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Review

Osteocalcin: A bone protein with multiple endocrine functions

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A B S T R A C T

Bones are now recognised as endocrine organs with diverse functions. Osteocalcin, a protein primarily produced by osteoblasts, has garnered significant attention. Research into osteocalcin has revealed its impact on glucose metabolism and its unexpected endocrine role, particularly in its undercarboxylated form (ucOC). This form influences organs, affecting insulin sensitivity and even showing correlations with conditions like type 2 diabetes and cardiovascular diseases. However, analytical challenges are impeding advances in clinical research.

Various immunoassays like RIA, EIA, ECLIA, IRMA, and ELISA have been developed to analyse osteocalcin. Recent innovations include techniques like OS-ELISA and OS phage Immuno-PCR, enabling fragment analysis. Advancements also encompass porous silicon for detection and ECLIA for rapid measurements. The limitations of immunoassays lead to ucOC measurement discrepancies, prompting the development of mass spectrometry-based techniques. Mass spectrometry increasingly quantifies carboxylated, undercarboxylated, and fragmented forms of osteocalcin.

Mass spectrometry improves routine and clinical analysis accuracy. With heightened specificity, it identifies carboxylation status and serum fragmentations, boosting measurement reliability as a reference method. This approach augments analytical precision, advancing disease understanding, enabling personalised medicine, and ultimately benefiting clinical outcomes.

In this review, the different techniques for the analysis of osteocalcin will be explored and compared, and their clinical implications will be discussed.

1. Introduction

Evolution conferred certain species with an advantage by endowing them with a skeletal structure. However, the skeleton remains highly energy demanding. Therefore, the skeleton needed a way to communicate with the energy system. Unfortunately, this mechanism was long overlooked, as scientists believed that the skeleton could not act as an endocrine gland [1]. Certain proteins produced by osteocytes and osteoblasts have since been found to possess an endocrine role within the bone. This group includes various proteins such as FGF-23 and osteocalcin (OC) [2].

The endocrine role of OC was discovered by Karsenty's team through the depletion of the OC gene in mice [3,4]. In these mice, hyperglycemia, hypoinsulinemia, reduced beta cells number, insulin insensitivity, and increased fat mass were observed. Interestingly, all these effects, resulting from the suppression of a protein previously known only for its involvement in bone, are related to glucose metabolism. Further research revealed the presence of insulin receptors in the bone and OC receptors in the beta cells of the pancreas. Thus, they discovered the role of bone in glucose metabolism, with OC emerging as the prominent player in this process [3]. However, there are conflicting results in the literature. When retested by Komory *et al.*, the findings were not consistent. The disparity appears to be attributed to the differences in the mouse strains used for the experiment [5].

OC is thus a small protein, almost exclusively synthesized by osteoblasts, first mentioned in 1975 [6]. This peptide is the most abundant non-collagenous protein in bones, consisting of 49 amino acids, and weighing approximately 5.9 KDa. It belongs to the group of gamma-carboxylated proteins (Bone Gla Proteins), with three possible carboxylations occurring at residues 17, 21, and 24. Gla Proteins are a group of proteins containing a gamma-carboxylation on a glutamine residue. Osteocalcin exists in various forms, with the carboxylated form (cOC)

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representing a state where all three carboxylation sites are fully carboxylated. The undercarboxylated form (ucOC) includes all osteocalcin forms with fewer than three gamma-carboxyglutamic acid (Gla) residues but more than zero. Uncarboxylated osteocalcin (unOC) represents the form without carboxylation. cOC, ucOC and unOC forms of osteocalcin are detectable in bone tissue as well as in the circulation [7].

The gamma-carboxylation process is catalyzed by an enzyme called gamma-glutamyl carboxylase (GGCX) and requires vitamin K as a cofactor [8].

As depicted in Fig. 1, carboxylation increases the affinity of OC for calcium ions on the surface of the hydroxyapatite (HAP) crystal in the bone matrix [9]. This is attributed to a conformational change in the presence of calcium, leading to the appearance of three alpha helices that enhance the affinity for HAP. cOC plays a crucial role in bone health and function, as it is involved in bone formation, resorption, and mineralization [5].

Total OC (tOC), a combination of both cOC, ucOC and unOC, serves as a bone turnover marker. Elevated levels of tOC have been found in patients with conditions such as osteoporosis and Paget's disease [10,11]. In patients undergoing hemodialysis, serum OC levels have also been found to be positively correlated with bone formation [12].

