.12	1.63 ± 0.10	.001
IL-8, pg/mL	2.13 (15.67)	2.21 (11.32)	.767
TNF-α, pg/mL	4.8 (11.4)	5.94 (9.22)	.096
Leptin, pg/mL	27797.14 ± 1742.16	40657.72 ± 2438.83	.000
Adiponectin, ng/mL	6719 (8912)	6138 (12982)	.441
Resistin, ng/mL	9.78 ± 0.52	10.11 ± 0.40	.730
Visfatin, ng/mL	1.09 ± 0.16	1.17 ± 0.08	.237
Total OC, ng/mL	15.92 ± 0.96	15.17 ± 0.47	.100
Gla-OC, ng/mL	12.68 ± 0.90	11.36 ± 0.39	.048
Glu-OC, ng/mL	3.23 ± 0.34	3.80 ± 0.24	.955
Gla-OC/Glu-OC, ng/mL	5.68 ± 0.81	3.83 ± 0.22	.281

Selection of 2 subgroups: nonobese and obese participants. AUC indicates area under the curve; GIP, glucose-dependent insulinotropic peptide; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HOMA-IR, homeostatic model assessment; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; IL-8, interleukin 8; MCP-1, monocyte chemoattractant protein 1; OC, osteocalcin; OGIS, oral glucose insulin sensitivity index; OGTT, oral glucose tolerance test; OLTT, oral lipid tolerance test; sPECAM-1, soluble platelet/endothelial cell adhesion molecule 1; sVCAM-1, vascular cell adhesion protein 1, TNF-α, tumor necrosis factor α.

*Significant difference between nonobese and obese group (unpaired t test or Mann-Whitney U test for nonnormally distributed variables), P < .05.

†Median (interquartile range); all such values.

‡Mean ± SEM; all such values.

ratio correlated: positively with fasting insulin and HOMA-IR index in obese subjects, negatively with inflammatory markers (hsCRP and sVCAM) in obese prediabetic participants, and inversely with sE-selectin in nonobese patients (Table 8).

4 | DISCUSSION

Our study, performed on a healthy nonobese control group and obese patients, demonstrated that despite a similar total osteocalcin level in blood, a significantly lower concentration of Gla-OC was found in obese patients compared with nonobese subjects. The decreased concentration of Glu-OC measured in the serum of obese patients characterized subjects with insulin resistance expressed by the increased: fasting insulin and glucose levels, postprandial (OLTT and OGTT AUC) glucose concentration, higher HOMA-IR value and with the decrease in the osteocalcin. The Gla-OC concentration was decreased

in patients with obesity and was associated with greater levels of the inflammatory markers: hsCRP and visfatin[24] as well as the E-selectin and s-VCAM, which are markers of endothelial injury. Of note, no correlation of osteocalcin forms with leptin, adiponectin or resistin was observed in our group of obese volunteers.

Our findings confirmed that osteocalcin is an osteoblast-specific secreted protein, which participates not only in bone remodeling but also in metabolism regulation. Bone remodeling is highly dependent on the energetic status of the organism. For example, anorexia nervosa and insulin-dependent diabetes mellitus are associated with osteoporosis, while a higher BMI is associated with increased bone mass.[25] The hormones implicated in the regulation of food intake and energy metabolism (ie, leptin, adiponectin and incretins) also regulate bone mass.[26-28]

High-fat diet (HFD) induces insulin resistance in osteoblasts and leads to a decrease in circulating levels of the active form of

TABLE 3 Comparisons of 2 subgroups with a higher and lower than median value of Gla-OC levels selected from the whole group of participating subjects

	Gla-OC >11.2 ng/mL (n = 66)	Gla-OC <11.2 ng/mL (n = 66)	P*
Gla-OC, ng/mL	14.99 ± 0.45[†]	8.46 ± 0.2	.000
Glu-OC, ng/mL	4.05 ± 0.24	3.3 ± 0.31	.008
Gla-OC/Glu-OC	5.02 ± 0.48	3.52 ± 0.23	.004
Total Osteocalcin, ng/mL	19.04 ± 0.47	11.78 ± 0.37	.000
Total Cholesterol, mmol/L	5.71 ± 0.12	5.28 ± 0.13	.014
LDL Cholesterol, mmol/L	3.78 ± 0.11	3.27 ± 0.10	.001
sPECAM-1, ng/mL	74.45 ± 2.03	67.4 ± 2.05	.012
Visfatin, ng/mL	0.95 ± 0.09	1.34 ± 0.12	.038
hsCRP, mg/L	1.14 (9.18)[‡]	2.61 (6.93)	.046
Urea, mmol/L	5.1 (3.3)	4.6 (2.7)	.024
Creatinine, mmol/L	66.3 (27.9)	63.3 (40.0)	.02
MDRD	88.9 (38.8)	94.5 (51.1)	.036

