Osteocalcin-GPRC6A: An update of its clinical and biological multi-organic interactions (Review)

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Abstract. Osteocalcin is no longer regarded as a molecule exclusive to bone remodeling and osteogenesis, but as a hormone with manifold functions. The discovery of the interaction of osteocalcin with the G protein-coupled receptor family C group 6-member A (GPRC6A) receptor has accompanied the characterization of several roles that this peptide serves in body regulation and homeostasis. These roles include the modulation of memory in the brain, fertility in the testis, fat accumulation in the liver, incretins release in the intestine and adaptation to exercise in muscle, in addition to the well-known effects on β-cell proliferation, insulin release and adiponectin secretion. The aim of the present review was to provide a practical update of the multi-organ effects that osteocalcin exerts through its interaction with GPRC6A and the clinical implications of this.

Contents

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Abbreviations: BGLAP, gene for osteocalcin; GPRC6A, G protein-coupled receptor family C group 6-member A; ucOC, undercarboxylated osteocalcin

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1. Bone produces osteocalcin

Bone is a dynamic tissue in constant remodeling (resorption and formation) and with a high capacity to regenerate. In addition to providing support to the body, protection for certain organs and enabling locomotion, bone produces molecules that act in an autocrine, paracrine and endocrine manner (1). One such molecule is osteocalcin, the endocrine function of which was discovered 10 years ago. Due to its extensive secretion during bone mineralization, osteocalcin was suspected to be exclusive to bone physiology. However, through studies performed in mice, the role of osteocalcin in metabolic modulation was elucidated (2,3).

Osteocalcin is a small protein (49 amino acids) encoded by the *BGLAP* gene synthesized by osteoblasts, and is present in two forms: Carboxylated (cOC) and undercarboxylated (ucOC). Only ucOC can signal as a hormone while cOC cannot (2,3).

ucOC and cOC can be measured in plasma separately or as the total osteocalcin (tOC), which includes the two forms independently of their degree of carboxylation, as well as recognizable fragments released when bone resorption occurs. Only 10-30% of the secreted osteocalcin reaches systemic circulation, while the remaining fraction is incorporated into bone matrix. In bone, cOC represents 15% of the non-collagen proteins of the matrix and contains three γ-carboxyglutamic acid residues. On the other hand, ucOC represents one third of tOC. The serum concentration of tOC has been considered a biochemical marker of osteogenesis that reflects the number and activity of osteoblasts (4,5).

2. Identification of the endocrine effect of osteocalcin and its action through GPRC6A

In 2007, Lee *et al* (6) demonstrated that ucOC increases the insulin secretion and proliferation of pancreatic β-cells, as well as adiponectin secretion from adipose tissue, thereby improving insulin sensitivity in mice. They also demonstrated that osteocalcin reduces fat mass and increases energy

expenditure by increasing the expression of genes involved in β-oxidation (*Pparα* and *Foxa2*) and in the electron transport chain (*Atp5a1, Atp5b, Mt-nd2, Cox* and *Cyc1*). This was the first irrefutable evidence of the participation of osteocalcin in carbohydrate, lipid and energy metabolism (6).

ucOC acts through binding to G protein-coupled receptor family C group 6-member A (GPRC6A). In fact, ucOC and testosterone are the only ligands of Gprc6a that have been validated using genetics *in vivo,* despite other ligands having been discovered *in vitro* (7-9). Although well stablished functions of OC through GPRC6A and GPR158 are presented later, a brief description of the expression, localization and function of GPRC6A in human cells and tissues is displayed next.

GPRC6A is expressed in several human, chimpanzee and small species tissues, including brain, lung, liver, heart, kidney, pancreas, skeletal muscle, placenta, spleen, ovary, testis, leukocytes, monocytes and adipocytes. However, the human ortholog GPRC6A is mostly retained intracellularly, in contrast to the cell-surface-expressed murine and goldfish ortholog (9,10).

This intracellular retention occurs in carriers of an insertion/deletion in exon 2 (SNP rs6907580 A/G/T) that eventually leads to a stop-codon early in the receptor sequence at amino acid position 57 (located in the third intracellular loop of GPRC6A), resulting in a non-functional receptor as reported by Jørgensen *et al* (10). According to this author, the functional variant is much more prevalent in the African population than in European and Asian populations, but further studies are required to elucidate the clinical significance of this allele variation among different populations (10).

