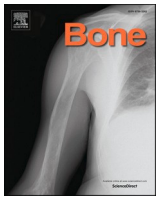
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Review Article



The role of bone in energy metabolism: A focus on osteocalcin

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ABSTRACT

Understanding the mechanisms involved in whole body glucose regulation is key for the discovery of new treatments for type 2 diabetes (T2D). Historically, glucose regulation was largely focused on responses to insulin and glucagon. Impacts of incretin-based therapies, and importance of muscle mass, are also highly relevant. Recently, bone was recognized as an endocrine organ, with several bone proteins, known as osteokines, implicated in glucose metabolism through their effects on the liver, skeletal muscle, and adipose tissue. Research efforts mostly focused on osteocalcin (OC) as a leading example. This review will provide an overview on this role of bone by discussing bone turnover markers (BTMs), the receptor activator of nuclear factor kB ligand (RANKL), osteoprotegerin (OPG), sclerostin (SCL) and lipocalin 2 (LCN2), with a focus on OC. Since 2007, some, but not all, research using mostly OC genetically modified animal models suggested undercarboxylated (uc) OC acts as a hormone involved in energy metabolism. Most data generated from in vivo, ex vivo and in vitro models, indicate that exogenous ucOC administration improves whole-body and skeletal muscle glucose metabolism. Although data in humans are generally supportive, findings are often discordant likely due to methodological differences and observational nature of that research. Overall, evidence supports the concept that bone-derived factors are involved in energy metabolism, some having beneficial effects (ucOC, OPG) others negative (RANKL, SCL), with the role of some (LCN2, other BTMs) remaining unclear. Whether the effect of osteokines on glucose regulation is clinically significant and of therapeutic value for people with insulin resistance and T2D remains to be confirmed.

1. Introduction

Improved understanding of diabetes as a metabolic disease evolved significantly following the discovery of insulin in the early 1900's. Since then, insulin has remained the only pharmacological treatment for people living with type 1 diabetes, and insulin resistance the most common therapeutic target for people living with type 2 diabetes (T2D). For decades, diabetes was viewed from the perspective of bi-hormonal regulation of glucose concentrations, decreasing in response to insulin, and increasing in response to glucagon. However, the discovery of other hormones derived from the gut and osteokines from bone has expanded our understanding of glucose homeostasis and energy metabolism. This review will briefly cover the traditional paradigm of insulin- and glucagon-mediated glucose regulation and its extension to gut-derived nutrient-stimulated (incretin) hormones, before providing a comprehensive review of the role of the circulating osteokine, osteocalcin (OC) in glucose metabolism.

2. Maintenance of glucose homeostasis

Whole body glucose regulation involves a complex interaction between several key tissues including the pancreas, skeletal muscle, liver

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and adipose tissue [1] (Fig. 1). Circulating glucose concentration reflects the 'rate of appearance' (glucose entering the blood stream from food digestion or organ release) versus the 'rate of disappearance' (glucose uptake and utilization by its targeted tissues). This balance is tightly regulated by glucagon and insulin (hormones produced by the α- and β-cells of the pancreas, respectively) [1]. In a fasting state, glucagon prevents hypoglycemia by stimulating gluconeogenesis (a pathway primarily occurring in the liver, and to a lesser extent in the kidneys, whereby glucose is formed from non-carbohydrate substrates), glycogenolysis (the breakdown of stored glycogen to glucose-1-phosphate and glucose) releasing glucose into the circulation for utilization as a fuel substrate in tissues. After a meal (post-prandially), blood glucose rises and in response, insulin is released by pancreatic islet β-cells to counter hyperglycemia, partially by suppressing hepatic gluconeogenesis and glycogenolysis and facilitating hepatic glycogen synthesis and promoting glucose uptake into other organs such as muscle. Transcriptional regulation of rate limiting enzymes and the modulation of enzyme activity through phosphorylation and allosteric regulation are involved [2,3]. Insulin is a peptide hormone that binds to its cell membrane receptors on target cells, acting as an anabolic hormone which promotes the synthesis of fatty acids and triglycerides in the liver and adipose tissue, increases muscle glucose uptake and glycogen storage in skeletal muscle and the liver, and activates/stimulates proteins in a wide variety of tissues (comprehensively reviewed elsewhere [4–7]). Insulin release from β-cells is augmented by incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) secreted from gut enteroendocrine cells in response to nutrient sensing. Insulin plays a crucial role in glucose metabolism by inhibiting hepatic glucose secretion [6,7], by increasing the number of glucose transporters 4 (GLUT4) in the cell membrane of skeletal muscle cells and by triggering the movement of GLUT4 from the cell cytoplasm to the membrane [5,8]. Under normal conditions, around 60–70 % of oral glucose ingested is disposed in skeletal muscle due to insulin action [9], making skeletal muscle a key target organ for intervention aimed at glucose control.

Insulin resistance is characterized by the inability of target tissues to increase glucose uptake in response to insulin, due to defects in insulin secretion, insulin action or both [1]. As such, the development of insulin resistance, due to obesity, physical inactivity and/or poor diet, can lead to the development of T2D. As a result, initial therapy for T2D in people who are overweight or obese and insulin resistant commonly involves metformin as an insulin -sensitizer [10]. Sodium-glucose cotransporter-2 (SGLT2) inhibitors promote renal excretion of glucose, lowering glucose concentrations with cardioprotective and renal benefits [11,12]. Dipeptidyl peptidase 4 (DPP-4) inhibitors represent a third class of oral agents used for T2D, which inhibit catabolism of GLP-1 and GIP. Formulations of GLP-1 resistant to catabolism, administered via weekly subcutaneous injection, act as incretins while slowing gastric emptying and inducing satiety, resulting in substantial weight loss, improved insulin sensitivity, and reduced risk of cardiovascular events in people with T2D [13]. The use of GLP-1 agonists has extended to treatment of obesity (and thereby prevention of T2D), with weekly subcutaneous administration of semaglutide reducing body weight by 15 % over 68 weeks [14]. A dual GLP-1 and GIP agonist, tirzepatide, is efficacious for treatment of T2D [15], and also obesity with optimal dosing achieving a 20 % reduction in body weight over 72 weeks [16]. A triple GLP-1, GIP and glucagon agonist shows even greater promise as obesity therapy [17]. These potent incretin-based pharmacotherapies achieve dramatic fat loss, but also result in significant loss of lean i.e., muscle mass. Thus, there is renewed interest in the preservation of muscle mass both as a bulwark against age-related frailty, and for the role of muscle as a metabolically active organ regulating glucose and energy metabolism.

Under normal circumstances, muscle is a major metabolically active site for glucose uptake and utilization. Muscle contraction (exercise)-stimulated glucose uptake and insulin-stimulated glucose uptake occur via two distinct intracellular signaling pathways [18–20]. This is

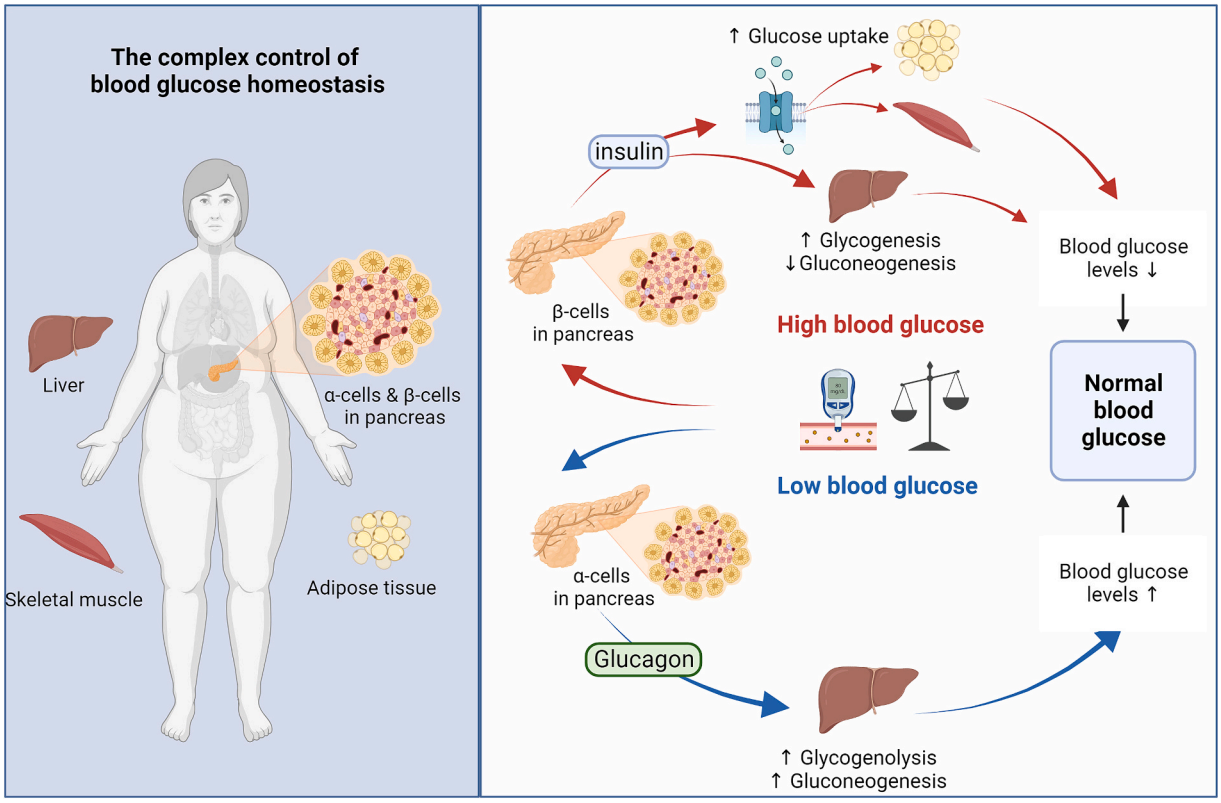


Fig. 1. Maintenance of normal glucose homeostasis by insulin and glucagon.
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important because the insulin dependent pathway in patients with T2D is impaired (insulin resistance) while the insulin independent pathway is intact. Indeed, muscle glucose uptake in patients with T2D is normal during exercise [21]. These data are clinically important as they highlight that insulin independent-stimulated muscle glucose uptake may be targeted for treatment in T2D, utilizing pathways that can "bypass" the ineffective insulin receptor. In addition to exercise, it is likely that other factors may activate similar insulin-independent cellular pathways to promote glucose uptake by skeletal muscle, offering possible future pharmacological targets for T2D. Given the musculoskeletal system arises from the same mesodermal origin during embryonic development, in recent years there has been a great deal of interest in the role the skeleton may play in regulation of glucose control, and metabolism more broadly.

3. Does bone have a role in energy metabolism?

Until recently, the skeleton was considered to have two major roles: protection and locomotion. To fulfil these roles, the skeleton must be both strong, to protect internal organs and prevent fractures, and light, to facilitate locomotion [22,23]. It is now recognized that bone is a highly dynamic organ, constantly remodeling and adapting to the environment. For instance, it is a mechano-sensing organ, with the ability to sense change in individual load (body mass change), and external and environmental loads (physical (in)activity), where a lack of loads (space flight, bed rest, immobilization) promote bone loss [24]. Further, bone has the ability to adapt based on the loads and forces placed upon it such as that experienced during exercise [24]. It is, therefore, well accepted that bone is a self-repairing and metabolically active organ, with capacity to change its mass, shape and properties in response to mechanical perturbations placed on the system. This adaptation is achieved via a process termed bone remodeling, with the bone cellular machinery responsible for the maintenance and integrity of bone material, composition, structure and strength.

Bone remodeling in the adult skeleton is a continuous process, involving the coordinated actions of both osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells) who work in tandem (coupled) to remove and replace pockets of bone [25,26]. This process is coordinated by both osteocyte- (mature long living cell) and osteoblast-secreted factors regulating osteoclastic activity and resorption [27]. Osteoclasts travel to bone surfaces via the circulation where bone resorption begins as a result of the secretion of hydrogen ions and hydrolytic enzymes. Osteoblastic cells communicate with osteoclasts and are recruited to resorption pits where they then secrete bone matrix proteins for the scaffolding of new bone [28]. Given bone remodeling requires energy, it is suggested that to some degree bone plays a role in whole body energy metabolism [29,30]. Over the past 10–15 years, several secreted osteokines have been implicated as endocrine regulators of energy metabolism as well as mineral homeostasis. This review will give an overview of key osteokines based on the available direct and observational evidence and provide a more focused overview on osteocalcin (OC) given the extensive research efforts on this hormone and the large evidence base available.

3.1. Bone turnover markers

Bone turnover markers (BTMs) are used widely in both the research and clinical practice setting and can be used in the diagnosis and management of several bone diseases including osteoporosis [28]. There is some correlative evidence that BTMs are lower in people with diabetes. For instance, a meta-analysis of 22 studies (20 cross sectional, 2 cohort studies) reported that C-terminal cross-linked telopeptide (CTX, a marker of bone resorption) and OC levels are lower in individuals with diabetes (Type 1 diabetes, T1D, and T2D pooled) compared to healthy controls [31]. There was large heterogeneity between studies included in this pooled analysis with different assays used to measure BTMs, and not all studies used fasting samples. In a prospective cohort study of 2966 community-dwelling older men, higher procollagen type 1 amino terminal propeptide (P1NP, a marker of bone formation) and CTX levels were related to lower diabetes risk [32]. The aforementioned data are limited by being associations in nature, rather than demonstrating causation, so future research should explore the direct effects of BTMs on glucose metabolism.

3.2. RANKL-OPG pathway

The receptor activator of nuclear factor-kB ligand (RANKL)/the receptor activator of NF-kB (RANK)/osteoprotegerin (OPG) axis is regarded as an essential signaling pathway modulating bone metabolism [33]. OPG serves as a soluble RANKL decoy receptor, mainly produced by osteoblasts. Denosumab, a monoclonal antibody against RANKL, is an effective anti-resorptive therapy for osteoporosis [34]. This pathway has also been shown to play a role in skeletal, smooth and cardiac muscle [35]. Emerging evidence now demonstrates that RANKL and RANK are found not only in bone, but also other organs including the liver and pancreatic β-cells, adipose and skeletal muscle and other tissues, suggesting that it may be involved in glucose metabolism [33].