The calculated median was considered. Gla-OC, indicates carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; hsCRP, high- sensitivity C reactive protein; MDRD, an estimated Glomerular Filtration Rate (eGFR) calculated from serum creatinine using the Modification of Diet in Renal Disease; sPECAM-1, soluble platelet/ endothelial cell adhesion molecule 1; LDL, low- density lipoprotein.

*Significant difference between Gla-OC groups (unpaired t test or Mann-Whitney U test for nonnormally distributed variables), $P <0.05$.

[†]Mean ± SEM; all such values.

[‡]Median (interquartile range); all such values.

TABLE 4 Comparisons of subgroups with a higher and lower than median value of Gla-OC levels selected from nonobese and obese subjects

	Nonobese (n = 34)		Obese (n = 98)		P*	P[†]
	Gla-OC <11.2 ng/mL (n = 13)	Gla-OC >11.2 ng/mL (n = 21)	Gla-OC <11.2 ng/mL (n = 53)	Gla-OC >11.2 ng/mL (n = 45)		Pa
Gla-OC, ng/mL	8.32 (2.17)	15.14 (4.55)[a]	8.68 (2.30)	13.52 (2.96)[b]	.000	$P = .000^a$, $P = .000^b$
Glu-OC, ng/mL	2.57 (0.48)	3.81 (2.88)	2.46 (1.74)	3.78 (2.89)[b]	.0179	$P = .0126^b$
Gla-OC/Glu-OC	3.37 (1.28)	3.98 (4.15)	3.43 (3.05)	3.60 (3.02)	.0866	N/A
Total Osteocalcin, ng/mL	11.85 (2.60)	19.59 (5.01)[a]	11.60 (3.06)	18.01 (3.39)[b]	.000	$P = .000^a$, $P = .000^b$
Total Cholesterol, mmol/L	4.89 (1.02)	5.68 (1.28)	5.10 (0.98)	5.52 (1.46)[b]	.0623	N/A
LDL Cholesterol, mmol/L	3.20 (0.45)	4.07 (1.65)	3.25 (1.22)	3.78 (1.13)[b]	.0108	$P = .0226^b$
sPECAM-1, ng/mL	68.25 (18.70)	72.78 (20.49)	64.68 (19.29)	81.01 (22.67)[b]	.0215	$P = .0292^b$
Visfatin, ng/mL	0.86 (0.85)	0.64 (0.49)	1.11 (0.99)[c]	0.80 (0.63)	.0332	$P = .0433^c$
hsCRP, mg/L	0.68 (0.84)	0.84 (1.06)	2.96 (3.54)[d]	1.48 (2.36)	.0016	$P = .0215^d$
Urea, mmol/L	3.90 (0.93)	4.80 (1.80)	4.85 (1.68)	5.40 (1.40)[e]	.0104	$P = .0049^e$
Creatinine, mmol/L	60.95 (6.55)	62.80 (9.90)	64.10 (10.15)	67.30 (8.25)	.1039	N/A
MDRD	93.97 (8.15)	88.89 (18.38)	94.59 (19.61)	88.86 (19.41)	.1326	N/A

The calculated median was considered. Gla-OC indicates carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; hsCRP, high-sensitivity C-reactive protein; MDRD, an estimated Glomerular Filtration Rate (eGFR) calculated from serum creatinine using the Modification of Diet in Renal Disease; sPECAM-1, soluble platelet/endothelial cell adhesion molecule 1; LDL, low-density lipoprotein; N/A, not applicable.

All values presented as median (interquartile range).

*P value for Kruskal-Wallis ANOVA by ranks test.

[†]Significant difference between groups (Dunn test), $P < .05$.

[a]Significant difference between nonobese Gla-OC subgroups.

[b]Significant difference between obese Gla-OC subgroups.