As three mRNA isoforms for Gprc6a have been identified (1365, 853 and 1165 bp), the functionality of the GPRC6A receptor may be dependent on a tissue-specific regulation mechanism, which is also the case for other receptors whose function and tissue-specific expression is regulated by alternative splicing (11). GPRC6A mRNA isoform 1 is highly expressed in the brain, skeletal muscle, testis and leucocytes; moderately expressed in the liver, heart, kidney and spleen; and lowly expressed in the lung, pancreas, adipocytes, placenta and ovary. Isoforms 2 and 3 are less abundant and are possibly naturally occurring splice variants (12). Therefore, although the pancreas and adipocytes express low levels of GPRC6A mRNA at the transcriptional level, these are the main organs of ucOC action, suggesting a different mechanism of regulation at other levels (translational and post-translational) or the existence of an ortholog receptor that also partially mediates the action of ucOC.

3. Downstream signaling pathways activated by the osteocalcin-GPRC6A interaction

At least two signaling pathways activated by the osteocalcin-GPRC6A interactions have been identified (Fig. 1): i) The IP3-Ca^{+2} pathway activated by the action of phospholipase C (PLC) that yields the secretion of insulin, adiponectin and possibly other hormones; and ii) the adenylyl cyclase-cAMP-PKA pathway that leads to the activation of the Mek-Erk cascade, thereby promoting functions in cellular proliferation, differentiation and modulation of insulin sensitivity (13).

The extracellular signal-regulated kinases (Erk) induce phosphorylation of CREB, which in turn binds to the cAMP response element (CRE) in the *Pparγ* gene. The *Pparγ* gene consequently leads to the transactivation of the adiponectin gene (*Adipoq*) by linking the Pparγ-Rxr heterodimer to the promoter region of the *Adipoq* gene, resulting in the synthesis of adiponectin (14). Signaling pathways are depicted in Fig. 1. In the pancreas, the binding of osteocalcin to GPRC6A also induces Erk phosphorylation and increases insulin synthesis (7). In Leydig cells, ucOC activates the ERK1/2 signaling pathway, increasing the intracellular calcium content and promoting the production of 25-OH Vitamin D (8).

Furthermore, osteocalcin promotes the nuclear translocation of activated Nrf2, while inhibiting the activation of JNK in the liver; these are two well-described pathways in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (15).

4. Osteocalcin target tissues

Independently of the tissues where GPRC6A is expressed or is functionally active and, of the discoveries of signaling pathways that it activates, there are clear effects of ucOC in certain tissues and cells that are summarized in Fig. 2 and are described in the following paragraphs.

Pi *et al* (16) reported that knockout mice for the GPRC6A receptor (-/-) and that those that do not express the *GPRC6A* gene in pancreatic islets ($GPRC6A^{\beta\text{-cell-cko}}$) have a smaller pancreatic islet size, lower insulin content, lower pancreatic weight, lower number of islets, lower insulin mRNA expression and lower insulin secretion in response to osteocalcin. Furthermore, these mice exhibit glucose intolerance with a non-altered sensitivity to insulin. In this way, it was established that the direct activation of GPRC6A by osteocalcin modifies β-cell proliferation and insulin secretion (16). These findings were confirmed by Wei *et al* (17), who demonstrated that osteocalcin promotes the proliferation of pancreatic β-cells during development and adulthood through GPRC6A in mice (14,17).

In rodent-derived cultured adipocytes, Otani *et al* (18) demonstrated that ucOC increases the expression of adiponectin by increasing cAMP through GPRC6A activation. This occurs thanks to intracellular ERK signaling, which leads to the expression of *Pparγ* and the subsequent production of this insulin-sensitizing hormone (14,18).

In skeletal muscle, osteocalcin binds to GPRC6A, favoring the uptake and catabolism of glucose and fatty acids during exercise. In line with this, it stimulates the release of interleukin-6 by the muscle, a molecule that modulates the secretion of ucOC in the bone, increases the hepatic production of glucose and stimulates the release of fatty acids from the adipocyte. During aerobic exercise, circulating levels of ucOC doubled at the time insulin reaches its lowest point. By contract, in aged mice, osteocalcin is required and sufficient to maintain muscle mass. Therefore, osteocalcin participates in the body's adaptation to exercise and aids in maintaining muscle mass (19,20).