RANKL is a major osteoblast mediated factor regulating osteoclast differentiation and maturation, and is a member of the TNF family that is involved in bone remodeling and particularly bone resorption [36]. However recent data suggested that the RANKL can act outside of bone on other targeted tissues including skeletal muscle, negatively affecting muscle mass and metabolism, which negatively impact insulin sensitivity [37]. A recent comprehensive review about RANKL biology and its interaction in the immune system has recently been published [36].

Higher serum OPG has been reported in both patients with metabolic syndrome (MetS) and T2D compared to controls [38–41]. Experimental evidence also suggests that the RANKL-RANK-OPG pathway may be involved in glucose regulation [42]. For instance, circulating and tissue OPG is increased in mice fed a high-fat diet (HFD), a suggested experimental model for MetS [40]. In a different study, serum OPG increased early after diabetes induction in Apolipoprotein E (ApoE)-null mice and littermate mice, which was related to high blood glucose levels [41]. Exogenous treatment of OPG in mice was found to induce proinflammatory changes in metabolic and adipose tissues, similar to the characteristics of a HFD mouse model [40]. Another study highlighted the potential importance of OPG in pancreatic β-cell physiology. This study demonstrated that the RANKL/RANK pathway impairs β-cell proliferation in both human and rodent islets, which OPG treatment was able to overcome [43].

Epidemiological and experimental evidence suggests that RANKL, a known mediator of osteoclast differentiation, may be involved in glucose metabolism [44]. In this study, it was shown in a prospective cohort (n = 844) that higher serum RANKL level is an independent predictor of T2D. Further, blocking of RANKL signaling in genetic and nutritional mouse models of T2D resulted in improved hepatic insulin sensitivity, plasma glucose levels, and glucose tolerance. This suggests that RANKL may be involved in the pathogenesis of T2D, and that anti-osteoporotic medications targeting RANKL signaling may have beneficial effects on glucose metabolism, such as a reduced risk of T2D when using denosumab for osteoporosis therapy [45].

3.3. Sclerostin

Sclerostin, predominately produced by osteocytes, is a negative regulator of bone formation via its action on the Wnt signaling pathway [46]. Romosozumab, an anti-sclerostin antibody, is used for osteoporosis therapy [47]. Recent findings suggest that sclerostin might be involved in the negative regulation of glucose metabolism. A cross sectional study reported that sclerostin levels are higher in T2D patients compared to controls, independent of age and sex [48]. Another study reported the sclerostin levels are higher in people with impaired glucose

regulation (pre-diabetes) compared to those with normal regulation, with sclerostin levels related to plasma insulin and insulin resistance as measured via the oral glucose tolerance test (OGTT) and euglycemic-hyperinsulinemic clamp [49]. In a prospective cohort study (n = 1778), sclerostin levels were associated with fasting insulin levels and HOMA-IR, but not associated with risk of incident T2D [50]. These human data suggest sclerostin may play a role in glucose metabolism, while causality has been assessed in several mechanistic studies using rodent models. For example, elevated glucose levels have been shown to directly regulate osteocyte function through sclerostin expression in vitro and in vivo using a diabetic rat model (rats treated with streptozotocin) [51]. Compared to mice fed a control diet, obese mice fed a HFD had lower bone mineral density and bone formation ratio alongside higher sclerostin expression [52]. In another study, compared to control littermates, mice deficient in sclerostin ($Sost^{-/-}$) have higher bone mass and lower visceral and subcutaneous fat mass, as well as improved insulin sensitivity [53]. During the hyperinsulinemic-euglycemic clamp, following injection of 2-[^{18}C]-deoxyglucose, compared to wild type litter mates, $Sost^{-/-}$ mice had increased insulin-stimulated skeletal muscle (gastrocnemius) glucose uptake at lower insulin infusion rates. For in-depth review of the potential role of sclerostin in glucose metabolism we refer readers to a recent review [54].

3.4. Lipocalin 2

Lipocalin 2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL) or 24p32, is a glycoprotein with a molecular weight of 25 kD. LCN2 has been linked to various functions, including neutrophil function [55], regulation of bone homeostasis in response to exercise/inactivity [56] and skeletal muscle regeneration [57]. LCN2 is secreted by multiple organs including adipocytes [58,59] and the kidneys [60], and expressed primarily by osteoblasts in the bone [61]. A thorough review linking LCN2 to metabolic and cardiovascular risk factors has been provided by Lin et al. [62].

Recently, it has been discovered that bone-derived LCN2 plays a role in appetite control and insulin secretion in mice [61]. Increased levels of circulating LCN2 can be used as a pathological biomarker for conditions such as acute kidney injury [63]. Evidence suggests that LCN2 is associated with diabetes [61,64–66]. Studies have also demonstrated that higher levels of circulating LCN2 are linked with obesity, insulin resistance, and dyslipidemia in type 2 diabetes mellitus (T2D) patients, indicating that LCN2 could serve as a serum marker for metabolic symptoms [67,68]. Animal models have also shown that LCN2 can have adverse metabolic effects [69–71]. The molecular mechanisms by which LCN2 affects glucose regulation is not yet fully understood, but it has been suggested via animal models that the bone-derived LCN2 exerts beneficial effects on pancreatic β-cells [61,66] and perhaps regulates brown adipose tissue to enhance thermogenesis and energy expenditure [72–74]. Evidence from cellular models also indicates contrasting direct metabolic effects of recombinant LCN2 in various cell types. In primary pancreatic islets, the administration of LCN2 at physiological levels enhanced insulin secretion in response to glucose [61]. Conversely, in mature 3T3-L1 adipocytes, treatment with LCN2 promoted fatty acid β-oxidation [75]. However, in H4IIe hepatocytes [76] and human adipose tissue [77], LCN2 treatment at similar concentrations actually induced insulin resistance, whereas knocking down LCN2 in 3T3-L1 adipocytes improved insulin sensitivity [76]. It is possible that in addition to varying tissue-specific activities, non-linear dose-response effects might account for the contrasting results, with interventions producing physiological or pathological levels of LCN2. Therefore, it is essential to explore whether varying exposure to LCN2 would lead to distinct metabolic outcomes in future investigations.

4. Osteocalcin

Osteocalcin (OC), a small polypeptide protein of 5.7 kDa, is the most abundant non-collagenous protein within the bone matrix [78]. It is expressed and produced primarily by osteoblasts and although its exact role in bone is unclear, recent evidence suggests it is involved in bone mineralization [78,79]. OC undergoes carboxylation at the 17, 21 and 24 glutamic acid (Glu) residues by γ-glutamyl carboxylase. This carboxylation, which involves vitamin K, increases the affinity of the protein to calcium ions which facilitates the alignment of the hydroxyapatite crystal in the bone matrix [79]. The decarboxylation of OC accrues during bone resorption, in a low pH environment [80] and is then released into the circulation.

In 2007, Karsenty et al. [30] were the first to report that ucOC functions as a hormone, produced by bone with modulatory actions at distant locations that could regulate energy homeostasis. They reported that OC knockout (KO) mice are characterized by reduced insulin sensitivity and glucose tolerance [30], while in a gain-of-function model, mice exhibited improved energy metabolism and resistance to diet-induced body weight gain and metabolic disorders [30]. Since then, using their genetically modified models, the Karsenty group has reported that ucOC is also involved in male fertility/testosterone regulation [81], muscle mass regulation [82], brain development [83] and cognition [83]. These papers have generated great interest as they opened the door for novel pharmacological approaches to treat multiple diseases such as obesity, T2D and muscle atrophy. While some have replicated these findings, this has not been consistently shown in several recent independent studies using OC-deficient rats [84] and other models of OC KO mice [79,85]. For instance in the study by Lambert et al. [84], in contrast to earlier OC-deficient mouse models, the OC-null rat showed similarly affected bone structure and function (increased trabecular bone and bone strength) but did not develop obesity, insulin resistance or glucose intolerance. The authors suggested that the rat model may be a more appropriate animal model system to investigate OC function, with data from mouse models having limited translation to humans related to genomic differences (discussed further in Section 5.1). Since this study, other labs using different OC-deficient mouse models have recently reported similar findings to the rat model, which are in contrast to findings earlier reported by Karsenty's group. For instance, Diegel et al. [85] reported that bone strength, serum glucose and male fertility in OC-deficient mice with $Bglap/2p^{Pro25fs17Ter}$ allele was not significantly different from wildtype mice. Further, Moriishi et al. [79] using an OC-deficient model by deleting Bglap and Bglap2, reported that OC is not involved in bone quantity, glucose metabolism, testosterone synthesis or muscle mass, but that it is required for bone quality and strength via the alignment of Bap crystallites.

It is not the intention of this review to answer the question why different OC-deficient rodents have such profound metabolic differences. This has been discussed previously [86–88]. Briefly, those authors concluded that genetic, post-translational and environmental factors likely account for much of the conflicting results related to function of G Protein-Coupled Receptor Class C Group 6 Member A (GPRC6A; the putative OC receptor) in animal models [86]. Additionally, the carboxylation status of OC, as well as its calcium coordination, influence OC structure and dynamics contributing to differences in the biological function of OC [89]. Instead, the aim of this review is to determine whether ucOC can act as a hormone, by focusing on its potential role in energy homeostasis using in vivo, ex vivo and in vitro data outside of OC KO animals.

5. Does ucOC have a direct effect on muscle glucose metabolism?

Skeletal muscle plays an essential role in whole-body glucose disposal and energy regulation [90]. It is the principal site of insulin-stimulated glucose uptake, transporting glucose from the blood to the myocyte, where it is then converted into an energy substrate for mechanical work (e.g., exercise) [91,92]. Also, it is a site of contraction-mediated glucose uptake, a process that can occur independently of

insulin. As a result of its functions, skeletal muscle has a key role in whole-body insulin resistance, and therefore is a likely target tissue for ucOC. However, exploring the direct effect of ucOC on whole body and skeletal muscle glucose metabolism in humans is difficult. Therefore, in vitro models using human tissue have been used. In recent years, mechanistic studies that examine the direct effect, or lack of effect, of ucOC on muscle have been conducted using a combination of in vivo, ex vivo and in vitro studies, with the majority of these studies performed in rodents or investigated using human cell lines (Table 1).

One of the first studies providing evidence for the direct effect of ucOC on whole-body glucose regulation in rodents, outside the Karsenty group, was published by Speranza et al. [97]. They reported that osteocalcin plays a critical role in the commonly reported dysregulation of energy homeostasis caused by glucocorticoid administration [97]. They firstly demonstrated that corticosterone (CS) treatment suppresses ucOC by >90 % in wild-type (WT) mice and this decrease was accompanied by the development of insulin resistance and impaired glucose tolerance. In addition, they treated transgenic mice with enzymatic disruption of the glucocorticoid signaling pathway in osteoblasts (Col2.3-:11β-hydroxysteroid dehydrogenase type 2 Tg mice), and found these mice, which had normal circulating levels of ucOC, were protected from glucocorticoid treatment-induced insulin resistance, glucose intolerance and abnormal weight gain [97]. This study provides potential support that osteoblasts and ucOC can directly influence whole-body glucose metabolism.

In an in vivo human model, we reported that exercise leads to increased circulating ucOC levels which in turn was associated with increased whole-body insulin sensitivity [115]. As such, we examined whether ucOC treatment could increase the insulin-sensitizing effects of exercise using an ex vivo rodent model of skeletal muscle contraction followed by ucOC treatment [105]. We reported that ucOC enhanced the insulin-sensitizing effects of muscle contraction in glycolytic muscle by 14 % (extensor digitorum longus, EDL), yet in contrast to our hypothesis, we did not observe changes in basal insulin sensitivity following ucOC treatment. We and others have reported that ucOC treatment in C2C12 myotubes enhances insulin-stimulated glucose uptake [105,114]. As such, it is possible that our previous work was limited by the use of intact muscle which may have inadvertently led to incomplete exposure of ucOC to the myocyte. In follow-up studies, muscles were cut longitudinally to enhance ucOC exposure. We then observed that ucOC alone (at physiological levels) increased basal muscle glucose uptake in mice EDL (fast-twitch) and soleus (slow-twitch) [106], as well as with insulin stimulation, in a muscle-specific manner [107]. It was also reported that ucOC treatment at similar levels can alleviate insulin resistance induced by corticosterone in both glycolytic and oxidative muscles [108]. Treatment with exogenous ucOC was shown to induce myoblast proliferation of C2C12 cells in vitro via PI3K/Akt and p38 MAPK pathway, and myogenic differentiation involving GPRC6A-ERK1/2 signaling, suggesting ucOC was essential for cell growth [112]. However, not all studies are in agreement, with some studies reporting limited or no effect of recombinant ucOC treatment on basal and insulin-stimulated glucose uptake and insulin signaling activity, as well as on glycolysis in mouse muscle tissue or cultured myotubes [100,103,104,113]. One paper reported that ucOC treatment (10 ng/mL daily, for 3 days) blunted insulin-stimulated glucose uptake in C2C12 myotubes [113]. While other studies using L6 muscle cells (1 h treatment 20 ng/mL ucOC) [110] and C2C12 myotubes (3-day treatment with 5 ng/mL ucOC) [114] have reported increased insulin-stimulated glucose uptakes following ucOC treatment. Differences in the model used, source (e.g., prepared within laboratory [114] versus purchased [113]) and dose of ucOC and insulin may explain discrepant findings. Table 1 summarizes the studies of the direct effects of ucOC on glucose metabolism.

Current studies have also explored the molecular mechanisms by which ucOC may directly enhance muscle glucose metabolism Fig. 2. Although the receptor/s of ucOC in muscle cells are still unclear, GPRC6A has been suggested to be one of the most promising candidates [82]. For instance, the knockdown of GPRC6A via RNA silencing in cultured muscle cells was found to abrogate the direct effect of ucOC on basal glucose uptake [93]. Some downstream targets of ucOC/GPRC6A cascade, including extracellular signal-regulated kinase (ERK), AMP-activated protein kinase (AMPK), c-AMP response element binding protein (CREB), and protein kinase C (PKC), have also been suggested to be potential mediators between ucOC and the activation of the insulin signaling pathway (see Fig. 2) [82,106,108]. However, it is likely that some unknown targets of ucOC remain to be discovered. Therefore, to delineate the underlying mechanisms, future studies with gene sequencing and quantitative proteomics and phosphoproteomics are warranted.