[c]Significant difference between nonobese Gla-OC >11.2 ng/mL and obese Gla-OC <11.2 ng/mL subgroups.

[d]Significant difference between nonobese and obese groups with Gla-OC <11.2 ng/mL.

[e]Significant difference between nonobese with Gla-OC <11.2 ng/mL and obese with Gla-OC >11.2 ng/mL subgroups.

osteocalcin (thereby decreasing insulin sensitivity in skeletal muscle and WAT).[29] Insulin resistance developed in osteoblasts is suggested to be the result of increased levels of free saturated fatty acids, which promote SMURF1-mediated insulin receptor (INSR) ubiquitination and its subsequent degradation in osteoblasts.[7] These experiments support the notion that insulin resistance in bone contributes to the deleterious

TABLE 5 Spearman rank correlation between Gla-OC, Glu-OC, and metabolic variables in the entire group of subjects participating in the study (n = 132)

	r	P
Gla-OC and BMI	−0.17	.047
Gla-OC and hsCRP	−0.18	.042
Gla-OC and visfatin	−0.19	.033
Gla-OC and total cholesterol	0.22	.011
Gla-OC and LDL cholesterol	0.3	.000
Gla-OC and urea	0.25	.004
Gla-OC and creatinine	0.28	.002
Gla-OC and MDRD	−0.31	.000
Glu-OC and fasting insulin	−0.18	.049
Glu-OC and HOMA-IR	−0.17	.058

Statistically significant correlations: $P < .05$. BMI indicates body mass index; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HOMA-IR, homeostatic model assessment; hsCRP, high-sensitivity C-reactive protein; MDRD, an estimated Glomerular Filtration Rate (eGFR) calculated from serum creatinine using the Modification of Diet in Renal Disease; LDL, low-density lipoprotein.

consequences of a long-term HFD on whole-body glucose homeostasis, in part because it decreases the activity of osteocalcin.[7]

Osteocalcin is γ-carboxylated on the glutamic acids (GLU) 13, 17, and 20 of protein in mouse, and on GLU 17, 21, and 24 in humans.[30] The carboxylation of osteocalcin and other Gla proteins occurs in the lumen of the endoplasmic reticulum and involves 2 enzymes γ-glutamyl carboxylase and vitamin K epoxide reductase (VKORC1),

which together constitute the vitamin K-dependent cycle. This posttranslational modification increases the affinity of osteocalcin for Ca^{2+} and therefore for hydroxyapatite, the mineral component of bone extracellular matrix (ECM). The vast majority of osteocalcin secreted by osteoblasts gets trapped in bone ECM.[30] In the serum, both the carboxylated and the undercarboxylated forms of osteocalcin are detected. The mechanism responsible for the differences in concentration and carboxylation status of osteocalcin has not yet been precisely elucidated. Possible mechanisms include certain hormones as well as both nutritional and non–nutritional-related factors such as: vitamin D, vitamin K or calcium.[16] Complex carbohydrate meals containing high amounts of fruits, vegetables and vitamins failed to significantly affect circulating concentrations of postprandial osteocalcin. Low as well as high carbohydrate meals resulted in decreased concentrations of osteocalcin over time. Henriksen et al[31] showed that oral ingestion of long chain triacylglycerides had no effect on osteocalcin concentration.

In our studies, a decreased level of Gla-OC in obese patients was observed. The Gla-OC concentration in serum inversely correlated with markers associated with low-grade inflammation in obesity: hsCRP and visfatin. Our results are in agreement with in vitro studies, which revealed anti-inflammatory properties of osteocalcin. In experiments conducted on primary-cultured adipocytes, Hill et al[15] showed that both forms of osteocalcin suppressed the secretion of tumor necrosis factor alpha into the media. However, only carboxylated osteocalcin suppressed IL-6 release. Both carboxylated and uncarboxylated osteocalcin increased the secretion of adiponectin

TABLE 6 Comparison between the subgroup of obese patients without biochemical markers of metabolic syndrome ("healthy obese") and obese patients with biochemical markers of metabolic syndrome (prediabetic patients)