In the liver of mice, osteocalcin has been proven to be a deterrent of NAFLD. Following intermittent and continuous intraperitoneal infusion, osteocalcin upregulates the

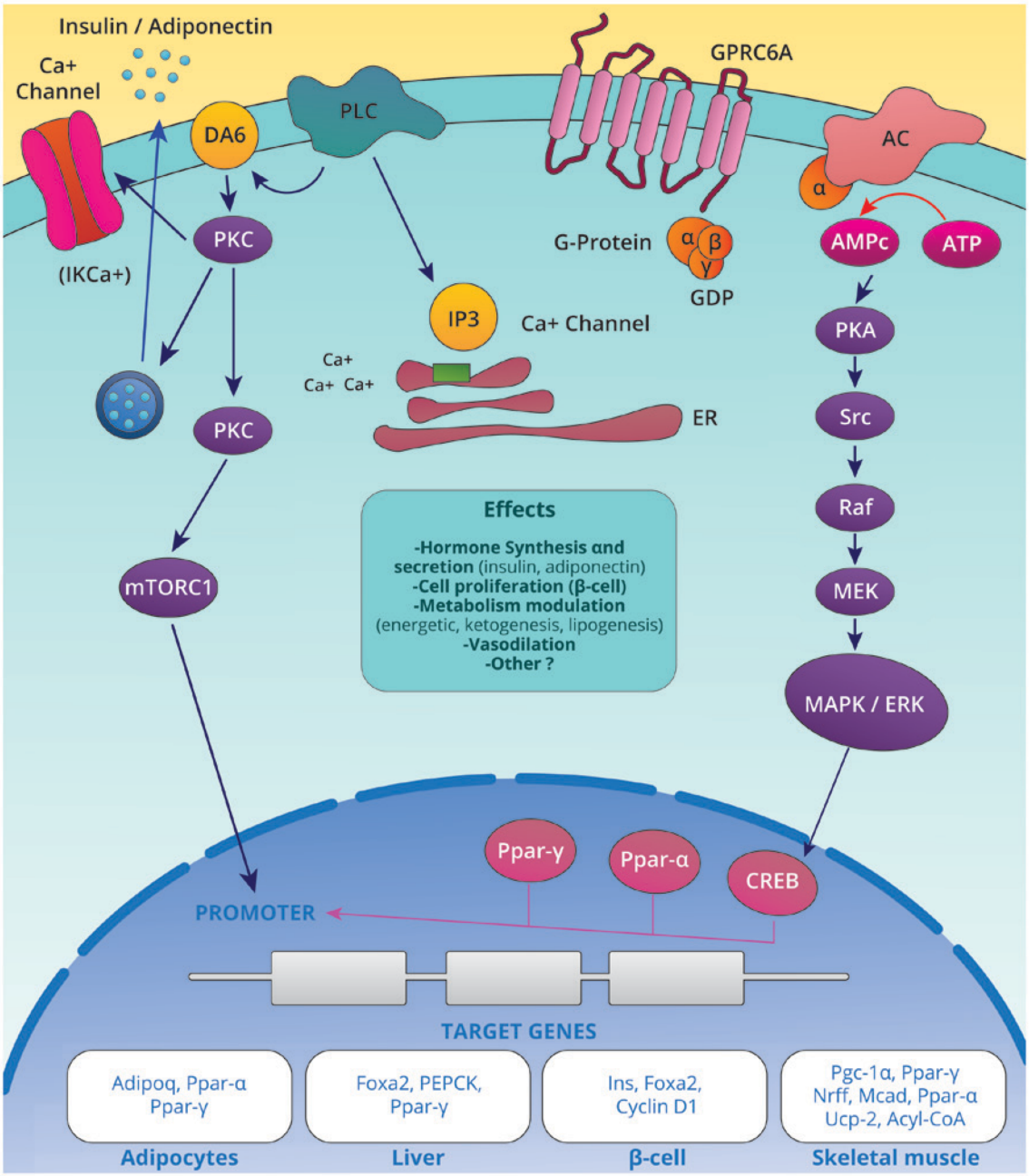

Figure 1. Signal transduction triggered by osteocalcin binding to GPRC6A and their final target genes and biological effects. GPRC6A, G protein-coupled receptor family C group 6-member A.

expression of antioxidant genes, including *Cat, Sod* and *Gpx*, which encode catalase, superoxide dismutase and glutathione peroxidase. Osteocalcin also decreases the content of triglycerides and reverses the histological damage in the liver of mice with NAFLD (15,20).

A potential interaction of ucOC with GPRC6A in blood vessels is possible but requires clarification, since identification of GPRC6A receptors in the aortic rings of rats and its activation by their agonist ornithine leads to the modulation of ion channels, including the intermediate-conductance Ca^{2+}-dependent K^+ (IKCa) channel, which in turn generates myocyte hyperpolarization, which may indicate a potential vasodilatory effect *in vivo* (17). As this is only an initial observation, vascular actions should be investigated in depth.

