Metabolism Open 20 (2023) 100264

Contents lists available at ScienceDirect

Metabolism Open

journal homepage: www.sciencedirect.com/journal/metabolism-open

Plasma undercarboxylated osteocalcin dynamics with glycemic stress reflects insulin sensitivity and beta-cell function in humans with and without T2DM

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ARTICLE INFO

Keywords:
Adipokines
Alkaline phosphatase
Hyperinsulinemia-euglycemic clamp
OGTT
Bone

ABSTRACT

This study aimed to better understand the relationship between bone-related biomarkers and nutrient stress in the context of metabolic health. We investigated plasma osteocalcin (OC) during an oral glucose challenge and experimental hyperinsulinemia in Type 2 diabetes (T2DM) and lean healthy controls (LHC). Older individuals with obesity and T2DM (n = 9) and young LHCs (n = 9) underwent a 75g oral glucose tolerance test (OGTT) and a 40 mU/m^2/min hyperinsulinemic-euglycemic clamp. Plasma undercarboxylated OC (ucOC) and total OC were measured at baseline, 60mins, and 120mins of the OGTT and clamp via ELISA. In addition, plasma alkaline phosphatase (ALP), leptin, adiponectin, Vitamin D and insulin were measured and indices of insulin sensitivity and β-cell function were derived. The T2DM group had lower ($p<0.05$) ucOC and ucOC:total OC ratio than LHC during both the OGTT and clamp. Further, baseline ucOC was positively correlated to indices of β-cell function and negatively correlated to indices of insulin resistance when both groups were combined (all $p<0.05$). Suppression of OC observed in T2DM may be related to glucose intolerance and insulin resistance. Similarly, our data suggest that the observed phenotypic differences between groups are likely a product of long-term glucose dysregulation rather than acute flux in glucose or insulin.

1. Introduction

Type 2 diabetes (T2DM) is a multifaceted disease with many related complications, most commonly, micro- and macrovascular disease [1]. However, evidence suggests that the skeletal system is also subject to pathological changes such as altered bone mineral density (BMD) [2], increased glycosylation of collagen [3], and decreased bone blood flow [4], leading to reduced bone quality and increased fracture risk [2,5]. The pathogenesis of the elevated fracture risk remains elusive, but is likely mediated through multiple complex and interrelated mechanisms sensitive to obesity, inflammation, hyperinsulinemia, and hyperglycemia [6,7].

In addition to regulating whole-body glucose homeostasis, insulin plays a critical role in mediating bone health as an osteogenic hormone. Insulin signaling cascades within osteoblasts triggers bone resorption via osteoclasts and activates osteocalcin (OC) by converting it to the undercarboxylated form (ucOC) [8], representative of fewer than three terminals that are γ-carboxylated. Interestingly, ucOC has been implicated as a regulator of glucose metabolism [9]. Once in circulation, ucOC has been demonstrated to stimulate β-cell insulin secretion [10], further highlighting the intimate relationship between OC and insulin. Independent of its effect on insulin secretion, ucOC enhances insulin sensitivity in peripheral tissues through stimulating adiponectin release, as shown in experiments utilizing heterozygote Osteocalcin +/- and Adiponectin +/- mice [11]. Adiponectin is an insulin-sensitizing agent that upon release from adipocytes and binding to adiponectin receptors

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https://doi.org/10.1016/j.metop.2023.100264
Received 6 October 2023; Received in revised form 15 November 2023; Accepted 22 November 2023
Available online 2 December 2023

1 and 2, increases AMP-activated protein kinase and peroxisome proliferator-activated receptor-alpha activity and upregulates fatty-acid oxidation and peripheral tissue glucose uptake [12].

Despite these known signaling pathways, the relationship between bone and insulin metabolism in humans remains unresolved. Serum OC is inversely associated with BMI and body fat percentage [13], and is markedly lower in individuals with T2DM [14,15]. However, the acute effect of insulin on OC and bone metabolism in humans has been minimal and inconsistent [16–18], especially in the context of T2DM. Further, differential responses of OC to physiological hyperinsulinemia (i.e. postprandial) compared to experimental hyperinsulinemia (i.e. hyperinsulinemic-euglycemic clamp) have not been examined and most of the research to date has come from epidemiological studies in humans and rodent experiments.