	Obese healthy (n = 29)	Obese with metabolic disturbances in prediabetes (n = 32)	P*
WHR	0.84 (0.23)[†]	0.92 (0.17)	.044
Fasting glucose, mmol/L	4.98 ± 0.06[‡]	5.69 ± 0.09	.000
Glucose OLTT AUC, mmol/L·min^{-1}	3282 (1173)	4218 (1390)	.000
Glucose OGTT AUC, mmol/L·min^{-1}	9240 (2168)	10176 (1620)	.000
Fasting Insulin, µIU/mL	13.11 ± 1.02	19.11 ± 1.59	.001
Fasting GIP, pg/mL	23.73 (73.50)	32.80 (103.70)	.044
OGIS, mL·min^{-1}·m^{-2}	381.59 ± 12.83	327.94 ± 12.83	.005
HOMA-IR	2.73 (4.69)	4.48 (7.51)	.000
Fasting TG, mmol/L	0.98 ± 0.05	1.53 ± 0.12	.000
TG OLTT AUC, mmol·L^{-1}·min^{-1}	2676.09 ± 180.80	4015.65 ± 305.51	.000
TG OGTT AUC, mmol·L^{-1}·min^{-1}	434.50 ± 25.16	673.07 ± 51.75	.000
HDL Cholesterol, mmol·L^{-1}	1.47 ± 0.04	1.29 ± 0.06	.005
Total osteocalcin, ng/mL	15.94 ± 1.10	14.41 ± 0.72	.432
Gla-OC, ng/mL	11.46 ± 0.86	11.37 ± 0.59	.834
Glu-OC, ng/mL	4.48 ± 0.57	3.04 ± 0.28	.025
Gla-OC/Glu-OC ratio	3.60 ± 0.47	4.35 ± 0.32	.040

AUC indicates area under the curve; GIP, glucose-dependent insulinotropic peptide; Gla-OC, carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment; OGIS, oral glucose insulin sensitivity index; OGTT, oral glucose tolerance test; OLTT, oral lipid tolerance test; TG, triglycerides; WHR, waist-to-hip ratio.

*Significant difference between obese healthy and obese prediabetic group (unpaired t test or Mann-Whitney U test for nonnormally distributed variables), $P < .05$.

[†]Median (interquartile range); all such values.

[‡]Mean ± SEM; all such values.

TABLE 7 Comparisons of 2 subgroups with a higher and lower than median value of Glu-OC level selected from the obese group of participating subjects

	Obese (n = 98)		
	Glu-OC >2.97 ng/mL (n = 49)	Glu-OC <2.97 ng/mL (n = 49)	P*
Glu-OC, ng/mL	5.35 ± 0.34[†]	2.08 ± 0.07	.000
Gla-OC, ng/mL	11.55 ± 0.50	10.92 ± 0.60	.297
Total osteocalcin, ng/mL	16.91 ± 0.60	13.01 ± 0.63	.000
Fasting insulin, mIU/mL	15.46 ± 1.16	17.81 ± 1.02	.030
HOMA-IR	2.96 (8.10)[‡]	3.96 (5.21)	.015
sE-Selectin, pg/mL	38.34 (35.92)	33.61 (84.39)	.260
hsCRP, mg/L	2.2 (8.10)	2.49 (7.90)	.734
Visfatin, ng/mL	1.12 ± 0.11	1.25 ± 0.12	.054
IL-6, pg/mL	1.50 ± 0.10	1.74 ± 0.18	.836
MCP-1, pg/mL	371.47 ± 15.97	362.93 ± 17.09	.610
sVCAM-1, ng/mL	624.90 ± 26.56	632.35 ± 24.48	.772
sPECAM-1, ng/mL	72.83 ± 2.39	71.63 ± 2.40	.521

The calculated median was considered. Gla-OC indicates carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HOMA-IR, homeostatic model assessment; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; sPECAM-1, soluble platelet/endothelial cell adhesion molecule 1; sVCAM-1, vascular cell adhesion protein 1.

*Significant difference between Glu-OC groups (unpaired *t* test or Mann-Whitney *U* test for nonnormally distributed variables), P < .05.

[†]Mean ± SEM; all such values.

[‡]Median (interquartile range); all such values.