In the testicle, ucOC binds to GPRC6A receptors in Leydig cells to induce testosterone biosynthesis. Furthermore, osteocalcin acts through a pancreas-bone-testis axis that regulates male reproductive functions by promoting testosterone production independently of and in parallel with the hypothalamus-pituitary-testis axis (21). Additionally, knockout mice for osteocalcin exhibit low testosterone serum levels and secretion by Leydig cells, in addition to microanatomical and functional abnormalities in the testis, epididymis, seminal vesicles and sperm count (22-24).

GPRC6A receptors have also been observed in the basolateral membranes of intestinal endocrine cells. Here, oral, intraperitoneal and intravenous osteocalcin exerts its action by binding to GPRC6A, which in turn increases the secretion of glucagon-like peptide type 1 (GLP-1) *in vitro*

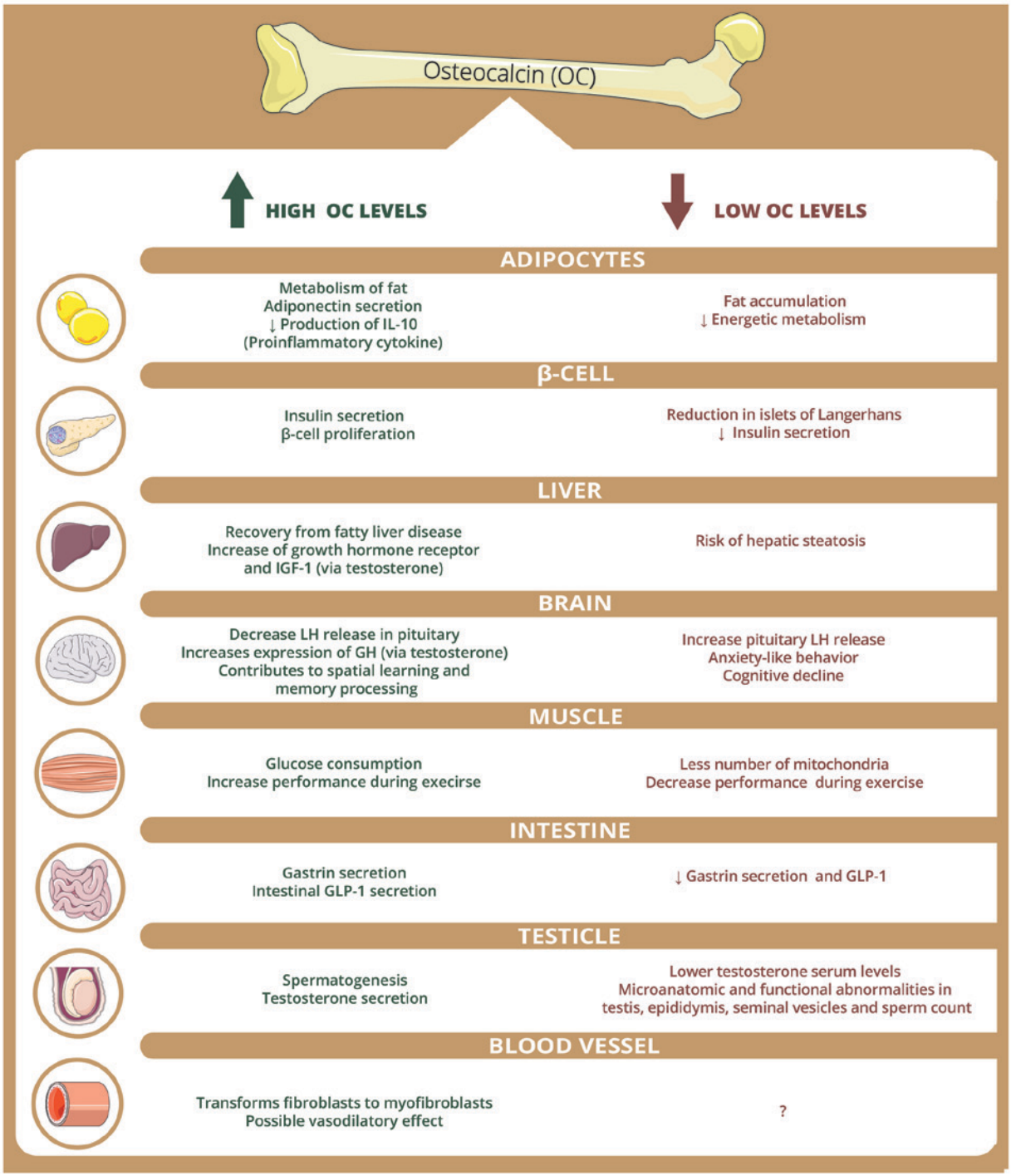

Figure 2. Target organs of OC action, and the effects of high and low serum or expression levels. OC, osteocalcin; GLP-1, glucagon-like peptide type 1.