To meet this need and to better understand the role of insulin resistance on key markers of bone health, we examined OC in response to an oral glucose tolerance test (OGTT) and to experimental hyperinsulinemia with euglycemia via hyperinsulinemic-euglycemic clamp. We were also interested in understanding the effects of acute glucose stimulation (with physiologically concomitant hyperinsulinemia) on plasma OC. Importantly, we studied both the active and total forms of OC, which in not routinely presented in the literature. Our study compared the response of older individuals with T2DM to that of young, lean, healthy, controls (LHC). The LHC group served as a standard of optimal human health. We hypothesized that compared to the LHC group, the T2DM group would have depressed total and undercarboxylated plasma OC at baseline. Further, compared to LHCs we expected T2DM individuals to have blunted ucOC responses to physiological and experimental hyperinsulinemia. Last, we hypothesized that ucOC would be related to measures of insulin sensitivity and β-cell function.

2. Materials and methods

2.1. Study design

Indices of bone health were examined in response to acute hyperinsulinemia and oral glucose challenge. All individuals were metabolically phenotyped into two distinct cohorts (LHC, T2DM) by use of clinical laboratory assessments, an oral glucose tolerance test (OGTT), and a hyperinsulinemic-euglycemic clamp after a period of metabolic standardization. Participant characteristics and experimental outcomes from these cohorts have been previously published [19–21], however this is the first report aimed at understanding bone biomarkers and interorgan cross-talk.

2.2. Participants

Participants (n = 18) were recruited from the greater Chicago area. Individuals in the LHC group were young, had a normal BMI (18.5-25 kg/m^2) and were free from known medical conditions, while individuals in the T2DM group were older, had obesity (BMI>30 kg/m^2) and had a clinical diagnosis of T2DM. All participants were non-smokers with normal kidney function and were screened using a medical history questionnaire, physical exam, and fasting blood work. Standard protocols were used to measure height and weight, and body composition was assessed using a dual energy X-ray absorptiometry (DEXA; Lunar, Madison, WI). Participants provided informed consent prior to enrollment and study procedures were approved by the University of Illinois at Chicago's Institutional Review Board (IRB # 2012-0362).

2.3. Metabolic control

Participants underwent a 72hr period of metabolic control prior to both experimental visits (OGTT, hyperinsulinemic-euglycemic clamp). Experimental visits were performed within one week of each other. This control period consisted of maintaining a 3-day diet record and refraining from over the counter supplements for 72hrs, purposeful exercise for 48hrs, and caffeine and alcohol for 24hrs. Participants fasted (no food or drink other than water) for 12hrs prior to each visit and were asked to withhold all medications the morning of each visit.

To control for muscle and liver glycogen stores, participants were instructed to consume approximately 55% of their kcals as carbohydrates on the day prior to the OGTT and hyperinsulinemic-euglycemic clamp. For the OGTT, a 3-day diet record was collected and it was verified that participants followed the dietary control recommendations. The night before the hyperinsulinemic-euglycemic clamp, participants were provided a balanced (55% carbohydrates, 35% fat, and 10% protein) dinner based on their estimated energy requirements, and diet records were collected for all other meals.

2.4. Glucose tolerance test

Glucose tolerance was measured by a 75g OGTT. Blood draws were performed at baseline and then every 30mins following glucose ingestion for 120mins total. LHCs were excluded if the OGTT indicated impaired fasting glucose or impaired glucose tolerance as defined by the American Diabetes Association [22].

2.5. Insulin sensitivity assessment

Whole body insulin sensitivity was assessed using a hyperinsulinemic-euglycemic clamp, as previously described [19–21]. Briefly, for a period of 120mins, insulin was infused at a constant rate (40mU/m^2/min) after a 10 min titrated prime while glucose (20% dextrose) was infused at a variable rate to clamp blood glucose at 90 mg/dL [23]. During the clamp procedure, blood glucose was measured every 5mins on a YSI glucose-lactate analyzer (YSI 2300; STAT Plus, Yellow Springs, OH), and additional blood samples were taken every 15mins for analysis of plasma metabolites. Clamp-derived glucose disposal rate (GDR) was calculated as described previously [24]. Subject glucose means (mg/dL) in the steady state period (~90-120 min) were 90 ± 1 with a CV of 5.9 ± 0.7 % for the LHC group and 88 ± 4 with a CV of 4.8 ± 0.7% for the T2DM group.