While the previous section focused on muscle, ucOC also affects glucose metabolism in other tissues. Other groups have provided evidence that ucOC improves human β-cell function [102]. Mouse kidneys transplanted with human islets and treated in vivo with a vehicle control (PBS) or ucOC (D-OC, 4.5 ng/h for 30 days post-transplant) augmented production of human insulin and C-peptide [102]. In addition, D-OC treatment in vitro of human islets in culture using a dose-response range of 1 to 15 ng/mL augmented insulin content and enhanced human β-cell proliferation [102]. Additionally, the effects of ucOC treatment on metabolic function (e.g., improved insulin sensitivity and glucose uptake or, activation of signaling pathways/proteins involved in glucose metabolism) has been shown in various organs including in vivo models of mouse liver [93,100] and adipose tissue [93,99,104], as well as in vitro in cells including mouse and rat adipocytes [99,103,104,110], hepatocytes [94], human umbilical vein endothelial cells [109], human aortic endothelial cells [111], and human islets [102] (Table 1).

To summarize, considerable experimental evidence exists to support the notion that ucOC has a hormone-like function where it can improve glucose metabolism and insulin sensitivity in muscle, and perhaps other organs. However, not all studies have consistently reported beneficial effects of OC treatment on energy homeostasis, with a select number of studies showing detrimental effects. There are a number of confounding factors that may influence data collection and interpretation, as discussed in Section 5.1.

5.1. *What are the potential confounding factors?*

Although we have minimized discussion of findings generated from genetically modified animals, there are some plausible explanations for conflicting findings reported. Differences of genetically modified animals with suppressed or overexpressed circulatory ucOC may contribute to the disparate findings in the literature (shown in Table 1). It is possible that compensatory mechanisms are activated, or the methods used to generate the specific or modified animal may inadvertently lead to several off-target effects. For instance, several studies have suggested that the CRISPR/Cas9 system, which has been used in two studies to delete *Oc gene* in rats and mice [84,85], can induce a substantial amount of off-target mutagenesis, generating undesired mutations at random sites and thus impacting precise gene modification [117,118]. Furthermore, the differences may also be attributed to the genetic variances inherent to the different mouse models used, as it has been shown that considerable strain-dependent differences in glucose metabolism exist in mouse strains frequently used for genetic manipulation [119]. Whether such differences are the source of discrepancies between studies is not clear, but in any case, highlights that caution must be taken when comparing data from different studies.

The discordance in current findings may have resulted from several common experimental confounding factors, such as the source of animals, administration techniques, the type and source of cell-lines, as well as treatment dose, duration and the muscle conditions (intact or split, resting or following contraction). One factor that needs emphasis is the source of recombinant ucOC used in these studies. Previous studies have reported the use of several types of house-made and commercial ucOC peptides, which may contribute to discordant findings.

Table 1
Direct effects of ucOC on glucose metabolism in various in vivo and in vitro models.

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
Genetically modified animals (excluding OC KO)					
LRP1 endothelial cell-specific inducible knockout mice [93]	OC (150 μg/kg p.day 2 w)	Not reported	↓	↔ INS	↑ *p*-IRS1, *p*-Akt & *p*-GSK3β (muscle, liver), & GLUT4 translocation (muscle)
KKAy mice [94]	ucOC (3, 30 ng/g per day, 4 w)	Not reported	↓	↑ GLU tolerance ↓ INS ↓ HOMA-IR ↓ hepatocyte lipidosis ↓ dyslipidemia	↑ INS stimulated *p*-IRβ, *p*-AKt, *p*-Foxo1 & *p*-GSK3β- liver. ↑ CD36- liver ↓ SREBP1c, ACC & FAS- liver ↓ MCAT- liver
ucOC/drug administration in vivo					
C57BL/6J mice [95]	Subcutaneous 14 d and 28 d osmotic pump recombinant OC (0.03, 0.3, 3, 30 ng/h) or vehicle	WT mice normal diet ↔ 3, 10, 30 ng/h OC dose-dependent ↓ fat pad mass	0.3 and 3 ng/h vs vehicle ↓ 30 ng/h ↔	0.3 and 3 ng/h vs vehicle ↑ serum INS ↑ INS secretion Improved GLU tolerance ↑ IS 0.03 or 10 ng/h ↔ serum INS, INS secretion, GTT vs vehicle 30 ng/h ↑ IS	Effects of OC on islet, β cell and adipocytes (ex vivo) *Islets:* OC 0.03 ng/mL ↑ Ins1, Ins2 OC >0.03 ng/mL INS expression progressively ↓ OC induced expression CyclinD2 and cdk4 *β cells:* OC 0.03–3 ng/mL ↑ Ins1, Ins2, CyclinD2 *White adipocytes:* ↔ adiponectin OC <1 ng/mL, ↑expression OC 10-30 ng/mL Brown adipocytes ↑ Pgca1α and Ucp1 OC > 3 ng/mL In vivo *Pancreas* 0.3 and 3 ng/h ↑ β cells proliferation 30 ng/h ↔ β cells proliferation ↑Mcad and Pparγ 0.3 and 30 ng/h ↑ adiponectin OC 0.3–30 ng/h *White fat* ↑Acyl-CoA, Ucp2 and Pparα OC 30 ng.h *3, 10, 30 ng/h OC* ↓ *Perilipin, Triglyceride lipase*
	Model of diet induced obesity: 1. Normal diet + implanted placebo pellets; 2. HFD + implanted OC (3 ng/h) 3. HFD + placebo pellets	HFD + placebo Obese HFD + OC Gained less body weight ↓ fat pads		HFD + placebo GLU intolerance INS insensitivity ↔ food intake HFD + OC ↓normal TGL Less GLU intolerant & more INS sensitivity vs HFD placebo ↔ food intake	HFD + OC vs placebo *Brown adipose* ↑ Pcg1α, Ucp1
	Model of obesity and GLU intolerance induced by hyperphagia. WT mice injected with gold thioglucose (GTG), implanted with pumps (OC 3 ng/h or vehicle)	GTG mice 3 ng/h OC ↓ body weight, fat mass vs vehicle		GTG vs OC or vehicle ↑ food intake to same as OC and vehicle GTG mice 3 ng/h OC vs vehicle ↓ TGL ↔ BGL, GTT, IS between PBS treated and GTG mice treated with 3 ng/h OC GTG mice+vehicle GLU intolerant INS insensitive	
C57Bl/6J mice fed normal chow or HFD [96]	WT mice normal chow: ucOC (3 or 30 ng/g/day), or vehicle, 16w	↔ body weight	3 ng/g/d 5w only ↓ 30 ng/g/d ≥5w ↓	4 & 8w treatment WT mice ↑ GLU clearance ↑ IS 16 w treatment 30 ng/g/day WT mice ↑ INS secretion	

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
				↑ IS ↑ GLU tolerance	
	8 w HFD v normal chow (ucOC 30 ng/g/day or vehicle)		HFD + vehicle ↑	HFD + vehicle vs normal chow ↑ serum INS GLU intolerance INS resistance ↓ heat production ↓ VO_2 and VCO_2	
		HFD + ucOC vs vehicle ↓ body weight	HFD + ucOC ↓	HFD + ucOC ↑ IS ↓fat pad weight ↑ VO_2 and VCO_2 ↑ heat production ↔ physical activity or food intake	HFD + ucOC 30 ng/g/day 14 w vs vehicle ↑ mitochondria number and size in skeletal muscle ↔ body temperature, Ucp1 or Pgc1α ↔ lipid accumulation liver ↑ TNFα in liver in mice fed HFD which was then normalized by ucOC
Col2.3-11bHSD2 Tg mice [97]	CS in WT & Tg mice (1.5 mg CS p/week), or placebo (28 d)	Tg mice with normal OC are protected from weight & fat gain seen in WT	Not reported	Tg mice are protected from developing IR & GLU intolerance compared to WT following CS treatment. Tg mice treated with CS vs Tg mice placebo: ↔ TGL ↑ Cholesterol	GC suppresses OC expression in osteoblasts: Attenuated reduction in OC levels in Tg mice correlate with protection again CS-induced metabolic dysfunction. Muscle & liver no diff in Gilz & Fkbp5 by GC treatment in WT & Tg mice
ApoE−/− mice fed chow or HFD [98]	OC (daily, 30 ng/g 12 w)	HFD ↓ BW	Chow ↓ HFD ↓	Chow ↓ TC & LDL-C ↔ GLU tolerance ↔ INS tolerance HFD ↓ TC, TG & LDL-C ↑ GLU tolerance ↑ INS tolerance	Chow ↔ TNF-α, IL-1 α, IL-12 p70 & IL-12 p40; ↑ *p*-PI3K, Akt & eNOS HFD ↓ TNF-α, IL-1 α, IL-12 p70 & IL-12 p40; ↑ *p*-PI3K, *p*-Akt & *p*-eNOS
Obese mice [99]	ucOC (3 ng/h, 4w)	↓ WAT ↔ BW	↔	↑ IS ↔ INS	Muscle ↔ GLUT4 protein & Slc2a4 gene expression WAT ↑ GLUT4 protein, Slc2a4 gene expression & *p*-Akt; ↔ Adipoq gene expression; ↓ Tnf, Il-1b, Il-6, Ccl2, Casp1 & Nlrp3 gene expression
Ldlr$^{-/-}$ mice fed WHFD [100]	ucOC (4.5 ng/h, 12 w)	Chow ↔ BW ↔ body fat WHFD ↔ BW ↔ body fat	Not reported	Chow ↔ INS tolerance ↓ liver fat content ↔ liver histology ↔ TGL ↔ cholesterol ↔ phospholipids WHFD ↑ INS tolerance ↓ liver fat content ↔ TGL ↔ cholesterol ↔ phospholipids ↓ pathological changes of NASH	Chow ↔ INS stimulated *p*-Akt in muscle; ↔ plasma AST WHFD ↑ INS stimulated *p*-Akt in muscle; ↔ INS stimulated *p*-Akt- liver; ↓ plasma AST; ↓ Cd68, F4/80 & Cd74 gene expression; ↔ MCP1, TNF, Nlrp3, Ciita & adiponectin gene expression- WAT; ↓ Cd68, Spp1 & Il1b Col1a2 & Col4a1- liver
STZ-induced diabetes fed HFD [101]	OC (30 ng/g, 12 w)	Control ↓ BW & abdominal fat mass Diabetes ↑ BW ↓ abdominal fat mass	Control ↓ Diabetes ↓	Control ↑ INS ↔ GLU tolerance ↑ IPGTT INS ↓ TC & LDL-C ↔ TG & HDL-C Diabetes ↑ INS ↑ GLU tolerance ↑ IPGTT INS	Not reported

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
				↓ TC, TG & LDL-C ↔ HDL-C	
Non-obese diabetic-severe combined immunodeficiency mouse model for in vivo function testing of grafted human islets [102]	D-OC (4.5 ng/h, 30 days)	Not reported	Not reported	↑ human INS ↑ C-peptide secretion	↑ β-cell proliferation (INS/glucagon & Ki67 staining)
C57BL/6 J mice fed HFD [103]	ucOC (3 ng/h, 28 d)	ND ↔ BW ↓ fat pad HFD ↓ BW & fat pad	Not reported	ND ↑ INS ↑ INS tolerance ↑ GLU tolerance ↓ TGL & FFA ↑ energy expenditure HFD ↓ INS ↑ INS tolerance ↑ GLU tolerance ↓ TGL & FFA ↑ energy expenditure	ND ↑ Foxa2, ↓ Pepck – liver; ↑ Pgc1α & Ucp1- adipose; ↑ Nrf1 & Mcad - muscle; ↔ mitochondria number, area & size - liver, adipose, muscle; ↔ Tnfα expression; ↔ *p*-ERK, *p*-eIF2α, *p*-IRE-1α & ATF6β/c-Jun- adipose, liver, muscle HFD ↑ Foxa2, ↓ Pepck- liver; ↑ Pgc1α & Ucp1- adipose; ↑ Nrf1 & Mcad- muscle; ↑ mitochondria number, area & size- liver, adipose, muscle; ↓ Tnfα expression; ↓ *p*-ERK, *p*-eIF2α, *p*-IRE-1α & ATF6β/c-Jun- adipose, liver, muscle.
C57BL/6J mice fed HFD [104]	ucOC (30 ng/g–1 BW, 8w)	ND ↓ BW ↓ fat-pad weight HFD ↓ BW ↓ fat-pad weight	ND ↓ HFD ↓	ND ↓ TGL ↓ FFA ↓ INS ↑ GLU tolerance ↑ INS tolerance ↑ energy expenditure HFD ↓ TGL ↓ FFA ↑ INS ↑ GLU tolerance ↑ INS tolerance ↑ energy expenditure	ND ↑ Pgc1α & Ucp1- adipose; ↑ Pgc1α & Mcad- muscle; ↔ mitochondria number & area- adipose, muscle; ↔ Atg7, p62 & LC3-II- adipose, muscle; ↔ autophagysomes number- adipose, muscle HFD ↑ Pgc1α & Ucp1- adipose; ↑ Pgc1α & Mcad – muscle; ↑ mitochondria number & area- adipose, skeletal muscle; ↓ Atg7 & LC3-II- adipose, muscle; ↑ p62- adipose; ↓ autophagysomes number- adipose, muscle
ucOC treatment in ex vivo muscles					
Eight-week-old male C57BL/6J mice [105]	ucOC: EDL (10 ng/mL, in the presence or absence INS, 60 μU/ml)	Not reported	Not reported	Contraction+ ucOC+INS ↑ muscle GU vs contraction+INS ucOC ↔ muscle GU from baseline ↔ resting IS ucOC post-ex vivo contraction (no INS) ↔ muscle GU vs contraction alone	Muscle GPRC6A expression Contraction+ucOC+INS ↑ *p*-AS160, Akt vs contraction+INS ucOC post-ex vivo contraction (no INS) ↔ *p*-Akt, *p*-AS160 vs contraction alone
Eight-week-old male C57BL/6J mice [106]	ucOC: EDL & soleus splits (0, 0.3, 3, 10, 30 ng/mL 1.5 h)	Not reported	Not reported	EDL ↑ basal GU (10 & 30 ng/mL) Soleus ↑ basal GU (0.3 & 30 ng/mL)	EDL *30 ng/mL:* ↑ *p*-MTOR, *p*-mTOR/*t*-mTOR ratio, *t*-AS160, *p*-ERK2; *3 ng/mL:* ↑ *p*-AS160, *p*-AS160/*t*-AS160 ratio; *3 & 30 ng/mL:* ↔ AMPKα Soleus *3 ng/mL:* ↑ *p*-AS160, *p*-AS160/*t*-AS160 ratio; *3 & 30 ng/mL:* ↑ *p*-ERK2 ↔ AMPKα; *30 ng/mL:* ↑ *t*-AMPKα
Eight-week-old male C57BL/6J mice [107]	ucOC: EDL & soleus muscle splits (0, 0.3, 3, 10, 30 ng/mL 1 h)	Not reported	Not reported	EDL ↔ IS GU Soleus ↑ basal & IS GU	EDL *30 ng/mL:* ↔ *p*-Akt, *p*-AS160,↑ Glut4 Soleus *30 ng/mL:* ↑ *p*-AS160, ↔ Glut4
Eight-week-old male C57BL/6 J mice [108]	Implanted CS slow-release pellets 3 days ucOC (30 ng/mL 1 h)	Not reported	CS vs Placebo ↑ ↑ during ITT (60, 90 min)	CS vs Placebo ↑ INS ucOC treatment ↑ GU EDL & soleus in placebo mice ↔ GU EDL & soleus in CS-treated mice ↑ IS GU EDL CS-treated mice ↔ IS GU soleus CS-treated mice	Placebo EDL *ucOC:* ↑ *p*-mTOR, *p*-AS160 *ucOC + INS* vs *INS* ↑ GPRC6A, *p*-PKCζ/λ CS EDL *ucOC + INS* vs *INS* ↑ *p*-mTOR, *p*-Akt, *p*-AS160/*t*-AS160 ratio, *p*-ERK2, *p*-ERK2/*t*-ERK ratio; ↓ AS160; ↔*t*-mTOR, *p*-mTOR/*t*-mTOR ratio, *p*-AS160, *t*-ERK2, *p*-AMPKα, *t*-AMPKα, *p*-AMPKα/*t*-AMPKα ratio, *p*-PKC