and its serum levels in mice (25,26). In fact, the concept of a bone-intestine axis is plausible since, in addition to the effect of ucOC on GLP-1 release, certain animal and human studies have demonstrated that GLP-1 stimulates bone formation (the stage where osteocalcin release occurs at a high rate) and reduce bone resorption (25,26). In addition, certain osteoblastic cell lines express the GLP-1 receptor, which is regulated in accordance to the glycemic level (27). Despite this, limitations include the few *in vivo* studies on this intestine-bone communication and the fact that osteocalcin (-/-) mice exhibit no decrease in serum GLP-1 levels (28).

In the brain, ucOC has several direct and indirect effects, including increasing growth hormone synthesis (29). ucOC serves an important role in memory increasing and decreasing anxiety by binding to the newly identified GPR158 receptor, as described by Prof. Karsenty's group (30,31). Additionally, regions of the brain involved in spatial learning and memory processing exhibit an intense accumulation of osteocalcin. Notably, the brains of mice with low levels of osteocalcin are consistently smaller than those where normal levels of osteocalcin are observed. Additionally, mice with defective production of osteocalcin exhibit higher rates of anxiety-like behavior and cognitive decline, and the two symptoms are

Table I. Comparison of total OC and ucOC concentrations between healthy and diabetic subjects.

A, Total OC (ng/ml)

Author, year	Diabetes	HS	P-value	(Refs.)
Pietschmann and Schernthaner, 1988	5.2	6.6	0.03	(34)
Rosato *et al*, 1998	2.5	4.4	0.0006	(35)
Akin *et al*, 2003	4.44	8.82	0.05	(36)
Achemlal *et al*, 2005	15.3	18.3	0.012	(37)

B, ucOC (ng/ml)

Author, year	Diabetes	HS	P-value	(Refs.)
Sanchez-Enriquez *et al*, 2017	1.5±1.4	2.3±1.8	<0.05	(38)
Díaz-López *et al*, 2013	3.57	4.45	0.009	(39)
Razny *et al*, 2016	3.04±0.28	4.48±0.57	0.025	(40)

OC, osteocalcin; ucOC, undercarboxylated osteocalcin; HS, healthy subjects.

fully corrected when osteocalcin is injected into the test subjects (32).

5. Importance of biological experimental findings regarding osteocalcin in human health and disease

There is abundant evidence of the pleiotropic effects of osteocalcin in animal and cellular models; however, replicating these findings in humans is paramount to embarking on meaningful translational research that will elucidate the therapeutic and/or prognostic value of this hormone. For this purpose, several studies have been conducted to assess and support the role of osteocalcin in human health and disease.

Recently, it was reported that the rs2274911 polymorphism in the GPRC6A gene is associated with insulin resistance in healthy weight and obese subjects independently of body mass index (BMI). Carriers of the risk allele A exhibited higher levels of fasting insulin, fasting plasma glucose, HOMA-IR and triglycerides, following correction for sex, age and ucOC levels (33). Furthermore, Oury *et al* (23) analyzed a cohort of patients with primary testicular failure and identified 2 individuals harboring the same heterozygous missense variant (SNP rs2274911; F464Y) in one of the transmembrane domains of GPRC6A, which prevented the receptor from localizing to the cell membrane. These patients exhibit glucose intolerance, insulin resistance and increased BMI (23). Therefore, the A risk allele of this variant predisposes to metabolic abnormalities and provides evidence of the importance of GPRC6A in human energetic metabolism, as suggested by seven studies (34-40) published in the last 10 years, comparing serum concentrations of osteocalcin among people with type-2 diabetes mellitus (T2DM) and the non-diabetic population. It is clear that lower levels of osteocalcin occur more frequently in T2DM when compared with healthy subjects (Table I). In fact, a recent meta-analysis also suggested that serum tOC levels may be lower among people with T2DM (41). Furthermore, patients with metabolic syndrome also have lower levels of serum tOC than healthy individuals, and an increase in serum tOC levels is associated with a significant mean increase in HOMA-B and a mean reduction of HbA1c, fasting plasma glucose levels, HOMA-IR and BMI (42). Additionally, a significant correlation between tOC and ucOC serum levels exists with markers of glycemic status and other cardio-metabolic parameters (43-50). Table II describes the correlation between tOC and ucOC and these glycemic and cardio-metabolic variables.