2.6. Plasma metabolites

Blood was collected in EDTA collection tubes at baseline, 60mins, and 120mins during the OGTT and hyperinsulinemic-euglycemic clamp. Blood was immediately centrifuged, and plasma was stored at -80C until further analysis. Plasma ucOC, total OC, adiponectin, and leptin were measured via commercially available ELISAs (all R&D, Minneapolis, MN, except ucOC: Takara, Shiga, Japan) per manufacturer's protocol. Insulin, C-peptide, total 25-OH vitamin D and alkaline phosphatase (ALP) were measured via automated clinical platform, and glucose was measured on a YSI glucose-lactate analyzer as mentioned above.

2.7. Calculations and statistics

In addition to whole body insulin sensitivity measured via the clamp, we calculated several insulin sensitivity and β-cell function indices from the OGTT to best characterize our cohort. Homeostatic model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function (HOMA-B) were calculated according to Matthews et al. [25]. Insulinogenic index (IGI), another marker of β-cell function, was calculated as 30min OGTT insulin minus fasting insulin divided by 30min OGTT glucose minus fasting glucose. Matsuda index was calculated as 10,000 divided by the square root of the product of fasting glucose multiplied by fasting insulin and mean glucose multiplied by mean insulin over the 120min OGTT [26]. Finally, the oral disposition index was calculated as $\Delta Insulin_{0\text{-}30mins}$ divided by $\Delta Glucose_{0\text{-}30mins}$ multiplied by 1/fasting insulin [27].

Statistical analyses were performed using SPSS Statistics 23 (IBM, Armonk, NY). Data were checked for normality by the Shapiro-Wilk test, and non-normally distributed data were Log10 transformed. Differences between group (LHC vs. T2DM) and time (baseline vs. 60mins vs. 120mins) were assessed via a two-way repeated measures ANOVA and subsequent Bonferroni post-hoc test. The ANOVAs were run on the Log10 transformed, normally-distributed, data; however, the associated figures represent raw data values. Sex, age, and BMI were tested as covariates in the ANOVA analyses to determine whether each variable significantly influenceds the relationship between either group or time on the dependent variable. Where covariates were significant, they were included in the ANCOVA and reported. An independent samples *t*-test was used to compare calculated health indices and clamp-driven changes between groups. To determine relationships between OC, ALP and plasma leptin, adiponectin, and indices of metabolic health, Spearman's rho nonparametric bivariate correlation coefficient was used. Significance was set at $p<0.05$ and all raw data are presented as mean ± SEM. The *, #, and & symbols reflect significant effects of group (LHC vs T2DM), time, or an interaction, respectively. If no symbol is present, there was no statistical difference compared to the control group.

3. Results

3.1. Participant characteristics

Baseline participant characteristics are reported in Table 1. By design, the T2DM group was older and had a greater BMI than the LHC group. Additionally, the T2DM group was more insulin resistant, glucose intolerant, had lower β-cell function, and had higher total BMD. The number of individuals in the T2DM group on the following medications for management of their T2DM was as follows: sulfonylureas (3), DPP-4 inhibitors (2), insulin glargine (3), and metformin (3). When medication use was cross-referenced against OC responses, no apparent effects of the medications were identified.

Table 1
Subject characteristics and metabolic data.

Variable (units)	LHC	T2DM	p value
n (M,F)	9 (3,6)	9 (5,4)	
Age (y)	28 ± 1	58 ± 4*	<0.01
BMI (kg/m^2)	22.0 ± 0.9	34.1 ± 2.1*	<0.01
Body Fat (%)	23.9 ± 2.0	40.2 ± 2.8*	<0.01
Bone Mineral Density (g/cm^2)	1.2 ± 0.04	1.3 ± 0.04*	0.02
Total 25-OH Vitamin D (ng/mL)	29.3 ± 1.8	28.1 ± 4.7	0.81
Fasting Insulin (μU/mL)	5.5 ± 0.7	7.9 ± 1.2	0.09
Fasting Glucose (mg/dL)	90 ± 4	129 ± 16*	0.03
Fasting C-peptide (ng/mL)	1.5 ± 0.1	2.0 ± 0.3	0.21
HbA1c (%)	5.3 ± 0.1	7.2 ± 0.6*	<0.01
OGTT Glucose iAUC (mg/dL/2hr)	2654 ± 428	10827 ± 1797*	<0.01
OGTT 120 min (mg/dL)	102 ± 4	225 ± 36*	<0.01
GDR (mg/kg/min)	9.8 ± 0.4	4.7 ± 0.6*	<0.01
Steady State Clamp Insulin (μU/mL)	62.0 ± 3.9	87.5 ± 6.5*	<0.01
HOMA-IR (AU)	1.3 ± 0.2	2.5 ± 0.4*	0.02
HOMA-B (AU)	76.6 ± 8.0	63.3 ± 14.1	0.42
Insulinogenic Index (IGI)	0.9 ± 0.1	0.3 ± 0.1*	0.01
Matsuda Index (AU)	8.6 ± 1.0	5.9 ± 1.5	0.14
Oral Disposition Index (mMol^{-1})	5.3 ± 1.1	2.3 ± 1.0	0.06