Although cOC exerts local actions in the extracellular matrix of bone, OC also has additional functions in the human body. For example, circulating unOC acts as an endocrine hormone in glucose metabolism, as shown in Fig. 1 [10]. Specifically, the administration of exogenous unOC to wild-type mice improves insulin sensitivity and insulin production, which mitigates the risk of type 2 diabetes [13]. Conversely, depletion of the OC gene in mice increases blood glucose, impairs glucose tolerance (GTT) and insulin sensitivity, reduces beta cell mass and pancreatic insulin content, and increases fat mass [14]. Several human studies have shown that a decrease in tOC is associated with a higher risk of type 2 diabetes, a higher body mass index, and lower insulin sensitivity. [11,15].

In addition to carboxylation, OC can also undergo fragmentation and degradation. These fragmented forms can be found in plasma and serum, and can be detected using immunoassays [16]. Degradation primarily occurs within the C-terminal region of the OC protein. This degradation process exhibits rapid kinetics, with some studies demonstrating that OC undergoes degradation in serum within a timeframe of less than 6 h at room temperature [16]. Immunoassays designed to target the intact form of OC may thus not effectively recognize the degraded fragments. Consequently, discrepancies arise in the assessed concentrations between immunoassays directed at tOC and those targeting the N-terminal mid-region [17–19]. The precise determination of OC concentration becomes challenging with immunoassays, as it becomes contingent upon factors such as degradation kinetics, sample preservation conditions, and the presence of pre-existing fragments in the sample.

OC is thus an intricately structured protein found in various isoforms, each with distinct conformations influenced by their capacity to bind calcium ions. These diverse conformations imbue OC with multifaceted functions, pivotal in both skeletal integrity and energy metabolism [20]. However, the protein's complexity poses challenges in elucidating its physiological roles, particularly concerning the unOC form, as existing literature exhibits discrepancies owing to methodological limitations in quantifying tOC, cOC, and unOC. Rapid degradation of OC in serum leads to the coexistence of intact and fragmented OC species in circulation, confounding the interpretation of data obtained from different assays. Consequently, comparability among results obtained from varied methodologies is hindered. Thus, there exists a pronounced deficiency in measurement techniques that lack the requisite specificity to yield conclusive findings regarding OC's physiological implications.

In the past decade, research has increasingly focused on detecting OC carboxylation states, largely facilitated by advancements in mass spectrometry [21–23]. Additionally, OC and other bone markers appear to play a role within a liver-bone axis, which has been investigated for its emerging significance in both non-alcoholic fatty liver disease (NAFLD) and osteoporosis [24,25]. Recent studies have also explored the link between OC and the central nervous system (CNS), as OC is implicated in neuronal structure, neuroprotection, and the regulation of cognition and anxiety. However, methodological variability has led to inconsistent findings and conflicting data in the literature. Despite this, evidence suggests a potential connection between OC and CNS function [26].

This article aims to address this shortfall by reviewing extant analysis methods and providing an overview of available immunoassays. Furthermore, it will expound upon recent advancements in mass spectrometry-based detection methods, which offer enhanced specificity in quantifying different OC fragments. Mass spectrometry emerges as a promising avenue for detecting and quantifying OC fragments with greater precision, potentially resolving existing ambiguities in understanding the molecule's biological significance due notably to its ability to detect carboxylations on a protein.