TABLE 8 Spearman rank correlation between Gla-OC/Glu-OC ratio values and biochemical variables in nonobese controls, obese, and obese with biochemical markers of prediabetes

	Spearman rank correlation Gla-OC/Glu-OC ratio					
	Nonobese (n = 34)		Obese (n = 98)		Obese with metabolic disturbances in prediabetes (n = 32)	
	r	P*	r	P*	r	P*
Fasting insulin	0.09	.63	0.26	.01	0.09	.63
Fasting glucose	−0.12	.51	0.11	.29	−0.05	.77
HOMA-IR	0.09	.61	0.26	.01	0.02	.92
OGIS	0.33	.09	−0.06	.60	−0.12	.54
Total cholesterol	0.07	.68	0.05	.64	0.02	.89
HDL cholesterol	0.05	.80	−0.13	.21	−0.24	.19
LDL cholesterol	0.07	.67	0.12	.26	0.01	.97
TG	0.18	.32	−0.09	.38	−0.16	.37
sE-Selectin	−0.38	.03	−0.09	.39	−0.09	.63
hsCRP	0.20	.27	−0.12	.23	−0.38	.03
Visfatin	−0.15	.39	0.03	.75	−0.24	.18
IL-6	0.10	.56	−0.13	.21	−0.30	.10
MCP-1	0.00	.99	0.04	.68	0.17	.38
sVCAM-1	0.00	1.00	−0.07	.51	−0.41	.03
sPECAM-1	0.18	.32	−0.02	.86	−0.09	.65
TNF-α	0.02	.90	−0.02	.84	−0.07	.75

Gla-OC indicates carboxylated osteocalcin; Glu-OC, undercarboxylated osteocalcin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein 1; sPECAM-1, soluble platelet/endothelial cell adhesion molecule 1; sVCAM-1, vascular cell adhesion protein 1, TG, triglycerides; TNF-α, tumor necrosis factor α.

*Significant Spearman rank correlation, P < .05.

and the anti-inflammatory cytokine IL-10. So far, human studies of the association between osteocalcin and inflammatory factors are still lacking. Although several human studies reported the inverse association between serum osteocalcin (without distinguishing between Gla-OC and Glu-OC) and hsCRP,[32–34] we are the first to show this association for the carboxylated osteocalcin form.

However, the mechanism linking hsCRP and bone metabolism is not well understood. The anti-inflammatory effect of Gla-OC could

be explained as a result of vitamin K action. It is well known that vitamin K has anti-inflammatory properties that could be mediated by carboxylated osteocalcin. On the other hand, an increase in systemic inflammatory response could be connected with osteocalcin carboxylation defect as a result of lowered serum vitamin K level. However, a recent study indicates anti-inflammatory action of vitamin K independently of osteocalcin carboxylation.[35] As regards association between visfatin and osteocalcin, Kacso et al[36] reported a negative correlation of Glu-OC with visfatin in type 2 diabetes patients with diabetic kidney disease. In our studies, we observed an association of Gla-OC but not Glu-OC with visfatin. However, subjects enrolled in our experiments were obese but did not develop diabetes.

Most of the in vitro and in vivo studies conducted so far indicate that osteocalcin endocrine function is regulated by its undercarboxylated form.[37,38] In contrast to Gla-OC, Glu-OC does not bind Ca^{2+} and, more importantly, does not require elevated Ca^{2+} concentration to fold into a helical structure. These observations suggest that at Ca^{2+} concentrations at approximately 1 mM, as normally found in cell culture media or in vivo in serum, only Glu osteocalcin will exhibit a helical conformation and presumably be able to activate its receptor(s) (ie, GPRC6A), providing a structural explanation for the lack of biological activity of Gla osteocalcin on glucose metabolism.[39,40] It acts on pancreatic β cells to increase insulin secretion, on muscle and WAT to promote glucose uptake and homeostasis, and on Leydig cells of the testis to favor testosterone biosynthesis after its binding to a GPRC6A receptor.[4,6,8,10,13,37] In animal models and in in vitro studies, it has been demonstrated that recombinant osteocalcin regulates insulin biosynthesis, by stimulation Ins1 and Ins2 gene expression in pancreatic islets, and is also a potent insulin secretagogue because of its ability to increase cytosolic Ca^{2+} levels.[12,13,40,41] This mechanism may contribute to the observed selected symptoms of insulin resistance in obese patients in our human study.