Data are presented as Mean ± SEM. LHC, lean healthy control participants; T2DM, participants with Type 2 Diabetes Mellitus; BMI, body mass index; HbA1c, hemoglobin A1c; OGTT, 75g oral glucose tolerance test; iAUC, incremental area under the curve; GDR, Glucose Disposal Rate as calculated from the hyperinsulinemic-euglycemic clamp; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; HOMA-B, Homeostatic Model Assessment of β-cell function. *$p<0.05$ via independent samples *t*-test.

3.2. Plasma osteocalcin, alkaline phosphatase and total 25-OH vitamin D

At baseline, plasma total OC, ucOC, and the ucOC:Total OC ratio were 25%, 51%, and 47% lower in T2DM compared to LHCs ($p = 0.035$, 0.001, and 0.001 respectively). During a 75g OGTT, there was a significant main effect of group (LHC vs. T2DM) on both Log10-transformed ucOC and the ucOC:Total OC ratio ($p = 0.004$, 0.002 respectively; Fig. 1A, C). In both cases, the T2DM group had depressed ucOC levels compared to the LHC group. However, we did not observe this group effect on Log10-transformed total OC (Fig. 1B) and did not see any effect of time on the OC variables.

During the hyperinsulinemic-euglycemic clamp, there were significant group, time, and interaction effects on Log10-transformed ucOC ($p = 0.019$, <0.001, 0.040 respectively). Post-hoc comparisons of the interaction effect revealed group differences within time at baseline and 60mins, but no group difference at steady state insulin stimulation (120mins). Further, insulin stimulation decreased plasma ucOC at both timepoints compared to baseline (Fig. 2A). We also report a similar main effect of time, and group x time interaction, for Log10-transformed Total OC ($p = 0.002$, 0.035 respectively; Fig. 2B). Last, the T2DM group had a lower ucOC:Total OC ratio during the clamp compared to controls (main effect, $p = 0.035$; Fig. 2C). When covariates of sex, age, and BMI were tested, none were found to significantly adjust the associations reported between group and time on the OC variables either during the OGTT or clamp (Figs. 1 and 2).

Baseline plasma ucOC at the OGTT was significantly correlated with many markers of metabolic health. With both groups combined, but not within either group individually, ucOC was positivity correlated to β-cell function as estimated by IGI (rho = 0.659, $p = 0.003$; Fig. 1D) and negatively correlated to HOMA-IR (rho = -0.547, $p = 0.019$). Similarly, both ucOC and the ucOC:Total OC ratio at baseline of the hyperinsulinemic-euglycemic clamp were positively correlated to insulin sensitivity via clamp-derived glucose disposal rate (rho = 0.507, $p = 0.032$; rho = 0.612, $p = 0.007$ respectively).

Despite changes in plasma OC, there was no group, time, or interaction effect on Log10-transformed plasma ALP during the hyperinsulinemic-euglycemic clamp ($p = 0.098$, 0.580, 0.357 respectively; Fig. 2D). No correlations between fasting ALP or the change in ALP with any of the insulin sensitivity or β-cell function health indices were observed. Similarly, no differences in total 25-OH vitamin D were observed between groups (Table 1) and no correlations were observed with OC, ucOC, ALP, insulin sensitivity, or β-cell function indices.

3.3. Plasma leptin and adiponectin

Given the group and insulin effects on plasma OC, and since ucOC regulates glucose metabolism through adiponectin [11], we investigated the effects of insulin stimulation (clamp) on adiponectin. There was no group, time, or interaction effect on unadjusted plasma adiponectin ($p = 0.055$, 0.056, 0.726 respectively; Fig. 3A). Age was a significant covariate for Log10-transformed adiponectin, revealing a significant difference between the groups after adjustment for age ($p = 0.01$; adjusted means of Log-transformed data are 4.023 and 3.463 for LHC and T2DM, respectively). Further, there were no correlations between plasma adiponectin and any of the metabolic health variables.