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
				↔ IS GU EDL or soleus placebo mice	Placebo soleus *ucOC* ↑ *p*-mTOR, *p*-mTOR/*t*-MTOR ratio, *p*-AS160, *p*-PKC *ucOC* + *INS* vs *INS* ↑ *p*-mTOR, *p*-AS160 CS- soleus *ucOC* + *INS* vs *INS* ↑ *p*-mTOR, *p*-mTOR/*t*-mTOR ratio, *p*-AS160, *p*-AS160/*t*-AS160 ratio, *p*-PKC; ↔ *t*-mTOR, *t*-AS160, *p*-AS160/*t*-AS160 ratio, *t*-GPRC6A, *p*-ERK2, *t*-ERK2, *p*-ERK2/*t*-ERK2 ratio, *p*-PKC
ucOC treatment in vitro cell culture					
Primary adipocytes (white and brown adipocytes), primary islets, MIN6 cells [95]	*Primary islets* 4 h treatment with OC (0.03, 1, 3, 10, 30 ng/mL) or vehicle *MIN6 cells* Treated with OC (0.03, 1, 3, 10, 30 ng/mL) or vehicle 4 h *Primary adipocytes* 4 h treatment with OC (0.01, 0.03, 1, 3, 10, 30 ng/mL) or vehicle	Not reported	Not reported	Not reported	Effects of OC on islet, β cell and adipocytes *Islets:* OC 0.03 ng/mL ↑ Ins1, Ins2 OC >0.03 ng/mL INS expression progressively ↓ OC induced expression CyclinD2 and cdk4 *β cells:* OC 0.03–3 ng/mL ↑ Ins1, Ins2, CyclinD2 *White adipocytes:* ↔ adiponectin OC <1 ng/mL, ↑expression OC 10-30 ng/mL Brown adipocytes ↑ Pgca1α and Ucp1 OC > 3 ng/mL
Mouse 3T3-L1 adipocytes [99]	ucOC (20 ng/mL, 24 h; pre-treatment: 20 ng/mL 6 h, followed by 20 ng/mL TNF for 18 h)	Not reported	Not reported	Not reported	24 h treatment ↑ expression of Slc2a4 & GLUT4; ↑ Adipoq expression; ↑ Akt phosphorylation after INS ucOC pre-treatment ↓ the NFKB subunit p65 activation in TNF-a induced cells; ↓ Tnf, Ccl2, Nfkb1; ↔ Adipoq; ucOC restored Slc2a4/GLUT4 content & ↓ expression of inflammatory genes after TNF-α challenge
HUVECs [109]	ucOC (5 ng/mL for 4 h), Tun (5 μg/mL for 4 h) INS (10 nM for 10 min) Wortmannin treatment Akti-1/2 treatment (10 μM for 4 h) Palmitate treatment (500 uM)	Not reported	Not reported	Tun ↓ GU ucOC+tun ↑ GU	ucOC+tun vs tun ucOC alleviated Tun-induced ER stress & improved INS signaling; ↓ protein expression ATF4 & CHOP; ↓ *p*-ERK, *p*-eLF2α; ↑ *p*-Akt, IRS-1; ↑ P13k activity in presence of IR Palmitate induced IR, ER stress & impaired INS signaling, ucOC reversed these effects. *ucOC treatment in palm cells* vs *untreated palm cells*↓ *p*-ERK, *p*-eLF2α, ATF4 & CHOP ↓ *p*-Y20 & *p*-Akt
L6 rat myotubes & adipocytes, 10 w old male C57BL/6 J mice & 150–180 g male Wistar rats [110]	OC (Adipocytes: 1 ng/mL cOC & ucOC, 1 h; L6 myotubes: 20 ng/mL cOC) INS stimulation	Not reported	Not reported	Rat adipocytes+cOC ↑ basal GU in cOC treated (dose-dependent) ↑ IS GU in cOC treated (dose-dependent) ↑ IS in cOC treated Rat adipocytes+cOC vs controls ↑ basal GLU oxidation ↔ IS GLU oxidation ↔ basal of IS lipogenesis, lipolysis or antilipolysis Mouse adipocytes +cOC & ucOC ↑ basal GU both cOC & ucOC ↑ IS GU both cOC & ucOC ucOC greater than cOC at ↑ basal GU & ↑ IS L6 myocytes+ cOC vs controls ↑ basal & IS GU	Rat adipocytes+ cOC & ucOC ↓ TNFα secretion in cOC & ucOC treated; cOC treatment ↓ IL-6 secretion, ↔ with ucOC; ↔ MCP-1 with cOC or ucOC Adipose tissue+cOC & ucOC ↔TNFα, IL-6, & MCP-1; ↑ secretion IL-10

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
HAECs [111]	ucOC (0.3–30 ng/mL) LA (100 μmol/L for 16 h) Wortmannin (100 nmol/L for 15 min)	Not reported	Not reported	Rat adipocytes & whole adipose tissue +cOC & ucOC ↑ Adiponectin secretion Pre-treatment ucOC (30 ng/mL) ↓ LA induced apoptosis in IS-HAECS	ucOC vs controls ↑ *p*-Akt, 0.5 to 4 h; ↑ *p*-Akt post ucOC treatment ≥3 - 30 ng/mL ↔ *t*-AKT Wortmannin prevented ucOC induced phosphorylation of Akt. Pre-treatment ucOC+INS before LA *INS*: ↑ *p*-Akt, whereas LA ↓ this IS ↑ *p*-Akt *ucOC + INS* restored *p*-Akt vs controls (pre-treatment wortmannin-blocked this effect) ucOC (0.3 to 30 ng/mL) ↑ *p*-eNOS, addition of wortmannin prevented this ucOC-induced phosphorylation of eNOS; ↑ NO levels vs controls Pre-treatment of ucOC on IS-HAECs LA-induced ↑ in apoptosis in IS-HAECs was significantly inhibited by ucOC pre-treatment. Pre-treatment with wortmannin abolished this anti-apoptotic effect of ucOC.
C2C12 mouse myotubes [105]	ucOC (0.3, 3, 10 & 30 ng/mL, 1 h) INS stimulation	Not reported	Not reported	↑ IS GU – dose-dependent 10–30 ng/mL	Expression GPRC6A detected; ↓ GPRC6A expression in siRNA(siGPRC6A) vs non-transfected, controls.
C2C12 myoblasts [112]	ucOC (doses 0-50 ng/ mL). PI3K inhibitor Wortmannin transfection GPRC6A siRNA	Not reported	Not reported	Not reported	C2C12 proliferation ↑ cell proliferation/ number (dose dependent); ↑ *p*-Akt & *p*-P38 MAPK (10 ng/mL, 24 h) myoblasts express GPRC6A protein & mRNA. Pre-treatment- wortmannin Akt phosphorylation attenuated; ↓ cell proliferation. Inhibition of P38 MAPK (SB203580) Inhibited effect of ucOC of cell proliferation; ↔ Akt; Wortmannin pre-treatment ↓ P38 MAPK; ↔ *p*-ERK with ucOC treatment between groups; C2C12 cell differentiation through *p*-ERK 1/2 (10 ng/mL) GPRC6A siRNA knockdown activation of Akt, P38 MAPK, ERK 1/2 inhibited; ↓ C2C12 cell proliferation. ucOC - C2C12 differentiation vs control ↑ myotubes size (larger); ↑ nuclei per myotubes; ↑ expression muscle-specific protein MyHC (0.1 to 50 ng/mL) ucOC treatment 10 ng/mL- cell myogenesis ↔ P13K/Akt & P38 MAPK pathways; ↑ *p*-ERK1/2; ↔ *t*-ERK Inhibition of ERK 1/2 (U0126) ↓ *p*-ERK 1/2 & expression MyHC; ↔ GPRC6A
C2C12 mouse myotubes [113]	ucOC (1, 10, 100 ng/mL, 72 h).	Not reported	Not reported	↔ basal GU all doses suppressed IS GU- 10 ng/mL ↔ glycolysis all doses	↔ *p*-IRS-1, *p*-Akt & Glut4: 10 ng/mL for 72 h in normal or IR cells induced by hyperinsulinemia.
Human islets in culture [102]	OC (0.3–1.0 ng/mL) & D-OC (1.0 to 15 ng/mL)	Not reported	Not reported	D-OC 7 & 14 d 7d: 1.0, 4.5 & 15 ng/mL ↑ INS content of GLU-	D-OC- 7 d SUR1 ↑ in islets cultured with 4.5 ng/mL 1.0 ng/mL: ↑ %β cell content vs controls

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
				stimulated islets 14 d: 4.5 ng/mL only ↑ INS content OC 0.3–1.0 ng/mL ↔ INS content vs controls	1.0 ng/mL: ↓ %α cell content vs controls ↔ PP cells
C2C12 mouse myotubes [114]	ucOC: GluOC at (5 ng/mL) with & without INS	Not reported	Not reported	GluOC treatment on IS GU INS: GU ↑ dose-dependent manner GluOC: enhanced IS GU	C2C12 myotubes GPRC6A presence GluOC treatment ↑ *p*-ERK dose dependent manner 0.1 to 30 ng/mL; H89 (PKA inhibitor) did not suppress GluOC-induced ERK phosphorylation; U0126 (MEK inhibitor) suppressed ERK phosphorylation below basal. U73122 (phospholipase C inhibitor) ↔ ERK phosphorylation; LY294002 (P13K inhibitor) ↔ ERK phosphorylation but inhibited basal Akt phosphorylation. GluOC pre-treatment 5 ng/mL + IS 20 m: ↑ *p*-Akt & *p*-ERK (no IS); 72 h: + IS ↑ *p*-Akt, ↔ *p*-Tyr; 24 h: ↔ IS Akt, ↔ IRβ GluOC pre-treatment + U0126 Inhibition of MEK abolished GluOC-mediated promotion of INS-induced Akt phosphorylation without affecting basal Akt phosphorylation
Mouse primary hepatocytes [94]	ucOC (0, 3, 30 ng/mL, 24 h) INS stimulation	Not reported	Not reported	Concentration dependent inhibition of hepatic GLU production with & without INS stimulation	Glycogen synthesis ↑ IS GSK3β; phosphorylation
3T3-L1 adipocytes, Fao liver cells & L6 muscle cells [103]	OC (5 ng/mL, 4 h) INS stimulation Tun Inhibitors: wortmannin, Akti-1/2, U0126, NF-kB NF-kB-p65 siRNA transfection XBP-1 siRNA transfection	Not reported	Not reported	Not reported	Adipocytes- Tun ↑ *p*-ERK, eIF2α & IR*E*-1α & expression ATF6β; ↓ IS IRS-1 & Akt OC- adipocytes, liver & muscle - Tun ↓ Phosphorylation of *p*-ERK, *p*-eIF2α & *p*-IRE-1α & expression ATF6β compared to tunicamycin alone; ↑ IRS-1 & Akt INS, Tun & OC with & without inhibitors↑ P13K activity & NF-k β-p65-DNA activity (liver cells under ER stress); Addition of wortmannin or akti-1/2 reversed OC effects on NF-k β-p65-DNA activity; Addition of U0126 ↔. Blocking NF-kB (pyrrolidone dithiocarbamate) & NF-kB -p65 siRNA ↔ OC protective effect on ER stress & impaired INS signaling induced by Tun. XBP-1 siRNA transfection in cells- Tun; ER stress induced, INS signaling impaired' Addition of OC treatment suppressed phosphorylation of *p*-ERK & increased IRS-1.
Mouse adipocyte 3T3-L1 cells & mouse C2C12 [104]	Pre-treatment Tun (5 μg mL' 4 h) then 5 ng mL OC for 4 h Palmitate treatment XBP-1 siRNA Autophagy inhibitor (3-methyladenine or Atg7 siRNA) Inhibitors: Akti 1/2, rapamycin, U0126, NF-kB	Not reported	Not reported	Not reported	Adipocytes & myocytes- tun ↑ ER stress; ↑ *p*-ERK & elF2α; ↓ IS tyrosine phosphorylation IRS-1 & Akt; ↑ autophagy (↑ Atg7 & LC3-II, ↓ p62) Adipocytes & myocytes- tun & OC ↓ *p*-ERK & eIF2α phosphorylation; reversed autophagy (Atg7, p62 & LC3-II); ↑ IS tyrosine phosphorylation IRS-1 & Akt; Restored phosphorylation of Akt & mTOR & maintained sensitivity of Akt to INS Adipocyte & myocyte- palmitate OC treatment alleviated autophagy, ER stress, & INS signaling

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Table 1 (*continued*)