Using an animal model of HFD-fed mice, Wei et al[7] determined that the severity of glucose intolerance and insulin resistance is in part a consequence of osteoblast-dependent insulin resistance. In obese individuals, total OC has been described to be associated with skeletal muscle but not hepatic insulin sensitivity, whereas undercarboxylated OC is uniquely associated with β-cell function only in individuals with impaired fasting glucose.[42]

Insulin resistance in osteoblasts led to a decrease in circulating levels of the Glu-OC observed in our human study, thereby decreasing insulin sensitivity in skeletal muscle. The underlying mechanism is not yet established, but besides promotion of insulin receptor ubiquitination (observed increased activity of SMURF1) and subsequent degradation in the proteasome, other mechanisms are possible. For example, the content in tissues of total diacylglycerols (DAGs), a group of lipid intermediate metabolites thought to account for lipotoxicity and insulin resistance, is significantly elevated under obesity. DAGs activate several serine kinases, such as c-Jun amino-terminal kinase, protein kinase C, and IκB kinase β (IKKβ), which phosphorylates inhibitory serine residues of the insulin receptor and insulin receptor substrates and thereby blocking insulin action.[43] IKKβ phosphorylation leads to the activation of nuclear factor-κB–mediated pathways, including the inflammatory one. These results underscore the involvement of bone (among other tissues) in the disruption of

glucose homeostasis resulting from lipotoxicity and involvement of insulin and osteocalcin cross talk in glucose intolerance.[4,6–8,10,13,44]

Along with this, is the recent demonstration that disrupting osteocalcin signaling in humans leads to glucose intolerance. Using mouse models, Oury et al found that osteocalcin and LH act in 2 parallel pathways and that osteocalcin-stimulated testosterone synthesis is positively regulated by bone resorption and insulin signaling in osteoblasts.[10] In humans, they analyzed a cohort of patients with primary testicular failure and identified 2 individuals harboring the same heterozygous missense variant in one of the transmembrane domains of GPRC6A, which prevented the receptor from localizing to the cell membrane. It was a point mutation in exon 4 of GPRC6A, resulting in F464Y amino acid substitution. This missense mutation affects a highly conserved residue, occurring in one of the transmembrane regions of the molecule and preventing its localization to the cell membrane, therefore resulting in a loss of function of GPRC6A. Patients harboring this substitution-mutation demonstrated higher fasting insulin and glucose levels as well as pathological OGTT parameters. Thus, insulin resistance in bone might contribute to whole-body insulin resistance in patients with type 2 diabetes.[10] Interestingly, it has recently been determined that the known detrimental effect of glucocorticoids on glucose metabolism could be in part explained by its negative action on osteocalcin production.[45] Recent publications in mouse models suggest that the protective effect of osteocalcin on obesity and insulin resistance might be, at least in part, due to its capacity to increase adiponectin release and energy expenditure in brown adipose tissue and skeletal muscle.[12,13,15] As mentioned previously, we did not observe any correlation between osteocalcin forms and adiponectin. Similar to our results, Wang et al[46] reported that total osteocalcin inversely correlated with HbA1c, and Glu-OC inversely correlated with fasting blood glucose. However, no significant correlation was found between osteocalcin and HOMA-IR.

The implementation of the Gla/Glu or Glu-OC/total OC ratio was suggested for finding an association between Glu-OC and metabolic syndrome parameters in children.[47] In our study, the presence of biochemical markers of prediabetes resulted in an increase in the Gla-OC/Glu-OC ratio value in comparison with healthy obese subjects. Of note, the Gla/Glu ratio value correlated positively with insulin resistance markers in obese patients and negatively with markers of inflammation in nonobese controls and in obese patients with metabolic disturbances.

However, the study has potential limitations, namely, an unequal number of both sexes and the small number of subjects in the study. Another limitation of the study is the small number of control group participants in comparison to obese subjects. So far, osteocalcin findings have shown that Glu-OC and Gla-OC are bystander markers rather than mechanistic ones. Data from human studies concerning these 2 markers are rather inconsistent, and their mechanism of action is not fully understood. Therefore, we postulate that the results of our study could be a base for further studies explaining the mechanism of osteocalcin action in larger groups of subjects. Our results argue for the suggestion that the decreased blood concentration of Glu-OC may be an early symptom of insulin resistance development in obesity, whereas the decreased level of Gla-OC seems to be connected with early symptoms of obesity associated inflammation in humans.

CONFLICT OF INTEREST

None declared.

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