While adiponectin is an insulin-sensitizing and anti-inflammatory adipokine [12], leptin is most well-known for regulating food intake and bodyweight [28]. However, leptin also modulates β-cell function and bone metabolism [29], which may contribute to abnormalities in T2DM due to the observed leptin-resistance in diabetes [30]. Thus, we assessed plasma Log10-transformed leptin and found a significant ($p = 0.003$) group effect (Fig. 3B), whereby the T2DM group had 21% higher plasma leptin than the LHC group. The difference between LHC and T2DM remained significant ($p = 0.009$) when covaried for age and BMI. Further, baseline ucOC at the OGTT was significantly correlated with baseline leptin (rho = -0.538, $p = 0.021$; Fig. 3D). This correlation was

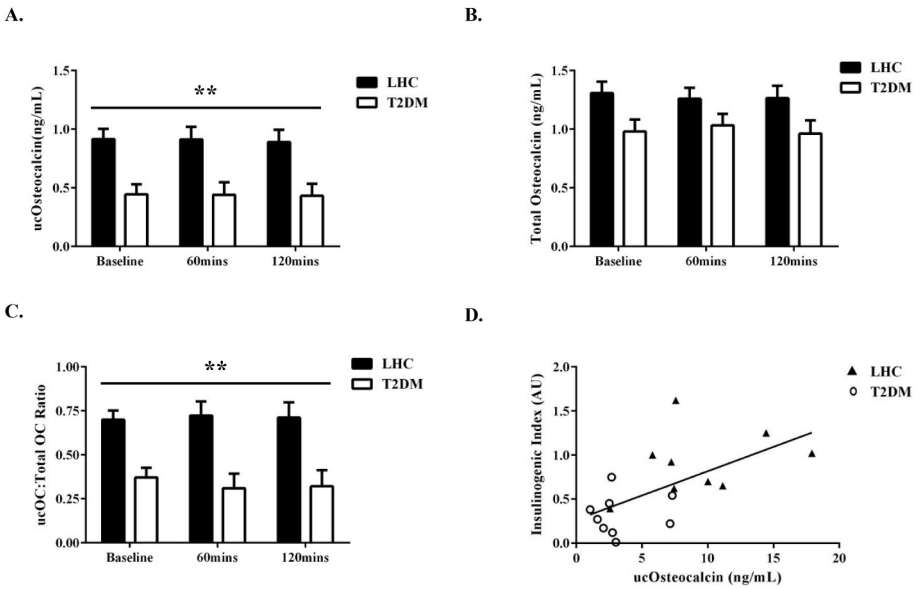

Fig. 1. T2DM present with lower undercarboxylated osteocalcin but oral glucose intake does not change plasma OC. Plasma ucOC (A), Total OC (B), and the ucOC: Total OC Ratio (C) during an OGTT are represented for each group. Log10-transformed data were analyzed via two-way ANOVA and raw values are presented as mean ± SEM. ** Main effect of group $p<0.01$. In both groups combined, baseline ucOC was correlated to the Insulinogenic Index, a marker of insulin secretion (D). The correlation was analyzed via Spearman's rho bivariate correlation coefficient, $p<0.05$.