Other studies have evaluated the association between osteocalcin serum levels and parameters of atherosclerosis. The majority of studies have reported a significant association between osteocalcin serum levels and determinations of carotid intima-media thickness (cIMT), brachial-ankle wave pulse velocity and carotid plaques in patients with diabetes and healthy subjects (43,44,51-55).

A recent observational study evaluated the association between osteocalcin serum levels and cognitive performance in healthy adults, demonstrating that they were positively correlated with measures of executive functioning and global cognition in older women. The authors reported that lower serum osteocalcin concentrations were associated with brain microstructural changes in the putamen, thalamus and caudate, as well as with poorer cognitive performance (56). These findings have therefore broadened the functions undertaken by osteocalcin to include the brain and neural processing.

Finally, two previous studies evaluated the association between osteocalcin and NAFLD in children and adolescents with and without obesity. Patients with NAFLD exhibited lower serum osteocalcin levels than those in the control group and the osteocalcin concentration were inversely correlated with liver enzymes and the severity of NAFLD. In addition, a serum osteocalcin level below 44.5 ng/ml was revealed to be a good predictor of hepatic steatosis severity with a sensitivity and specificity of 80% (57,58). Furthermore, normoglycemic

Table II. Correlation coefficients of total OC and ucOC serum levels with glycemia, HbA1c, insulin and HOMA-IR, blood pressure, lipids and cIMT.

	Total OC			Undercarboxylated OC		
Variable	r	P-value	(Refs.)	r	P-value	(Refs.)
Glucose	-0.213	0.009	(45)	-0.283	0.006	(38)
	-0.085	0.025	(44)	-0.220	NS	(59)
HbA1c	-0.140	0.208	(46)	-0.228		(60)
	-0.023	0.573	(44)	<0.001		
Insulin	-0.243	0.003	(45)	-0.108	<0.001	(60)
HOMA-IR	-0.005	NS	(47)	-0.349	<0.05	(61)
				-0.144	<0.001	(60)
SBP	-0.068	0.001	(48)	0.277	0.049	(38)
DBP	-0.077	<0.001	(48)	0.450	0.003	(38)
% body fat	-0.240	<0.05	(49)	-0.311	0.048	(38)
BMI	-0.258	<0.001	(48)	-0.310	0.046	(38)
c-HDL	0.097	<0.001	(48)	0.150	0.030	(50)
Apo-B/Apo A-1	-0.01	0.850	(50)	-0.160	0.020	(50)
cIMT	-0.145	0.041	(43)	-0.33	<0.01	(59)

OC, osteocalcin; ucOC, undercarboxylated osteocalcin; HS, healthy subjects; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; c-HDL, cholesterol contained in High Density Lipoproteins; NS, non-significant; cIMT, carotid intima-media thickness.

postmenopausal women with NAFLD exhibited significantly lower serum osteocalcin levels than controls and the serum osteocalcin levels exhibited a negative correlation with the fatty liver index values, even following adjusting for confounding factors (51). In males, NAFLD is negatively associated with serum osteocalcin (53). These observations highlighted the role of osteocalcin as a potential protector against NAFLD development and deterioration, as well as a marker of its progression.

In conclusion, the increasing volume of evidence regarding the multi-organ effect of ucOC, supported by *in vivo* and *in vitro* findings, indicates the requirement for deeper approaches to clarify its participation in human health and disease, as well as to test its therapeutic potential. On the other hand, the validation of ucOC as a prognostic or pathogenic marker for metabolic-endocrine disorders remains to be fully elucidated since no universal standardized method for its measurements, nor any reference values, have been established. Therefore, the medical-scientific community must continue to advance efforts to clarify the participation of ucOC in human health and disease and the clinical implications of this.

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Availability of data and materials

The datasets used during this review are available from the corresponding author on reasonable request.

Authors' contributions

MCD-F and RF-DL performed the literature search, interpreted the results and wrote the manuscript. JRV-B conceived the review, performed the literature search, interpreted the results, wrote the manuscript and gave final approval of the version to be published.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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