Experimental overview		OC effect on metabolic function			Potential mechanisms
Model	Treatment	Body composition	Blood glucose	Metabolism	
					Transfection with XBP-1 siRNA & OC treatment XBP −/− cells adipocytes & myocytes ↓ expression levels of Tun induced Atg7 & LC3-II protein & *p*-ERK phosphorylation & ↑ expression p62 & IRS-1 phosphorylation. Transfection 3-methyladenine & Atg7 with OC treatment adipocytes ad myocytes ↓ 3-methyladenine- & atg7-induced *p*-ERK phosphorylation & ↑ IRS-1 INS, Tun & OC treated adipocytes & myocytes with inhibitors Akti 1/2 & rapamycin: nullified protective effect of OC. U0126: did not reverse effects of OC on autophagy or ER; pyrrolidone dithiocarbamate: reversed protective effects of OC on autophagy, ER stress & INS signaling

Key: ACC, acetyl-CoA carboxylase; Akt, protein kinase B; ApoE−/−, apolipoprotein E-deficient; Akti-1/2, protein kinase inhibitors; AMPKα, activated protein kinase α; AS160, Akt substrate of 160 kDA; AST, aspartate aminotransferase; ATF6β, paralogue of unfolded protein response (UPR); BGL blood glucose; BW, body weight; Casp1, caspase 1; Ccl2, chemokine (C-C motif) ligand 2; CD, cluster of differentiation; Ciita, class II transactivator; cOC, carboxylated osteocalcin; CS, corticoid steroid; d, day; D-OC, decarboxylated osteocalcin; EDL, extensor digitorum longus; eKO, endothelial cell specific inducible knockout; eIF2α, eukaryotic initiation factor-2α; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinases; F4/80, marker of murine macrophages; FFA, free fatty acid; FAS, fatty acid synthase; Fkbp5, FK506 binding protein 5; Foxo1, forkhead box protein O1; Foxa2, forkhead Box A2; GC, glucocorticoid; Gilz, glucocorticoid-induced leucine zipper; GLU, glucose; GluOC, undercarboxylated osteocalcin; GLUT, glucose transporter; GPRC6A, G protein-coupled receptor class C group 6 member A; GSK3β, glycogen synthase kinase-3 β; GU, glucose uptake; h, hour; HAECS, human aortic endothelial cells; HDL-C, high-density lipoprotein cholesterol; HFD, high fat diet; HOMA-IR, homeostasis model of insulin resistance; HUVECs, human umbilical vein endothelial cells; IL, interleukin; INS, insulin; IPGTT, intraperitoneal glucose tolerance test; IR, insulin resistance; IRβ, insulin receptor β; IRE-1α, inositol-requiring enzyme 1 α; IRS1, insulin receptor substrate-1; IS, insulin sensitivity; Ki67, Ki-67 protein; LA, linoleic acid; LDL-C, low-density lipoprotein cholesterol; $Ldlr^{-/-}$, low-density lipoprotein receptor knockout; LRP1, low density lipoprotein receptor-related protein 1; Mcad, Medium chain acyl-CoA dehydrogenase deficiency; MCAT, malonyl CoA-acyl carrier protein transacylase; MCP1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; MyHC, Myosin heavy chains; MTOR, mechanistic target of rapamycin; NASH, Nonalcoholic steatohepatitis; ND, normal diet; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nfkb1, nuclear factor–κB; Nlrp3, NLR family pyrin domain containing 3; Nrf1, Nuclear respiratory factor 1; OC, osteocalcin; *p,* phosphorylation; Pepck, phosphoenolpyruvate carboxykinase; Pgc1α, peroxisome proliferator-activated receptor-gamma coactivator α; PI3K, phosphatidylinositol3-kinase; PP cells, Pancreatic polypeptide cells; RNA, ribonucleic acid; Si, silenced; Slc2a4, solute carrier family 2 member 4; Spp1, secreted phosphoprotein 1; SREBP1c, sterol regulatory element binding protein 1c; STZ, streptozotocin; SUR1, sulfonylurea receptor 1; *t,* total; TC, total cholesterol; Tg, transgenic; TGL, triglyceride; TNF-α, Tumor necrosis factor-α; Tun, tunicamycin; ucOC, undercarboxylated osteocalcin; Ucp1, mitochondrial uncoupling protein 1; w, week; WAT, white adipose tissue; WT, wild type.

Another potential source of difference is the use of ucOC under normal/healthy conditions versus use under pathological conditions. There is a substantial amount of evidence that ucOC is capable of improving insulin action in insulin resistant muscle, without altering basal glucose handling and signaling activity, in both in vivo and in vitro models [100,103,104,108]. As such, it is likely that ucOC will have a favorable effect under pathological conditions, including those with elevated blood glucose or insulin resistance compared to those with normal blood glucose levels and insulin sensitivity. However, this hypothesis should be confirmed in the future. Although current results are promising, the magnitude of the effect and clinical relevance and translation to humans has yet to be fully realized.

6. Does osteocalcin regulate energy and glucose metabolism in humans?

6.1. What are the limitations of current investigative tools?

Conflicting findings in observational or clinical trials in OC-focused human studies may be influenced, in part, by several underlying factors e.g., age, sex, clinical characteristics, medications used or menopausal status amongst other potential factors. For instance, compared to placebo, 1 year of calcium supplementation in 1368 older women has been shown to decrease tOC and ucOC levels by 17 % and 22 %, respectively, with no change in fat mass or glycated hemoglobin (HbA1c) [120].

Furthermore, throughout the literature there is a large variation in the different assays used to assess and measure tOC and ucOC. Currently, there is no optimal method for the measurement of ucOC. Commonly, a hydroxyapatite (HAP) binding method proposed by Gundberg et al. [121] or a direct determination for Glu-OC by an ELISA specific for fully uncarboxylated OC is used, each with limitations. The HAP binding method is based on the lower affinity of ucOC to the HAP compared to fully carboxylated OC. The method is complex, and levels are highly dependent on technical details such as antibodies used, specific binding capacity of the HAP, amount of apatite used, or specific ELISA used. This method does allow the expression of ucOC as a percentage of tOC (ucOC % or ucOC/tOC), as ucOC is measured on the same sample before incubation with HAP after measuring tOC. This may be more clinically informative. There is an available combination kit that recognizes Gla-OC (carboxylated OC) and uncarboxylated OC but neither of these kits recognizes undercarboxylated OC. There are some instances where ELISAs can report ucOC levels higher than those of tOC, which may suggest non-specific binding and an overestimation of ucOC. In the last several years, new ELISAs to assess ucOC in humans were developed and validated. These include the ELISA developed by Lacombe et al. [122] which used monoclonal antibodies against human ucOC. The ELISA was validated in two cohorts, people with and without T2D, this ELISA is

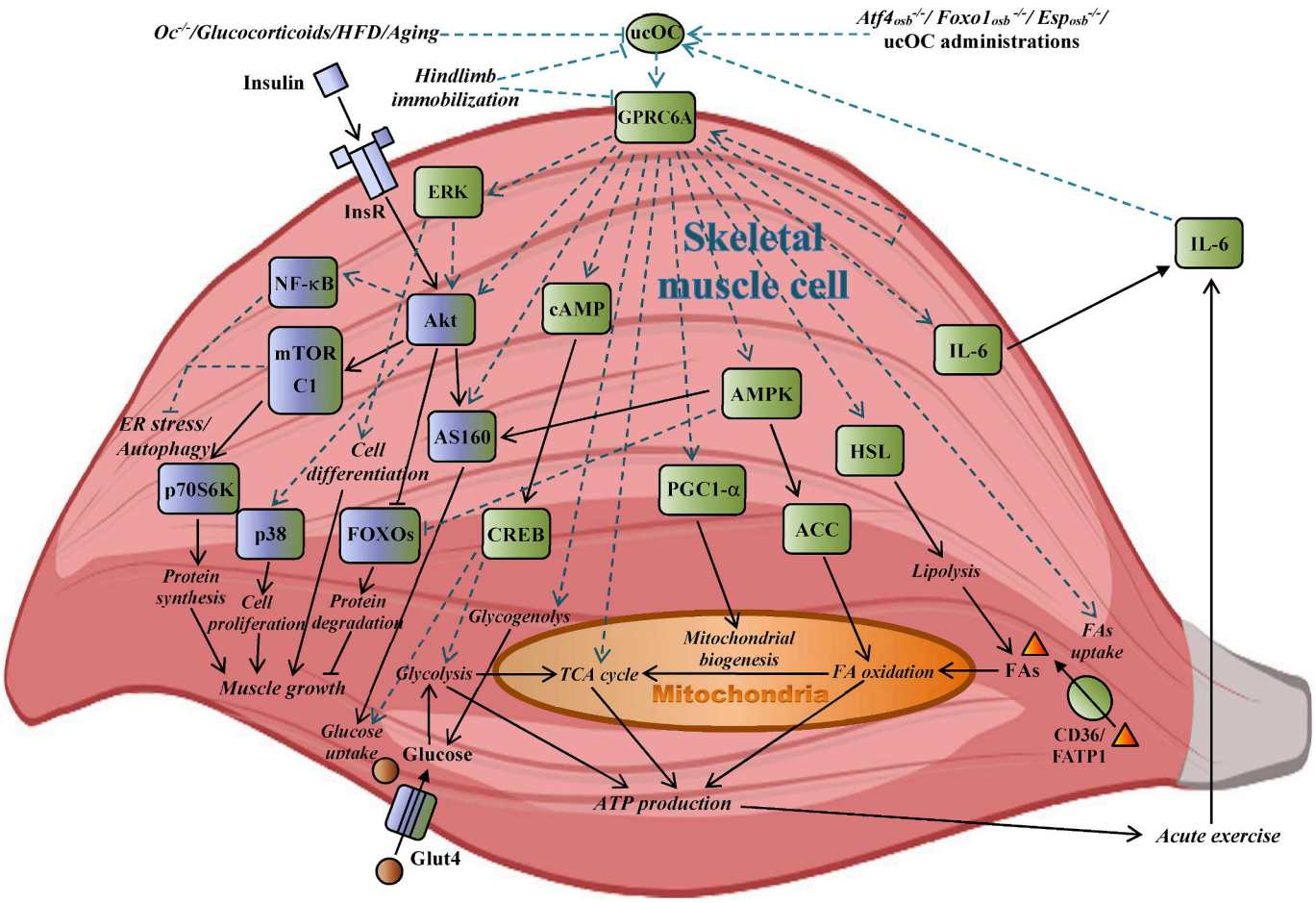


Fig. 2. Suggested signaling pathways underlying the effects of ucOC on muscle energy metabolism ucOC regulates muscle energy metabolism via signaling pathways involving GPRC6A as the receptor. The ucOC-induced enhancement of glucose uptake (both non-insulin-stimulated and insulin-stimulated), glycolysis, and insulin-stimulated glycogenesis is mediated via a sophisticated signaling network including GPRC6A, ERK, Akt, AMPK, AS160, GSK-3, the cAMPK/CREB axis, and Glut4. The activity of other downstream targets of Akt such as mTORC1 and NF-κB are also enhanced, attenuating ER stress and autophagy accompanied with insulin resistance. The utilization of other intracellular carbohydrate sources, such as glycogen, is elevated via the ucOC/GPRC6A cascade. Furthermore, ucOC increases FA uptake via enhancing the expression of FA transporters including CD36 and Fatty acid transport protein 1(FATP1), and also enhances lipolysis via enhancing the activity of HSL. In addition, ucOC favors mitochondrial biogenesis and mitochondrial functions in terms of TCA cycle and FA oxidation, via the upregulation of PGC1-α and the AMPK/ACC axis. Lastly, the ucOC/GPRC6A cascade self-amplifies the signaling strength by increasing the expression of GPRC6A. Overall, ucOC-induced enhancement of transport and utilization of nutrients contributes to the production of ATP. During exercise, ucOC levels are considerably increased via the ucOC-IL-6 reciprocal regulation, leading to increased ATP production to fulfil the energy demands of muscle cells. In animals with ucOC administration, ucOC is enhanced, leading to higher levels of energy metabolism and ATP production. On the contrary, in glucocorticoid-treated, HFD-fed and old animals of which ucOC is reduced, energy metabolism and ATP generation are impaired.

Green boxes represent downstream proteins of the ucOC/GPRC6A cascade. Boxes with both blue and green colors represent proteins that are regulated by both proximal insulin signaling cascade and ucOC/GPRC6A cascade.

→: well-studied activation or enhancement.

- - →: recently suggested activation or enhancement.

- - ⊣: recently suggested inhibition.

⟶: translocation.

This figure has been adapted from Lin et al. [116].

commercially available at Bio Legend Inc. A study by Bonneau et al. [123] evaluated the ability of widely used ELISAs to identify an assay to assess the γ-carboxylation of human OC on its first Glu residue (Glu17), the residue that then generates undercarboxylated Glu-17 (produced by the acidic environment generated by osteoclasts), that is then released into the blood stream. They report that the Intact OC ELISA (ALPCO) was appropriate to measure tOC as it detects full length OC regardless of γ-carboxylation levels, further Takara's Gla-type OC assay reliably quantified OC γ-carboxylation on Gla17. In absence of an assay to detect Glu17-OC they normalized Gla-17 to tOC (Gla17/tOC ratio) and showed in 129 non-diabetic postmenopausal women that a higher Gla17/tOC ratio was associated with lower insulin sensitivity (hyperinsulinemic-euglycemic clamp, and OGTT) and higher insulin resistance (HOMA-IR) supporting the role of ucOC as a hormone influencing insulin resistance in humans. The HAP method currently provides the most clinically relevant measurement of this protein and its forms. Tighter control of bio sample handling, and pre-analytical factors will assist with direct comparison between studies such as controlling for feeding, and diurnal effects. Given the high sensitivity of OC to such factors, studies that have accounted for this are more likely to reflect the circulating levels of this bone-peptide and its forms. Notwithstanding this, there is still useful information from a number of studies investigating the effects of OC in humans, while comparing different studies may be difficult and the limitation of for instance cross-sectional studies, interpretation of OC within studies that have used adequate controls or have repeated measures is possible. It should be noted, that similar to these limitations

discussed, the way in which these bio samples are collected, stored and incubated may also apply to samples obtained from animals, and the fasting period prior to sample collection should be standardized to enable direct comparison between studies. Elucidating the direct effects of ucOC on glucose regulation is essential to our understanding of the function of this bone hormone in general, as it could have clinical implications in identifying new mechanistic targets and treatment avenues for metabolic conditions such as T2D.