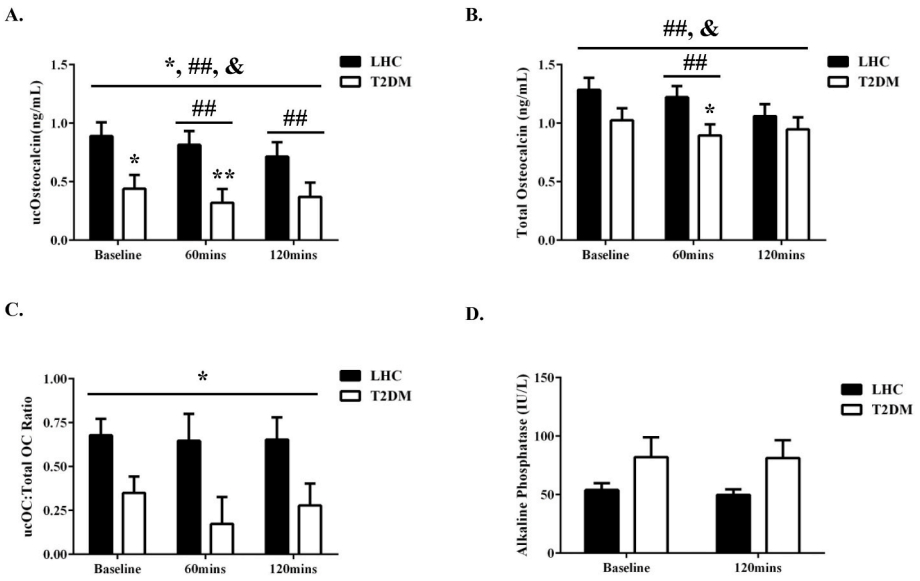

Fig. 2. Insulin-stimulation and T2DM impact plasma osteocalcin but not alkaline phosphatase. Basal and insulin-stimulated plasma ucOC (A), Total OC (B), the ucOC:Total OC Ratio (C) and ALP (D) are represented for each group. Log10-transformed data were analyzed via two-way ANOVA and raw values are presented as mean ± SEM. * Main effect of group or post-hoc difference between groups within time $p<0.05$; ** post-hoc difference between groups within time $p<0.05$; ## Main effect of time or post-hoc difference from baseline $p<0.01$; & Interaction effect $p<0.05$.

not significant when assessed within each group.

The leptin-to-adiponectin ratio serves as an indicator of adipocyte health and has been proven to be a better indicator of metabolic disease compared to either leptin or adiponectin alone [31]. For these reasons, we investigated the leptin-to-adiponectin ratio in the context of diabetes, insulin-stimulation, and in relation to OC, ALP, and other markers of insulin sensitivity and secretion. There was a significant ($p = 0.003$, Fig. 3C) group effect on the Log10-transformed leptin:adiponectin ratio, which remained significant ($p<0.001$) when covaried for age and BMI. More specifically, individuals with T2DM had a 73% higher leptin:adiponectin ratio compared to LHC, suggesting poorer adipocyte health.

4. Discussion

T2DM is a multifaceted disease that when uncontrolled, results in numerous complications including disorders to the skeletal system. Diabetes-related bone disease presents as weakened bone structure and function and its pathophysiology is related to whole body glucose and insulin regulation. Here, we investigated the effect of insulin and glucose via a hyperinsulinemic-euglycemic clamp and oral glucose via a 75g OGTT, respectively, on markers of bone metabolism in two distinct groups: LHC and T2DM. As expected, the T2DM group had lower plasma ucOC and ucOC: Total OC ratio than the LHC group, which agrees with previous meta-analysis work on this topic [15]. Depressed ucOC not

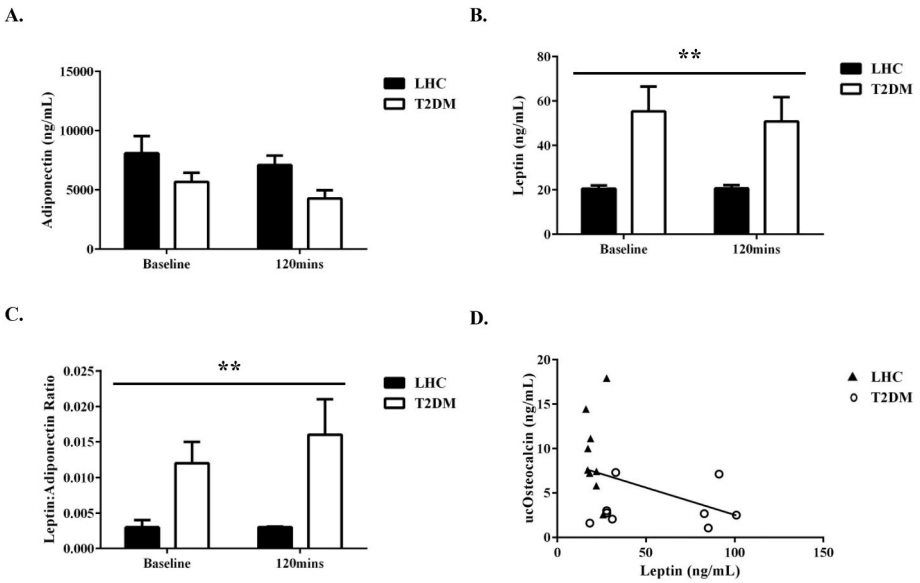

Fig. 3. Group effects on leptin and the adiponectin:leptin ratio and the relationship between leptin and OC. Adiponectin (A), Log10-transformed leptin (B), and the leptin:adiponectin ratio (C) show changes between experimental insulin stimulation and the LHC and T2DM groups. Data were analyzed via two-way ANOVA and raw values are presented as mean ± SEM. ** Main effect of group $p<0.01$. In both groups combined, basal leptin was correlated to baseline ucOC during the OGTT (D) according to Spearman's rho bivariate correlation coefficient, $p<0.05$.