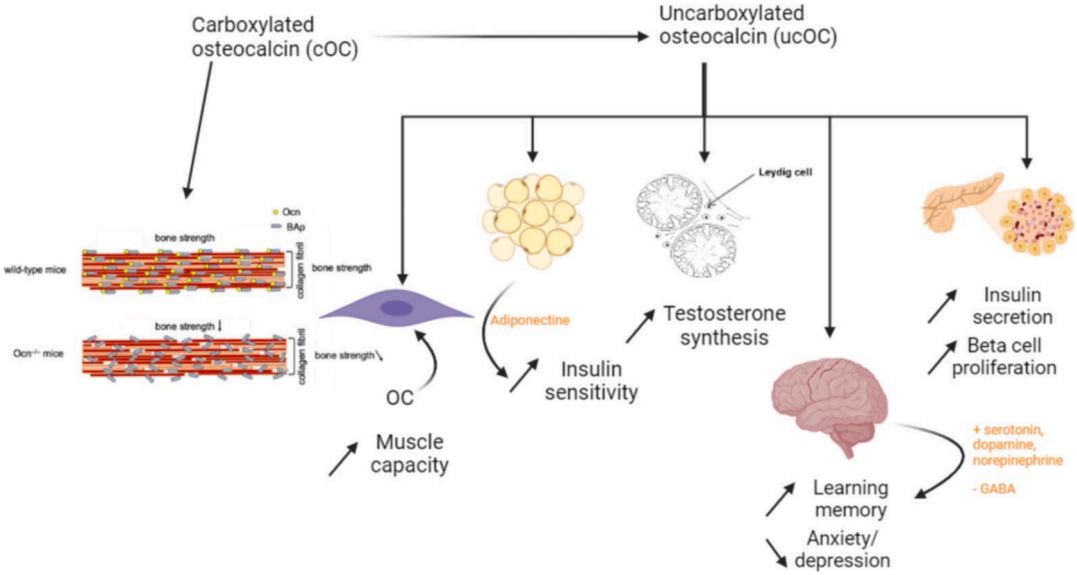

Fig. 1. Roles of OC [4].

2. Immunoassays

Immunoassays have been extensively employed for the analysis of OC. Several types of immunoassays have been developed, including radioimmunoassays (RIA), enzyme immunoassays (EIA), electrochemiluminescence immunoassays (ECLIA), immunoradiometric assays (IRMA), and enzyme-linked immunosorbent assays (ELISA). The different tables presented in this review provide a comprehensive comparison of the studies discussed in this review.

2.1. RIA and pre-RIA techniques

Before the advent of RIA, OC analysis and characterization were conducted using acrylamide electrophoresis gel and isoelectric focusing. Bone Gla-Proteins (BGP) were isolated from demineralized bones of various species through gel filtration [27]. However, this technique lacked specificity as it detected all BGPs present in the sample [6]. Consequently, RIA was introduced as it offered enhanced specificity and sensitivity.

RIA for OC was first developed by Price *et al.* in 1980, employing bovine OC (bOC) as the basis [28]. Due to the amino acid sequence similarities between bOC and human OC (hOC), hOC demonstrated 100 % cross-reactivity with bOC [28,29]. Consequently, the concentration of hOC was determined using bOC immunoassays.

Despite the presence of cross-reactivity, RIA already demonstrated good sensitivity, e.g. the study of Patterson-Allen *et al.* described earlier showed a sensitivity of 50 pg/mL [9]. These results are remarkable, and most of the other assays were also highly sensitive, with measurements primarily below the nanogram per microliter range. Srivastava's team achieved a sensitivity of 0.9 ng/mL, while Carstanjen's study reported a level of 200 pg/mL [30,31]. All the results are summarized in Table 1.

Although RIA was sensitive, it had drawbacks such as the instability of ^{125}iodine-OC. The use of radiolabeled proteins also required special considerations. As an alternative to RIAs, other assays were rapidly developed, including EIA using osteocalcin-β-D-galactosidase conjugates. This EIA demonstrated better sensitivity, with 50–100 pg/mL, but cross-reactivity between bOC and hOC still persisted. A comparison with RIA showed similar results between both methods [32].

2.2. IRMA

In addition to RIA, IRMA methods were also utilized for determining OC concentrations. Garnero *et al.* conducted a comparative study between IRMA and RIA to determine the different immunoreactive forms of OC [19]. The comparison involved three IRMA assays and three RIA assays. The sensitivities varied, with the most sensitive RIA assay exhibiting a sensitivity of 0.023 ng/mL and an intra-assay CV of 4.5 % [19]. Dumon *et al.* developed an IRMA against synthetic hOC with a sensitivity of 0.3 ng/mL, an intra-assay CV of less than 5 %, and an inter-assay CV of less than 7 % [33]. The results are presented in Table 2.

Table 1
Comparison table of the developed RIA.