6.2. *What is the observational evidence linking OC promotion of glucose regulation in humans?*

A large number of cross-sectional studies show that higher circulating levels of tOC and ucOC (Table 2, *for methodological detail see Online Supplementary Tables 1 and 2*) are associated with better glycemic control, and lower BMI and fat mass in numerous cohorts including older adults, T2D and those with metabolic syndrome [124–150]. This is supported by a recent meta-analysis of 39 observational studies (23, 381 people), that reported higher tOC and ucOC were related to lower fasting blood glucose (BGL) and HbA1c, albeit all analyses were derived from cross-sectional data [151]. Compared to controls, many cross-sectional studies (Table 2*)* show that individuals with abnormal glucose regulation (i.e., T2D, metabolic syndrome or insulin resistance) have up to 50 % lower tOC and ucOC (Table 2). Findings of a meta-analysis of 22 observational studies (20 cross-sectional and 2 cohort studies) reports that tOC levels are lower in patients with T1D compared to healthy controls (n = 674 T1D, n = 513 controls), but with no difference in the level of tOC in patients with T2D compared to controls (n = 1706 T2D, n = 1700 controls) [152]. However, in this pooled analysis of studies, a large number of different methods were used to assess tOC, and not all studies used fasting samples. While these data are limited by their cross-sectional design, several, but not all, longitudinal prospective cohort studies report similar findings. These studies demonstrate that a relationship exists between lower tOC and ucOC with long-term risk of poorer glucose metabolism (i.e., insulin resistance, higher fasting plasma glucose) across different populations (follow up range: 2 to 4 years) [129,153,154]. Moreover, numerous studies show that reduced serum tOC or ucOC is related to increased risk of future T2D development (follow-up range: 3 to 12 years) in various populations [139,155–158]. A meta-analysis reported that the pooled risk estimate for T2D in those with the highest tOC (quartile 4) levels, compared to the lowest tOC levels (quartile 1), was 0.23 (95 % CI 0.12–0.46) for cross-sectional studies (six studies, n = 6974) and 0.89 (95%CI 0.78–1.01) for cohort studies (three studies, n = 1662), adjusted for several risk factors [159]. It should be noted that there was large heterogeneity across the cross-sectional and cohort studies included in this meta-analysis, and with limited available large prospective cohort data at the time it may explain the non-statistically significant pooled confidence interval for the cohort studies.

While many observational studies support a link between lower ucOC and/or tOC with poorer glucose metabolism (Table 2), not all studies have observed a similar relationship [138,140–144,160–164]. For instance, in a prospective cohort study including 1071 middle-aged healthy adult female and male twins, tOC (as well as other bone markers, CTX and P1NP) was not associated with glucose metabolism, nor changes in glucose homeostasis or the development of T2D over the subsequent 12 years. [162] The participants in this study were healthy and relatively young, with only 33 (3 %) incident T2D cases at follow up. This limited the capacity to explore this relationship between tOC with adverse glucose homeostasis, and ucOC was not measured. These findings were similar to that reported previously by a large longitudinal study (n = 1635, aged 21–70 years) [164] who reported that tOC, ucOC and the ucOC/tOC ratio was not associated with risk of incident T2D over a 10 year follow up period. Findings from two other recent longitudinal studies similarly reported no evidence of a relationship between baseline ucOC or tOC with risk of incident T2D over 8 years in older

Table 2
Correlative link between tOC, ucOC and glucose metabolism.

Osteocalcin forms	Study design and population	Levels compared to the reference group
tOC	Cross sectional study design Type 2 diabetes [32,124–126,156,158,166–177] Pre-diabetics [134,172] Metabolic syndrome [127,137,156,171,178–187] Type 2 diabetics with metabolic syndrome [150] Middle-aged men [188] Older adults [138] Prepubertal overweight children with pre-diabetes [189] Postmenopausal women with impaired fasting glucose [136] Women with GDM during pregnancy [190] Premenopausal women with insulin resistance [191] or high fasting blood glucose [149] Type 2 diabetics with varying degree of HbA1c [192] Non-alcohol fatty liver disease [193,194] Acromegalic patients [195] Adults with coronary heart disease [196] Non-diabetics with first degree relatives with type 2 diabetes [197] Trauma patients with high HbA1c [198] Healthy elderly with high fasting plasma glucose [129]	↓
	Prospective cohort/longitudinal study design Type 2 diabetes [135,139,155,165,199,200] Pre-diabetics [163] Middle-aged men [143] Women with GDM postpartum [142] Cross sectional study design Pregnant women with GDM [201] Cross sectional study design Type 1 diabetics [202,203] Obesity [204] Type 2 diabetics [122,203] Metabolic syndrome [149,205] Pre-diabetics [134] Middle-aged adults with different degrees of glucose tolerance [206]	↑
	Type 2 diabetics not taking antidiabetics [177] Polycystic ovarian syndrome [207] Obese men [160]	↔
ucOC	Prospective cohort/longitudinal study design Older men with high cardiovascular risk [154] Gestational diabetes during pregnancy [142,208] Cross sectional study design Type 2 diabetes [32,122,126,166,209,210] Metabolic syndrome [178,205] Type 2 diabetics with metabolic syndrome [150] Obesity [204] Middle aged men [188] Hemodialysis patients with T2D [209] Prepubertal overweight children with pre-diabetes [189] Patients with metabolic syndrome and diabetes [132]	↓
	Prospective cohort/longitudinal study design Type 2 diabetes [139,165] Cross sectional study design Polycystic ovarian syndrome [207]	↑
	Cross sectional Type 1 diabetics [202] Type 2 diabetics with varying degree of	↔

(*continued on next page*)

Table 2 (*continued*)

Osteocalcin forms	Study design and population	Levels compared to the reference group
	HbA1c [192] Middle-aged adults with different degrees of glucose tolerance [206] Adults with varying degrees of glucose regulation [133] Older adults [138]	
	Prospective cohort/longitudinal study design Type 2 diabetes [155] Older men with high cardiovascular risk [154] Gestational diabetes during pregnancy [142,208] and postpartum [142]	

Table note: this table includes studies that either include controls, or includes observational cohort studies that explore and compare characteristics by upper and lower levels of the biomarker of interest by e.g., tertile allocation and the comparison of those in the highest versus the lowest tertile.

adults ($n = 338$) [144] and over 5 and 10 years in elderly Japanese men ($n = 1700$) [165].

Observational data generally support the link between OC and glucose metabolism. However, there are many limitations in making a conclusive statement as the bulk of this data is driven by cross-sectional studies. Many studies have only measured tOC, probably due to methodological difficulties described earlier (Section 5.1) in the measurement of ucOC. Some studies are also retrospective in nature, and many of the studies have not been designed with tOC or ucOC as the primary outcome, hence methodological limitations can affect interpretation of findings (such as OC measured in non-fasting samples, or OC measured in samples that have been in long-term storage). The longitudinal studies discussed that report on ucOC measured using the HAP method do not support a relationship between ucOC and risk for incident T2D [144,164]. A consensus for the methodological assessment of ucOC should be reached and then be implemented in future research. Lastly, the measurement of OC may be dependent on many factors (dietary intakes of calcium and vitamin K and possibly others which have not yet been fully realized) which in the majority of studies have not been considered, further limiting our understanding of OC (Section 5.1).

6.3. Are OC levels different based on sex and is this related to CVD?

Whether there are differences in circulating levels of tOC and ucOC based on sex, and across the life phases has not yet been explored in large cohorts. Age-referent ranges across the adult male lifespan for all OC forms and ratios have been reported previously [211]. They show that tOC and ucOC (HAP method) follow a u-shape pattern with age, while ucOC/tOC follows a positive linear increase with age. Whether similar relationships are found in women is unknown. Sex-based differences will be important to delineate, as it does appear that circulating levels of tOC and ucOC are influenced by sex. An exemplar study including 96 healthy young adults (74 males, 22 females) reported that tOC, cOC and ucOC (HAP method) are higher in males compared to females at baseline [212]. Compared to females, males had ~34 % higher tOC (females: 20.1 ± 5.5 μg/L, males 30.5 ± 10.9 μg/L) and ~ 29 % higher ucOC (females 8.4 ± 2.9 μg/L, males 11.8 ± 4.3 μg/L). Despite different baseline tOC and ucOC levels, the increase in tOC and ucOC after a single session of high intensity exercise was similar between sexes. Although it should be noted that the sample size in this study is small, and the proportion of sex was not balanced. Furthermore, two separate but comparable large cohort studies using the same laboratory, technician and assay methods (HAP method) involving 2966 older men aged 70–80 years [32] and 1368 older women ≥70 years [120], revealed that older women had ~24 % higher tOC (older men: 20.8 ± 12.6 μg/L versus older women: 27.3 ± 10.9 μg/L) and ~15 % higher ucOC compared to older men (older men: 11.0 ± 5.0 μg/L versus older women: 13.0 ± 5.7 μg/L). However, direct comparisons within the same study are required to confirm these observations, and the physiological relevance of potential sex-based differences in OC remains unclear.

CVD's are a leading cause of death worldwide in both men and women [213]. However, our understanding of how CVD develops in women compared to men remains incomplete. Traditional risk factors for CVD vary in how much they contribute to CVD risk between sexes. For instance, women who have diabetes have triple the risk of CVD mortality, whereas men have only double the risk [214]. Compared to men, after experiencing their first CV event women have worse in-hospital mortality and a higher 30-day, 1-year, and 5-year risk of death [215,216]. They are also 50 % more likely to suffer a second heart attack within 12 months [217]. Whether sex-based differences in CVD risk could be attributed, in part, to variations in OC levels are unknown. One study has reported in 3542 older men (70–89 years) that tOC levels predict all-cause and CVD-related mortality, yet the relationship is u-shaped, meaning those older men with the lowest, and the highest tOC levels were at increased risk [218]. This data supports the suggested association of tOC with glucose metabolism and therefore CVD risk, it may however be related to either reduced or increased bone turnover that is often associated with ageing or being a marker of other underlying health issues or poorer health outcomes. Future research should confirm whether a similar relationship is found in women and with ucOC.

6.4. What interventions manipulate ucOC levels with subsequent metabolic changes?

Although there are challenges to studying the direct effects of ucOC on human metabolism in vivo, other strategies using pharmacological and non-pharmacological approaches can be employed. These approaches alter metabolism and, albeit indirectly, ucOC (Fig. 3). One such approach is to manipulate glycemic control (e.g., hypoglycemic drugs or lifestyle interventions including exercise and diet) and to observe changes in tOC or ucOC levels, or conversely manipulate ucOC levels directly or indirectly (e.g., vitamin K supplementation, glucocorticoids) and to observe the effects on glycemic control. These approaches will be discussed in the sub-sections below.

6.4.1. What are the effects of dietary-induced weight loss with or without exercise on ucOC and glucose metabolism?

Data on studies comparing effects of diet, with and without exercise on ucOC are conflicting. The majority report that ucOC increases following a dietary only intervention, but whether this is related to subsequent beneficial effects on metabolic outcomes is unclear.

In obese non-diabetic men, 4 months of restricted daily calories (1500 k/cal) increased ucOC (EIA method) without altering tOC, and decreased BMI (−5 %), fasting blood glucose and insulin resistance (HOMA-IR) [219]. The increase in ucOC was not related to the post-intervention improvement in BMI, fasting blood glucose or HOMA-IR. Although ucOC was related to triglyceride levels, these were not significantly different pre- to post-intervention. This study did not have a control group, the diet was self-selected, and participants were asked to consume 2 servings of green vegetables daily. Such vegetables are known to be high in dietary vitamin K1 [220], which may affect the carboxylation of OC (a vitamin K dependent protein), and therefore it is not clear whether changes in ucOC were a result of weight loss or the dietary intake. Similarly, a secondary analysis of a 12-month RCT in obese older adults reported that following a calorie restricted diet (10 % diet-induced weight loss group, 500–700 kcal/day deficit with 1/g/kg/day protein) ucOC and tOC levels were increased. Whereas ucOC was unchanged and tOC decreased after exercise (multi-component), both were unaltered by the diet-exercise combination [221]. Although both diet alone and combination diet-exercise groups improved insulin

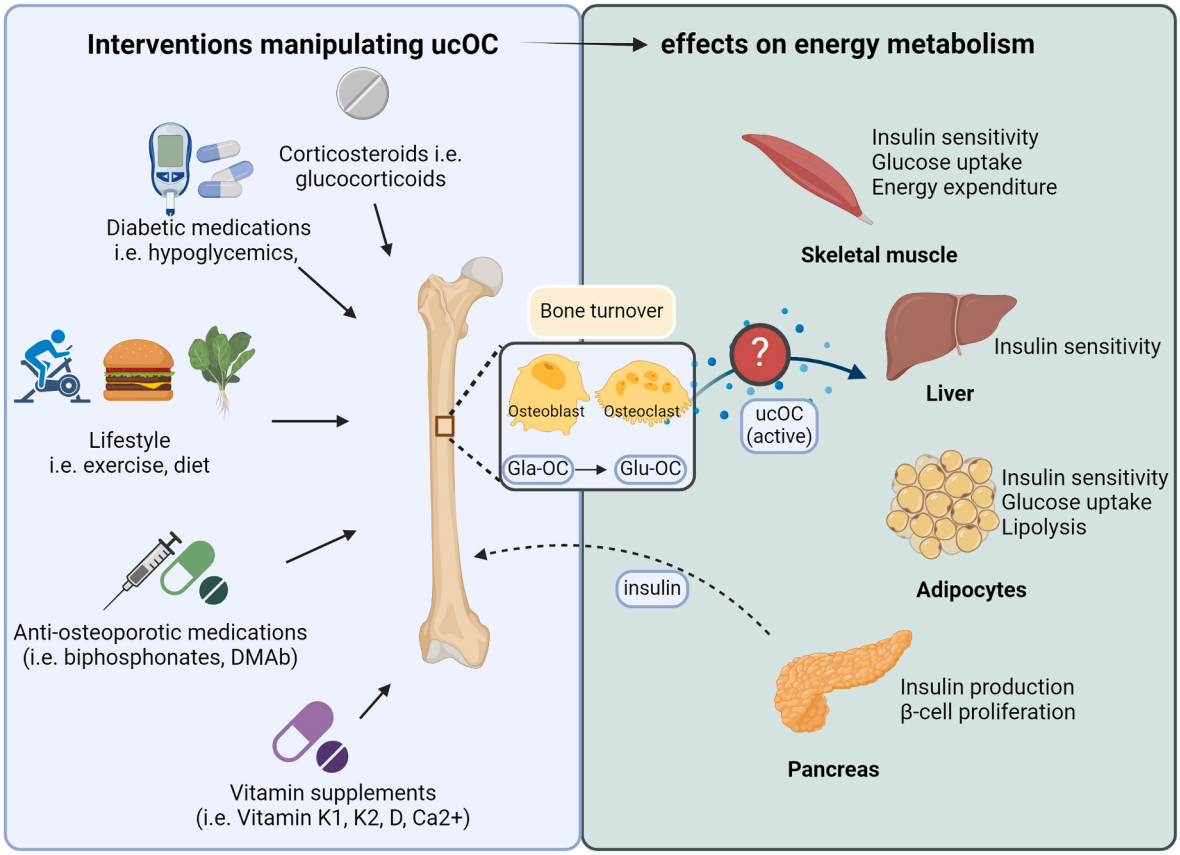


Fig. 3. Interventions that manipulate ucOC and glucose metabolism.
Created with BioRender.com.

secretion, only in the diet alone group did ucOC levels predict 20.2 % of the variance in insulin secretion (as measured by the disposition index) [221]. It was hypothesized by authors that weight loss-induced bone resorption (a trigger of ucOC release) is prevented by exercise. They also demonstrated that changes in bone profile (change in BMD, P1NP and CTX) accounted for the variance in ucOC [221].