only associates with poor bone turnover but suboptimal glucose metabolism. From prior work we know that both ucOC and OC directly increase basal and insulin-stimulated glucose transport in muscle and adipose cells [32], signal insulin release by pancreatic β-cells, and increases the secretion of adiponectin [11]. Our present work supports OC's role in these insulin-sensitizing processes as we show strong relationships between baseline ucOC and both clamp-derived glucose disposal rate and OGTT-derived insulinogenic index and further, a negative relationship between ucOC and HOMA-IR. These data are novel because unlike previous studies [16,17] because our work spans both physiological (OGTT) and experimental (clamp-derived) metabolic perturbations and can be applies across the metabolic spectrum, rather than specifically to the absence or presence of diabetes.

Mechanistic evidence suggests that not only is OC release from osteoblasts an insulin-dependent process, but that once active, ucOC leads to further insulin secretion [8]. These data lead us to hypothesize that ucOC would increase with insulin stimulation, however in contrast to our hypothesis we report decreased plasma ucOC and total OC under conditions of the hyperinsulinemic-euglycemic clamp. Further, there was no change in the ucOC:Total OC ratio, which signals a uniform decrease with insulin. This effect may be due to compensatory mechanisms in response to the experimental hyperinsulinemia, whereby individuals downregulate their endogenous production of ucOC to prevent further increases in plasma insulin through OC-stimulated β-cell secretion of insulin. Our finding of no difference in ucOC between T2DM and LHC at steady state insulin, further supports this and may suggest that the LHC group is able to adapt and compensate to a larger degree than the T2DM group. In addition, our results agree with previous work in healthy elderly women that reported a 35% and 22% decrease in ucOC and Total OC respectively with a 2hr hyperinsulinemic-euglycemic clamp [16].

We were also interested in understanding the effects of acute glucose stimulation (with physiologically concomitant hyperinsulinemia) on plasma OC. In the present study we report no effect of a 75g oral glucose load on ucOC, total OC, or the ucOC:Total OC ratio. These findings combined with the experimental insulin-stimulated dynamics of OC suggest that acute fluctuations in glucose and insulin do not explain the large baseline differences in ucOC between LHC and T2DM. Further, our correlation analyses suggest that the long-term metabolic status (i.e. insulin resistance) of an individual is more closely associated to plasma ucOC and bone metabolism than the short-term hyperglycemic or hyperinsulinemic excursions that may occur during the day.

Despite well-established evidence of elevated ALP in T2DM [33], we report no statistically significant differences in ALP by group or with insulin stimulation. It is also possible that the group differences were masked by the glucose-lowering effects of diabetes medication, as ALP has been previously associated to fasting glucose levels [34]. Further, our reported changes in OC but not ALP, may be reflective of the temporal changes underlying the pathology of diabetic bone disease. Meaning that, depressed OC and impaired bone turnover may precede increased ALP and subsequent pathological mineralization of soft tissue. Despite evidence that soft tissue mineralization occurs at a greater rate with longer duration of T2DM [35] and poor glucose control [36], this time course remains speculative. A longitudinal study design is needed to answer this question.

Finally, we report higher plasma leptin and a higher leptin:adiponectin ratio at baseline and across the hyperinsulinemic-euglycemic clamp in T2DM compared to LHC. Since OC modulates peripheral insulin sensitivity through adiponectin [11], we also expected to see depressed adiponectin in the T2DM group, however we reported no group differences in the unadjusted means. We believe we were underpowered for this outcome, as its likely that the p value of 0.055 would reach significance with a greater *n* size, especially given that adiponectin is statistically different between groups when covaried by age. However, we only measured total adiponectin rather than the multiple isoforms found in human circulation (high molecular weight, hexameric middle molecular weight, trimeric low molecular weight, and albumin-binding low molecular weight) [37], which may have impacted our results. Our data of elevated leptin and a high leptin:adiponectin ratio in the T2DM group confirms previously established phenotypes of diabetes [28,31,38]. However, our data expands beyond this as we report a negative relationship between plasma ucOC and leptin. This, combined with leptin's powerful role in inhibiting bone formation [29,39], may help explain the mechanisms underlying weakened bone structure in T2DM.

This study investigated OC and ALP dynamics in humans under insulin stimulation, and OC change during an OGTT. The strengths of this study include the measurement of insulin sensitivity with the gold

standard hyperinsulinemic-euglycemic clamp, further characterization of insulin secretion via OGTT-derived indices, the metabolic control period prior to testing, and the use of two distinct metabolic groups. Further, we measured both total OC and ucOC along a dynamic time course, which allowed us to more appropriately look at osteocalcin in the context of regulating glucose metabolism and insulin sensitivity.