Authors	Immunoassay	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Carstanjen *et al. 2003* [30]	RIA	Equine OC	3.68–127.31 ng/mL	0.2 ng/mL	Intra-assay: 6.2 % Inter-assay: 8.2 %	Higly specific, no cross-reaction	93.88–107.9 %
Jtippner *et al.* 1986 [68]	RIA	hOC 37–49	/	/	/	Highly specific, detect intact OC	/
Patterson-allen *et al.*1982[9]	RIA	Intact OC	0.1–4.0 ng/mL	<50 pg/mL	Intra-assay: 6.2 % Inter-assay: 6.3 %	Cross-reactivity with calf and human	>95 %
Price *et al.* 1980[28]	RIA	Calf OC	/	/	/	Cross-reactivity to human: 100 %	/
Srivastava *et al.* 2000 [31]	RIA	Synthesised (NH2-CSDQYGLKTAYKRIYGITI-COOH)	0.9–80.0 ng/mL	0.9 ng/mL	Intra-assay: <10.0 % Inter-assay: <7.0 %	/	81–111 %
Srivastava *et al.*2002 [41]	RIA	unOC	3.1–400.0 ng/mL	6.25 ng/mL	Intra-assay: <15.0 % Inter-assay: <15.0 %	Non-specific binding: <5%	94–113 %
Tanaka *et al.* 1986 [32]	EIA	bOC	1.6–50 ng/mL	0.1 ng/mL	Intra-assay: 3.4–7.1 % Inter-assay: 4.8–12.6 %	Cross reaction with hOC and partially rat	81.4–95.5 %
Taylor *et al.* 1988, 1990[10,69]	RIA	Mid region hOC, unOC	/	20 pg/tube	Intra-assay: <10.0 % Inter-assay: <10.0 %	Low cross reactivity to bOC	<94 % hOC

Table 2
Comparison table of developed IRMA.

Authors	Immunoassay	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Dumon *et al.* 1996[33]	IRMA	Synthetic hOC 7–19 hOC 37–49	2 to 200 ng/mL	0.3 ng/mL	Intra-assay: <5.0 % Inter-assay: <7.0 %	/	/
Garnero *et al.* 1994[19]	A. IRMA 2 site B. IRMA “ C. IRMA “ D. RIA comp E. RIA comp F. RIA comp	A. Intact B. N-terminal/ mid C. mid/C-terminal D. N-terminal E. mid F. C-terminal	A. 0.3–200 ng/ mL B. 0.4–300 ng/ mL C. 0.5–150 ng/ mL	A. 0.3 ng/mL B. 0.4 ng/mL C. 0.5 ng/mL D. 0.081 ng/ mL E. 0.023 ng/ mL F. 0.32 ng/mL	Intra-assay: A. ≤ 3.5 % B. ≤ 3.8 % C. ≤ 3.6 % D. ≤ 4.0 % E. ≤ 4.5 % F. ≤ 5.2 %	/	A / B 96–103 % C / D / E / F /
	IRMA 2 site	N-terminal/ mid	0.4–300 ng/mL	0.4 ng-mL	Intra-assay: ≤4.0 % Inter-assay: ≤6.0 %	Not with ovine and bOC	96–103 %
Nakatsuka *et al.* 1991 [70]	IRMA	tOC	1.0–64.0 ng/mL		Intra-assay: 2.3—-2.4 % Inter-assay: 2.2–5.2 %	bOC	104–116 %
Ostoemeter Biotech[49]	IRMA	Mid/N-terminal OC	/	0.5 ng-mL	Intra-assay: 3.7–4.5 % Inter-assay: 3.9–6.7 %	/	/

2.3. ELISA/Immunofluorometric assay

At the time of ELISA development, the physiological reference values of OC were known and recognized, but the medical community had not universally accepted the clinical significance of the protein as a biochemical marker for bone turnover. One of the reasons for this was probably the use of a two-site immunoassay that only measured tOC and might not detect fragmented OC [14]. Knapen *et al.* analyzed the differences between competitive and sandwich immunoassays and concluded that sandwich immunoassays, due to their better coverage of the molecule, were able to exclude fragment detection. Sandwich methods were also reported to be more sensitive [34]. However, the distinction between tOC and the mid N-terminal sequence remained problematic. There was a significant lack of specificity, as demonstrated

Table 3
Comparison table of ELISA/fluorometric assays.