Conversely, 20 weeks of caloric restriction resulting in a 12.5 % loss in body weight (400 kcal/day deficit) with and without aerobic exercise (moderate or vigorous) in women did not alter tOC, ucOC or the ucOC/tOC ratio [222]. Although, in this study, the women received supplemental vitamin K to maintain OC carboxylation, to test whether ucOC was required for weight loss. Given weight loss occurred despite no change in OC forms, the researchers hypothesized OC may be unrelated to weight loss. Other studies using vitamin K similarly do not support the association between the change in ucOC with a change in body weight or body fat [161,223]. It is possible that vitamin K may assist in glucose regulation independently of ucOC, however the direct evidence is predominately shown in animal studies with data in humans conflicting, limited to the observational nature in design and different methods used to assess vitamin K [224,225].

In summary, evidence suggests that weight loss may increase ucOC, but findings also suggest that weight loss can occur independent of changes in OC. None of these studies accounted for vitamin K, which is known to impact OC carboxylation. Considering dietary Vitamin K1 has been shown to be significantly inversely related to the ucOC/tOC ratio, a marker of vitamin K status [226], and that dietary and supplemental calcium (total) intakes have been shown to have a linear relationship between calcium intake and tOC and ucOC [120], these factors should be accounted for in studies measuring OC. Lastly, there is some evidence that bariatric procedures such as biliopancreatic diversion (BDP) which is known to induce substantial weight loss and improve glucose control in patients with T2D, dramatically increases ucOC levels (by 257 % and 498 % at 3 and 12 months, respectively) and this increase in ucOC was associated with improved insulin resistance [227]. Although the study was prospective in design, the sample size of this study was relatively small ($n = 16$) and heterogenous, therefore findings should be confirmed in larger populations.

6.4.2. *What effect does exercise have on OC and glucose metabolism?*

Exercise is a cornerstone strategy for the prevention and management of T2D and osteoporosis [228,229]. Exercise affects bone health, in part by modulating bone turnover markers including tOC and ucOC [115,230–233], and improves insulin sensitivity and glycemic control. Even a single bout of exercise increases insulin sensitivity for up to 48 h after exercise is complete [234,235]. Exercise is also an effective means to build lean mass and reduce fat [236].

Most acute (single-bout) exercise studies have reported a transient increase in ucOC in young adults, middle aged obese men, and older women [115,212,230,237], and this increase in ucOC has been shown to be related to decreased post-exercise glucose levels [230]. Conversely, acute exercise in older adults, irrespective of mode (aerobic or resistance), decreases tOC without altering ucOC [238]. Although, following exercise, the percent change in tOC and ucOC was inversely related to the percent change in glucose levels. Further, higher ucOC at baseline has also been shown to be related to higher whole-body insulin sensitivity at rest and following acute exercise in middle-aged obese men [115]. These findings led researchers to hypothesize the existence of a feed-forward loop, whereby acute exercise increases ucOC with accompanied improvements in glucose homeostasis and insulin sensitivity [115,230]. Given nutrient intake and feeding (i.e., oral glucose tolerance test [OGTT]) lowers tOC and ucOC, studies were undertaken to examine whether acute exercise could attenuate the postprandial

suppression of tOC and ucOC. These exercise studies, irrespective of exercise intensity, did not alter postprandial suppression of tOC and ucOC [237,239]. However, the acute exercise bout was performed prior to, and not after insulin and glucose infusion or the OGTT. When exercise was performed in the postprandial period (1 h after meal consumption) this prevented the postprandial-induced suppression of tOC and ucOC in overweight and obese adults, at least with moderate intensity exercise [240]. Researchers hypothesized that elevated insulin and glucose levels following high-intensity exercise may partially explain the lack of change in serum tOC and ucOC [240]. Overall, although not all studies report significant changes in ucOC following acute exercise, findings from these studies suggest that a relationship exists between post-exercise ucOC and improved glycemic control.

The effects of chronic, long-term exercise on tOC and ucOC levels are conflicting. Some studies report either no change in ucOC and/or tOC [212,221,241], increased ucOC and/or tOC [242–244] or decreased tOC [245]. A recent meta-analysis of 22 RCTs (pooled $n = 1027$ participants' $n = 632$ people in the intervention group, $n = 411$ people in the control group) reported an overall increase in ucOC with exercise with no change in tOC [246]. Once removing studies that included dietary interventions with exercise, the overall effect of exercise was an increase in tOC levels. It should be noted that the pooled analysis exploring ucOC included only 4 studies (6 study arms, $n = 171$) compared to 18 studies for tOC (27 study arms. $n = 948$). Additionally, it included healthy and clinical populations combined which may limit interpretation of pooled effects.

Whether chronic changes in tOC or ucOC after regular exercise are related to the improvement in glucose metabolism remains unclear. One study reported no such relationship between tOC and glucose, insulin or HOMA-IR following 8 weeks of aerobic exercise in postmenopausal women; however, ucOC was not measured [245]. In obese men with MetS who were randomized to perform 12 weeks of aerobic, resistance or concurrent aerobic and resistance exercise, ucOC levels were increased after all training modes [243]. The greatest increase in ucOC was seen with aerobic and concurrent groups versus the resistance alone interventions, with similar effects shown for both glucose and HbA1c. This suggests that the aerobic component of the exercise groups may have had a greater effect on ucOC and metabolic responses than resistance exercise. However, whether the exercise-induced change in ucOC is related to these metabolic beneficial effects was not explored. Others reported that 8 weeks of running exercise increases tOC, ucOC and the ucOC/tOC ratio in obese young healthy males [242]. The exercise-induced improvement in insulin resistance (HOMA-IR) was related to the change in both tOC and ucOC after multi-variable adjustment [242]. Overall, it appears that chronic exercise increases ucOC [246] and this increase in ucOC may be related to improved insulin resistance [242], although data is limited and this should be explored in other populations and using different exercise modes. In line with findings of a previous systematic review reporting that responses of bone remodeling markers (including tOC and ucOC) to acute exercise may depend on the exercise type, intensity and population tested [233], future acute and chronic exercise studies should explore different exercise types, intensities and durations, as well as performed in different populations on tOC, cOC, and ucOC responses and subsequent metabolic changes.

6.4.3. Does vitamin K effect OC and glucose metabolism?

Carboxylation of OC occurs via vitamin K and as such the ucOC/tOC ratio represents a marker of vitamin K status [247–249]. One non-invasive intervention to manipulate ucOC that has evolved is via supplementation or dietary intervention of vitamin K which, even after very short intervention periods (2 to 4 weeks), can decrease circulating ucOC and ucOC/tOC [250–255]. Some of these studies have reported beneficial effects on glucose metabolism such as increased insulin sensitivity following vitamin K1 [254] and K2 supplementation [255]. Compared to placebo, 4 weeks of phylloquinone supplementation in pre-menopausal women with prediabetes increased cOC and decreased ucOC levels, improved glycemic control (decreased 2-hour post OGTT glucose and insulin) and insulin sensitivity [254]. No relationship was found for the change from baseline of cOC, ucOC or tOC with these metabolic variables. However, they also reported that the phylloquinone supplementation lowered %ucOC, and this decrease in %ucOC was related to decreased 2-hour post-OGTT glucose levels [254]. It should be noted that cOC and ucOC in this study were measured by two separate EIAs, with the total OC calculated as the sum of cOC and ucOC, hence, % ucOC should be interpreted with caution. A cross-sectional study ($n = 3658$ healthy adults from Japan) reported that a daily diet rich in vitamin K is related to lower ucOC, and lower ucOC is associated with a higher HbA1c [256]. However, others report no change in fasting serum glucose or insulin concentrations despite a reduction in ucOC following daily treatment of phylloquinone for 12 months in postmenopausal women [252]. Similarly, 4 weeks consumption of increased green leafy vegetable intake, which are known to raise vitamin K1 levels, decreased ucOC but this was not related to cardiometabolic measures such as plasma glucose, lipid concentration, blood pressure or arterial stiffness [257]. One study reported that three months of vitamin K2 supplementation in patients with T2D increased cOC and tOC without changing ucOC, and decreased blood glucose and the HOMA-IR [258]. Although other studies have shown that cOC, not ucOC, is related to insulin resistance (HOMA-IR) [138,259], associations were not explored in this study [258] and it may suggest future studies should consider not only tOC and ucOC, but also cOC. The relatively small sample size and assay of ucOC may also limit interpretation of the findings. Of note, in many of these studies' serum vitamin levels or dietary intakes were not always measured at baseline and therefore individual responses may be different based on baseline levels.

It is possible that vitamin K may have a protective effect on diabetes independently of OC. Indeed, it has been shown in a large cohort including 54,787 Danish residents (median age 56 years) that diets with higher dietary intakes of Vitamin K1 are associated with lower risk of diabetes [260]. The potential role of the y-carboxylation of specific proteins in β-cells, which may explain how vitamin K can protect against T2D, has been reviewed extensively elsewhere [261].

Another method to manipulate vitamin K levels is the use of warfarin. Warfarin has been used for over 50 years as an anticoagulant medication and its mechanism of action includes binding to the enzyme vitamin K 2,3-epoxide reductase, which reduces clotting capacity and 'thins' the blood [262]. Warfarin increases ucOC levels via vitamin K, which is also involved in OC carboxylation [263]. As such, warfarin treatment would be expected to improve glucose regulation, however any effects are not yet clear. For instance, it has been reported that in patients with atrial fibrillation receiving oral anticoagulants, those on apixaban, dabigatran or rivaroxaban had lower diabetes risk than those on warfarin [264]. As there was no placebo group, it is not clear whether individuals undergoing warfarin treatment had lower or higher risk of diabetes compared to no treatment. Another study reported that adding warfarin to sulphonylureas treatment in patients with T2D increases the risk of hypoglycemia-related hospitalization [265]. Whether this severe hyperglycemia is driven by higher ucOC is unknown as this was not measured. Nevertheless, it will be important to assess the effects of warfarin on ucOC and the potential clinical implications of such interaction. Future studies should report whether participants were undergoing warfarin treatment to assist with data interpretation.

6.4.4. Do hypoglycemic medications affect OC and the relationship with glucose metabolism?

Common methods to improve glycemic control in patients with T2D include the use of lifestyle interventions (e.g., diet and exercise) and glucose-lowering medication. In men with poorly controlled T2D (HbA1c >9 %) who were treated with oral hypoglycemics or insulin, tOC increased with improved glycemic control (HbA1c of <8.3 %) [266]. At baseline, serum tOC was also associated with HbA1c, but not plasma glucose. The authors suggested that chronic hyperglycemia in

diabetic patients may reduce bone formation, but with no change in urinary hydroxyproline that there may be less effect on bone resorption. Further, there was no relationship shown between serum C-peptide (an indicator of insulin secretion) and tOC in the whole cohort, nor those treated with glucose-lowering medication, suggesting that this change in tOC is unrelated to insulin secretion per se, but rather glycemic control [266]. It should be noted that this study was relatively small ($n = 16$), and considering only tOC was measured, together with other confounding factors previously highlighted, it is unclear whether this change in tOC is indeed driven by ucOC or cOC, which may reflect different pathophysiology. Japanese patients ($n = 50$) with poorly controlled T2D (HbA1c >6.5 %) who achieved improved glycemic control following one month of treatment, had increased tOC levels, while ucOC levels were unchanged the ucOC/tOC ratio decreased [267]. Baseline serum C-peptide, fasting plasma glucose, and HbA1c levels were not related to changes in tOC, ucOC or ucOC/tOC following improving glycemic control, whereas the decrease in HbA1c was related to the increase in tOC. That same study also demonstrated that a higher baseline serum adiponectin was related to changes in tOC, ucOC and urinary N-terminal cross-linked telopeptide of type 1 collagen (NTX) [267]. In multiple regression analysis, after adjusting for confounders (age, gender, diabetes duration, BMI and serum creatinine) this relationship between tOC and urinary NTX was maintained. This led those authors to conclude that serum adiponectin may be able to predict subsequent improvement in bone remodeling changes related to glycemic control [267]. Similar to the previously discussed study, it should be noted that the sample size was relatively small (n = 50) and was performed in Japanese individuals who had a relatively low BMI for individuals with T2D (mean ± SD BMI for the whole group was 24.0 ± 5.3 kg/m^2) compared to other studies where individuals with T2D generally have greater BMI levels, such as the large trial involving 5047 participants with T2D who had a mean ± SD BMI of 34.3 ± 6.8 kg/m^2 [268]. Thus, the ability to extrapolate results to Caucasian populations may be limited. Although the authors show that changes in bone markers and adiponectin were not different in insulin versus non-insulin treated patients, classes of diabetic medications may have differing effects on bone remodeling and adiponectin. The study also included patients who were treated with diet alone, with caloric restriction or certain dietary factors likely to influence OC forms, particularly ucOC [120,219,221,257,267]. Similarly, in another study 8 weeks treatment with hypoglycemic medications in adults with T2D (HbA1c 7.0–10.9 %) resulted in higher tOC and reduced glucose variability [269]. Multiple stepwise regression analysis indicated that higher baseline tOC best predicted the subsequent improvements in glucose variability during glucose-lowering treatment. As per previous studies discussed, this study is limited by its small sample size, ucOC was also not measured. The study suggests also that different glucose-lowering agents may have different effects on tOC, which requires further exploration. Considering the limited evidence overall, it is possible that improved glycemic control may be related to higher tOC levels.