However, this study is not without limitations. While we utilized various measures of insulin sensitivity and markers of beta-cell function, we did not include any direct non-invasive measures of beta-cell function or mass [40]. Also, our plasma ALP measurement was not specific to bone-derived ALP. Even though bone-specific ALP is the primary contributor to dysregulated ALP in diabetes, without measuring the bone, hepatic, and renal contributions separately, the translation of our ALP findings is limited. Additionally, we did not account for the potential influences of vitamin K on our findings. Future work should include these measures as the undercarboxylation of OC is a vitamin K dependent process [9]. Similarly, we did not evaluate any potential effects of diabetes medications on our outcomes of interest as it is likely that when examined individually, these medications may have direct effects on osteocalcin metabolism. Our sample size was relatively small, with only 9 individuals in each group and these groups were not matched on age, weight, or sex. With regard to age, our experimental groups were different by design, with the T2DM group being older than the LHC group. The purpose of this was to study a cohort of T2DM beyond the average age of diagnosis [41], and to discern long-term hyperglycemia versus acute hyperglycemia. However, we cannot ignore that aging has been shown to modulate both circulating OC and ucOC [42,43]. For example, lower total circulating OC, but not circulating ucOC, was associated with a higher HOMA-IR in older adults [44]. Similarly, previous clinical reports have found that low OC was associated with impaired glucose metabolism in men and premenopausal women only, and that no association was found in women $\geq$50 [45]. Finally, our two groups had difference BMIs by design, but we cannot ignore previous literature showing correlative relationships between OC and BMI, at least in young men with type 1 diabetes [46]. In an effort to recognize this important body of work on the impact of sex, age, and BMI on OC function, we tested these variables as covariates and in all of our OC outcomes. While we found no significant influence of age, sex, or BMI on group differences in OC, it may be possible that due to our small sample size we are unable to discern the true influences of each of these factors. Therefore, inferences should be interpreted with caution until larger study designs can verify our results.

From a translational perspective, species specific differences in OC exist between mice and humans at the genomic and protein levels [9], complicating the interpretation of human observations on OC's relationship to insulin and glucose metabolism based on pre-clinical literature. While there are several rodent models that also report ucOC as the mechanism by which the skeleton maintains glucose homeostasis [11,47], as well as a clinical investigation describing the ability of acute exercise to increase circulating ucOC in a manner associated with insulin sensitivity [48], conflicting data also exists. In experiments conducted by Hill et al. [32], OC increased glucose transport and insulin sensitivity in cultured adipocytes and muscle cells regardless of its carboxylation status, suggesting that ucOC may not be exclusive as the active isoform. Further, Lambert et al. reported OC knockout rats (generated using CRISPR technology) did not develop glucose intolerance or insulin resistance [49]. The OC-null rats also had better trabecular bone volume, thickness, and density measured using microCT. Altogether these data point to a future need for mechanistic studies to fully verify and elucidate the role and activity of different OC isoforms on insulin dynamics in a species-specific manner.

In conclusion, our study shows marked decreases in plasma ucOC and the ucOC:Total ratio in T2DM compared to LHC, likely representing both reduced bone turnover and impaired glucose metabolism. Further, the lack of OC response to the OGTT, depressed ucOC with insulin stimulation, and no group difference in ucOC at steady state insulin, suggest that acute flux in glucose or insulin are not responsible for the large phenotypic difference in ucOC with T2DM. But rather, our data suggest that it is the long-term metabolic dysfunction (low GDR, IGI, and leptin; high HOMA-IR) in T2DM that is associated with lower active OC and likely subsequent skeletal complications.

Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT author statement

Conceptualization: KNZF, EMB, KKD, JMH
Methodology and investigation: KNZF, EMB, JTM, BKB, VRM, KAV, JMH
Formal analysis: KNZF, EMB, KKD, JMH
Resources: KAV, JMH
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Project administration: JMH
Funding acquisition: JMH, EMB

Declaration of competing interest

The authors have no conflicts to report.

Acknowledgements

This work was supported in part by UIC Honors College Undergraduate Award (EMB), UIC Helen Barton Scholarship (EMB), CTSA Award ULRR029879 (JMH), American Diabetes Association 1-14-JF-32 (JMH), NIH R01DK109948 (JMH). Special thank you to Eileen Weinheimer-Haus for manuscript editing.

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