Authors	Immunoassays	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Bhadricha *et al.* 2019 [39]	Sandwich ELISA	Human tOC	/	1 ng/mL	Intra-assay: 8.6 % Inter-assay: 9.4 %	/	92.38–105.96 %
Eick *et al.* 2020[40]	ELISA on dried blood spots	tOC (1–49)	0.2 to 10 ng/ mL	0.34 ng/mL	Intra-assay: 8.3 % Inter-assay: 14.8 %	tOC	96–102 %
Ferron *et al.*2010[51]	ELISA	Human cOC	6.3 to 400 ng/ mL	/	/	Cross reactivity with unOC	/
Fu *et al.* 1999[71]	ELISA	Rat OC		0.1–0.15 ng/ mL	Intra-assay: <9.0 % Inter-assay: <9.0 %	/	97–116 %
Funaoka *et al.* 2010 [72]	Sandwich ELISA	Total Rat cOC	0.1–100 ng/ mL	0.1 ng/mL	Intra-assay: ≤4.9 % Inter-assay: ≤5.9 %%	bOC:0.08 % Glu rat OC:0.38 %	89.4–103.7 %
Kuronen *et al.*1993 [14]	Sandwich ELISA	Human tOC	0.3–23 ng/mL	0.2 ng/mL	Intra-assay: 1.8–6.2 % Inter-assay: 2.0–5.9 %	/	/
Parviainen *et al.*1994 [38]	Sandwich ELISA	Human tOC	0.5–40.0 ng/ mL	<1 ng/mL	Intra-assay: <2.3 % Inter-assay: <2.5 %	/	105 %
Prakash *et al.*2005 [37]	Indirect ELISA	Human tOC	2.5 –160 ng/ mL	2.5 ng/mL	Intra-assay: 7–11 % Inter-assay: 12–15 %	/	95 –104 %
Rahimi *et al.* 2016 [45]	Competitive ELISA	Human tOC	/	0.1 ug/mL	/	/	89 %
Ylikoski *et al.* 1998 [36]	Dual-Label Immuno-fluorometric Assay	hOC, mid	0.5–80.0 ng/ mL	0.1 ng/mL	Intra-assay: 5.0– 11.0 % Inter-assay: 3.5–9.4 %	Cross-react with tOC	88.4–122 %

by studies comparing different commercial and in-house assays that showed no agreement between these different assays [35].

Ylikosky *et al.* presented a promising solution by developing a dual-label immunofluorometric assay capable of measuring both tOC and the N-terminal mid-fragment. This innovative assay was among the pioneers in employing fluorescent probes for precise OC measurement [36].

When it comes to ELISA, the majority of assays are designed to detect intact OC through sandwich immunoassays that bind to both the C-terminal and N-terminal regions of the protein. Consequently, measuring only the mid N-terminal sequence of OC posed a challenge. However, the immunoassays developed by Bhadricha *et al.*, Prakash *et al.*, and Parviainen *et al.* demonstrated sensitivities in the nanogram per microliter range [37–39], but the most sensitive ones were primarily around 0.1 ng/mL [14,40]. Most of these assays were sandwich immunoassays targeting OC, regardless of its state of carboxylation. The results are presented in Table 3.

During the late 1990 s and early 2000 s, numerous immunoassays were developed, as evident from the different tables. The key advancement in the new assays was the reduced analysis time, which helped prevent sample degradation during analysis. Immunoassays for OC were also developed for the serum of various species, including horses and rats [38,41].

2.4. Newly developed immunoassays

In the 2010 s, novel techniques emerged, such as open sandwich ELISA (OS-ELISA). In contrast to traditional sandwich ELISA, OS-ELISA overcame the limitation of requiring two epitopes for small peptides or fragments, which previously made their analysis impossible. OS-ELISA utilized only parts of the complete antibody (VH and VL domains) to reduce steric hindrance. The combination of variable domains with maltose-binding protein (MBP), created through *E. coli* transformation, enabled the detection of smaller proteins with two epitopes while preserving the sensitivity of ELISA. This technique, when coupled with microfluidic chips, facilitated reduced analysis time (from 4 h to 12 min) and required smaller sample volumes (from 50 μL to 5 μL) [42].

Another related approach is OS phage Immuno-PCR, developed by Dong *et al.*, in which the VH domain is linked to a displaying phage instead of MBP. The amount of phage in the OS-ELISA is proportional to the concentration of OC. After the ELISA, PCR is performed to detect the DNA of the phage. The highly specific and powerful signal amplification provided by Immuno-PCR allows for a significant increase in sensitivity, sometimes up to 10,000-fold [43,44].

The solid phase of OC detection has also undergone optimization. Rahimi *et al.* replaced the solid phase with porous silicon, resulting in an improved detected signal by up to 20 times [45].