6.4.5. *Do glucocorticoids effect OC and glucose metabolism?*

Glucocorticoids (GC) are a principal treatment of chronic inflammatory disorders such as rheumatic diseases. GC treatment is associated with increased risk for hyperglycemia and worsening of pre-existing diabetes or GC-induced diabetes [270]. Additionally, those receiving GC-treatment, even at low doses, have been shown to have lower serum levels of tOC [271,272]. This observed decrease in tOC levels in GC treated patients is associated with an increased likelihood of developing T2D [273]. A recent study showed that GC decreased total OC and procollagen type 1 N-terminal propeptide (PINP) in a dose-dependent manner and that these changes were related to the GC-induced adverse effects on glucose and lipid metabolism [274]. It has also been shown that endogenous glucocorticoids have negative effects on muscle mass [275]. This relationship between GC-induced decreases in tOC and associated adverse effects on glucose metabolism (e.g., development of insulin resistance) supports the mechanistic findings earlier discussed by Brennan-Speranza et al. [97].

6.4.6. *Do osteoporotic treatments effect OC and glucose metabolism?*

Another intervention that suppresses bone remodeling is antiresorptive therapy, commonly used as first line treatment for osteoporosis such as bisphosphonates and denosumab [DMAb] which have been shown to affect glucose metabolism [276,277]. Bisphosphonate use has been shown to decreases tOC and ucOC [278–280]. Due to the link between OC and glucose metabolism, one hypothesis is that bisphosphonate or DMAb treatment may also affect glucose metabolism. Some observational studies report that adults treated with antiresorptives have a decreased risk of developing T2D [281–285] but not all studies support this [286–290]. Data obtained from three RCTs including osteoporotic postmenopausal women reported that treatment with antiresorptives did not affect fasting glucose or risk for T2D [286], but these trials did not present ucOC data. Osteoporotic patients treated with risedronate exhibited decreased levels of tOC and ucOC, but this was not associated with changes in glucose metabolism [287]. Similar findings were reported following DMAb treatment in postmenopausal osteoporotic women with and without diabetes [288–290]. Findings of these studies may not have been limited by the small sample sizes or underpowered for the outcome of interest. Recently, in a larger study adequately powered for the outcome incident T2D, has since shown that compared to oral bisphosphonate use, denosumab use is associated with lower incident T2D risk in adults with osteoporosis [45]. As such, it appears that a modest reduction in ucOC following antiresorptive treatment may not be sufficient to effect whole-body glucose metabolism. This is in contrast to the hypothesis that lower ucOC is associated with increased risk of T2D. The reason for this is still not clear. However, it is possible that these medications have effects on other pathways that compensate for the lower ucOC, and as such there is no change in blood glucose levels. More studies are needed to identify these mechanisms. In contrast, glucocorticoids, which dramatically suppress OC contributes to the development of abnormal glucose metabolism [291].

In women with hypoparathyroidism or osteoporosis, parathyroid hormone (PTH) treatment, an anabolic bone-agent, was reported to increase tOC and/or ucOC levels [292–295]. However, these studies delivering PTH treatment led to conflicting results regarding the relationship between the change in OC with glucose metabolism. For instance, PTH treatment in postmenopausal osteoporotic non-diabetic women increased tOC and ucOC, and this was related to decreased blood glucose levels [295], but most studies report no link [292–294] or did not measure metabolic outcomes [292].

7. Does osteocalcin have a role in lipid metabolism?

While there is significant knowledge surrounding the direct effect of OC on skeletal muscle insulin resistance and glucose uptake, less is known about its effect on lipid metabolism. OC has been implicated in lipid metabolism due to inverse correlations between OC and BMI [296,297], fat mass [129] and particularly visceral fat [146], with effects being both direct and indirect.

Much of its role likely comes from its influence on adipocytes, where ucOC interacts with its putative receptor, GPRC6A, to initiate a molecular cascade that culminates in an increased expression of the adipokine adiponectin. Human osteoblasts express the adiponectin receptor, which stimulates osteoblast proliferation, differentiation and mineralization [298,299]. Adiponectin has also been shown to affect skeletal muscle metabolism by increasing fatty acid oxidation [300]. This increase in fatty acid oxidation is due, in part, to the activation of peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α) and an increase in mitochondrial biogenesis [96,301].

There does also appear to be direct effects of OC on skeletal muscle lipid metabolism, with Mera et al. reporting that free fatty acid (FFA)

uptake is promoted by OC [82], utilized within the increased mitochondrial content. IL-6, which is released from skeletal muscle in response to exercise and inflammation, promotes the release of ucOC from bone, creating a positive feedback loop to increase the uptake of FFA [302], thus reducing the substrates for lipogenesis [82]. This may help explain the inverse relationship of ucOC and obesity [129,146,178,296,297,303,304], with OC directly correlated with serum adiponectin [169,305,306].

The role of ucOC does appear to increase the use of lipids for metabolism. ucOC has been shown to decrease lipid accumulation in adipose tissue [126] and either oral administration or intraperitoneal injection of ucOC decreases the size of adipocytes in mice [307]. ucOC increases the expression of peroxisome proliferator-activated receptor γ (PPARγ), leading to adipogenesis [307], as well as increasing lipolysis via the enhancement of expression of hormone sensitive lipase [308].

These effects of ucOC are not restricted to adipose tissue, as the accumulation of triglycerides in liver cells subsequent to high fat diet is prevented by ucOC [96,309]. Interestingly, some authors suggest that this may not be due to the upregulation of lipid metabolism, but secondary to decreasing oxidative stress and inflammation [309]. Given the link of IL-6 with increased ucOC release, this may be a method to dampen inflammation and maintain insulin sensitivity and glucose and lipid metabolism.

Taken together, it does appear that OC contributes to increased lipid metabolism and reduced fat mass, directly via increased fat availability and mitochondrial-induced fat oxidation, or secondary to reduced inflammation and oxidative stress. Pregnancy stimulates maternal fat accumulation as fat metabolism is essential for fetal growth [310]. Given that ucOC limits adipose lipid accumulation [126], examining ucOC in pregnancy is a useful design to tease out the effects of ucOC on lipid metabolism. ucOC levels are lower in pregnancy, suggesting a reduction in lipolysis. Indeed, expression of the GPRC6A receptor is reduced, while various lipogenesis markers are elevated [311]. Hormone sensitive lipase (HSL) expression is also reduced. Thus, the decline in ucOC and associated lipid metabolism regulators would support fat accretion for fetal growth. Increasing ucOC acutely decreases adipose mass, but triglyceride (TG) levels and lipolysis are unaffected in pregnancy [311]. However, TG levels and lipolysis are decreased in non-pregnant rats, suggesting TG levels during pregnancy are maintained to ensure fetal growth as a protective mechanism. This highlights that in isolation, ucOC increases lipolysis and fat oxidation, both directly and indirectly, although the overall contribution of skeletal muscle remains under investigated. ucOC is associated with lower levels of body fat, but there is a complex interplay between organs dependent on (patho)physiological conditions.

8. Does osteocalcin have a role in energy metabolism at the liver?

The liver plays a major role in fat metabolism and glucose regulation and like skeletal muscle, can develop insulin resistance leading to lack of inhibition of glucose release following a meal which contributes to hyperglycemia. A high fat diet can lead to nonalcoholic fatty liver disease (NAFLD). There is some evidence to suggest that ucOC influences liver function and can protect insulin resistance, hepatic inflammation and activates autophagy caused by a high fat diet in hens [312,313].

ucOC has been shown to prevent NAFLD in mice fed a high-fat diet, as well as KKAy mice, a model that is characterized by obesity hyperglycemia and insulin resistance. This protective effect may be largely through the prevention of insulin resistance, via mechanisms that include a reduction in the level of pro-inflammatory markers, and profibrotic genes through the regulation of the nuclear factor-like 2 and or c-Jun N-terminal kinase (JNK) pathways [94,100]. In addition, OC reduced the expression of proinflammatory and profibrotic genes (Cd68, Mcp1, Spp1, and Col1a2) in liver [100]. Similar results were reported by Jing Du et al. [309] who also reported that OC can protect against NAFLD in male C57/BL6J mice from diet-induced hepatic triglyceride accumulation and liver injury, via mechanisms that involved increased levels of oxidative stress (malondialdehyde and 8-isoprostaglandin F2a) as well as a higher ratio of oxidized to reduced glutathione in the liver. Furthermore, OC treatment activated Nuclear factor-E2-related factor-2 (Nrf2) nuclear translocation and up-regulated the expression of antioxidant enzyme genes (superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase) and also inhibited the activation of ROS (reactive oxygen species)-JNK cascade in the liver [309,314]. ucOC treatment in primary chicken hepatocytes improved metabolic outcomes including a reduction in triglycerides and lipid droplets, and also reduced ROS concentration and inflammatory markers including IL-6 and TNF-α [314]. Similarly, some of the effects of ucOC in this study were via its effect on the ROS-JNK signaling pathway [314] (and also reviewed here [313]).

In humans, it was reported in a sub-cohort of liver biopsy participants ($n = 196$) from the Shanghai Changfeng Study, that serum tOC and ucOC quartiles were inversely associated with liver steatosis, inflammation, ballooning and fibrosis grades in men and women [315]. After adjustment for multiple confounding factors, male and female participants with the lowest quartile of tOC levels at baseline had more severe liver steatosis (ORs of 7.25 95%CI 1.07–49.30 and 4.44 95%CI 1.01–19.41, respectively). For ucOC, the relationship remained significant in females, but not males, after multivariable adjustment (OR = 3.96 95%CI 1.09–14.30 and OR = 5.10 95%CI 0.97–26.82). In a larger prospective cohort study ($n = 2055$), median 4.2 year follow-up, the females but not the males with the lowest quartile of tOC at baseline had higher risk to develop NAFLD (HR 1.90 95%CI 1.14–3.16) and lower chance to achieve NAFLD remission (HR = 0.56 95%CI 0.31–1.00) [315]. Unfortunately, ucOC was not measured in this larger study, therefore limiting interpretation of tOC. In mice, OC alleviated hepatic steatosis and reduced hepatic sterol regulatory element binding protein 1 (SREBP-1) and its downstream proteins expression. It is likely that these effects of ucOC in liver are mediated by the GPRC6A receptor [316].

The cellular mechanism of ucOC regulating liver function and preventing NAFLD is not clear, but it may include the mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), hedgehog, AMP-activated protein kinase (AMPK), JNK and peroxisome proliferator-activated receptor (PPARs), as reviewed by Wenjun Tu et al. [313].

9. Conclusion

Emerging evidence demonstrates that bone has a role in energy metabolism and there may be multiple osteokines that are involved in glucose regulation. For instance, a recent multi-omics analysis identified 375 potential candidate osteokines involved in inter-organ communication that could be explored in future research [317]. Specifically, there is corroborative evidence from different independent research groups to demonstrate that ucOC acts as a hormone, released from bone and impacting other organs and tissues such as liver, skeletal muscle and fat tissues, which has the capacity to increase muscle insulin sensitivity and modulate glucose regulation in mice. In humans, the evidence supporting the role of tOC and ucOC in glucose regulation arises mostly from cross-sectional data and a limited amount of longitudinal data, with some indirect evidence from interventional studies. However, the data are inconsistent and future research should focus on designing well-powered studies with tOC and ucOC as the primary outcomes, taking into account confounding factors. While current evidence from demonstrates that ucOC has an effect on glucose metabolism and can function as a hormone, this data is mostly derived from rodent models. Data from human studies are not yet conclusive and the direct causal link remains unclear, as this data is limited by its association in nature. Therefore, the magnitude of the effect and clinical implications in humans remain largely unknown. Overall, evidence is supportive of the concept that bone is involved in energy metabolism, some of these

osteokines contribute in a beneficial way (ucOC and OPG), some negatively (RANKL and SCL), while the role of others (LCN2, P1NP and CTX) remain unclear. Whether the effect of osteokines on energy metabolism and muscle can be harnessed therapeutically for treatment of T2D remains to be determined.

10. Future directions

Based on the current evidence, particularly data generated from human cross sectional, observational and clinical trials, it is extremely difficult to dissect association from causality. If we are to truly determine the role of OC in energy metabolism, then future research should consider the following points:

- Data generated from well-powered epidemiological studies are required, where findings can then pave the way for clinical trials and potential Mendelian randomization studies, to assess the identified associations for causality. We also need such epidemiological studies to uncover confounding factors of OC such as dietary and supplemental intakes of calcium and vitamin K, which can then be considered in the design of future clinical trials.
- Clinical trials need to be designed with OC as a primary outcome, and ucOC measured preferentially using the HAP method. Many trials reporting on OC to date are retrospective in design; that is samples have been measured following trial completion, introducing methodological limitations and restricting interpretation.
- For interventional studies (e.g., diet or exercise) we need large, randomized controlled trials, performed in both sexes. These trials need to account for confounding influences on OC, such as dietary and supplemental calcium intakes, vitamin K intakes and potentially other dietary factors.
- Given it is now known that OC exists in multiple forms, measurement of tOC alone limits our understanding of this bone peptide. Future research should endeavor to also measure ucOC to enhance interpretation. It should be noted that many additional forms of OC may exist that are not yet fully realized. Therefore, focused efforts to improve our measurement of these forms will progress this field.
- There is some correlative evidence for a link between OC and high-density lipoprotein (HDL) following a Mediterranean diet [318]. Whether OC can regulate HDL, or vice versa, should be examined.
- Lastly, to further our understanding on OC generally, we need preclinical and animal models examining the direct effects of OC (reporting both beneficial and adverse effects, including toxicology studies) across different organs and tissues that express the putative receptor for ucOC, including fat tissues, liver, skeletal muscle, pancreas, and blood vessels which may pave the way for clinical trials looking at OC treatment in non-human primates and humans.

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Cassandra Smith: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Xuzhu Lin:** Writing – review & editing. **Lewan Parker:** Writing – review & editing. **Bu B. Yeap:** Writing – review & editing. **Alan Hayes:** Writing – review & editing. **Itamar Levinger:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

None of the authors have a conflict of interest to declare.

Data availability

No data was used for the research described in the article.

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