Electrochemical immunoassays (ECLIA) have also been employed for measuring OC concentrations. Kim *et al.* developed an ECLIA based on the microwave-mediated immobilization of antibodies and the antibody-antigen reaction. This approach utilized a membrane for pretreatment and a custom-made electrode for measurement, enabling low-cost and user-friendly immunoassays for target proteins [46]. Additionally, the analysis of OC in dried blood spots using an immunoassay was recently developed by Eick *et al.* The sensitivity was 0.1 ng/mL, and the intra/inter-assay CVs were below 8.25 % and 14.8 %, respectively. The recovery was also excellent, as shown in Table 4[40].

In conclusion, numerous immunoassays have been developed for OC analysis, employing various techniques and strategies to enhance sensitivity and specificity. Currently, most available kits are commercial ELISA-based assays, known for their high sensitivity. While these kits are predominantly manual [47,48], automated options have also been developed, such as the "IDS-iSYS N-Mid Osteocalcin" assay [49,50]. The immunoassays that are mostly used and have been developed in the last decade are present in Table 5. However, mass spectrometry is increasingly being used as it provides information on the different fragments present in a sample and allows for more precise quantification of cOC, ucOC, and unOC. Moreover, as explained in the next section, the quantification of unOC is particularly important, but the available immunoassays lack accuracy.

3. Uncarboxylated osteocalcin analysis

The analysis of unOC has become increasingly important due to its apparent involvement in different physiological mechanisms [3]. Two main methods are used to measure circulating unOC. The first is the HAP binding assay, which involves using HAP to bind cOC, which is then removed by centrifugation. UnOC can then be measured in the serum supernatant using commercially available immunoassays (RIA or ELISA) [13]. This method is indirect and provides semi-quantitative results as it does not precisely quantify the serum concentration of unOC or cOC [51]. The second method involves direct immunoassays that use antibodies specifically targeting unOC.

3.1. UnOC immunoassay

Different immunoassays have been developed to assess the concentration of unOC. Until 2020, the immunoassay developed by Takara Shuzo Co. (Kyoto, Japan) was the only commercially available kit for measuring unOC [52]. The sensitivity of this kit was 0.25 ng/mL, with intra-assay and inter-assay CVs of 5.2 % and 8.3 %, respectively. However, it overestimates large unOC fragments, leading to inaccuracies in the determination of unOC or the ratio of unOC to tOC [13].

Since 2020, a new commercially available kit developed by Lacombe *et al.* recognizes unOC. This ELISA kit uses capture antibodies (mouse monoclonal antibodies 8H4) specific to the C-terminal region of hOC (amino acids 30 to 49) and detection antibodies (mouse monoclonal antibodies 4B6) specific to the mid-region of human unOC (amino acids 12 to 28). Antibodies were generated by immunizing mice with full-length, bacterially-produced recombinant human unOC. Hybridoma clones were subsequently established from polyethylene glycol-fused

Table 4
Comparison table of newly developed immunoassays.

Authors	Immunoassay	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Dong *et al.* 2012[43]	phage-based open-sandwich immuno-PCR immunoassays	OC C-terminal 7	/	<16 pg/mL LOQ: 61 pg/mL	/	/	
Hasan *et al.* 2013[44]	Open-sandwich immuno-PCR Immunoassay	OC C-terminal 7	/	100 fg/mL	/	/	/
Ihara *et al.* 2010[42]	Micro open sandwich ELISA	BGP C-7	/	1 ng/mL	/	Cross reactivity with albumin	/
Kim *et al.* 2018 [46]	Membrane-Based Microwave-Mediated Electrochemical Immunoassay	Intact OC	0.5–300 ng/mL	100 pg/mL	Intra-assay: 2.63–7.67 % Inter-assay: 1.20–6.59 %	Cross reactivity with CTX: 13.1 % P1NP: 28.5 % PTH: 17.1 %	/

Table 5
Comparison table of immunoassays used and developed in the last decade.

Authors	Immunoassays	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Takara (Glu-OC MK-118; Takara Bio Inc., Otsu, Shiga, Japan)	ELISA	unOC	0.25—–8 ng/ mL	0.25 ng/ mL	Intra-assay: 4.6 % Inter-assay: 5.7 %	Cross reactivity to cOC (5 %) bOC (1.7 %)	71–124 %
ImmunodiagnosticSystems (N-MID® Osteocalcin; ImmunoDiagnosticSystem ltd., Boldon, UK)	EIA	Human OC	2–200 ng/mL	2 ng/mL	/	/	/
Cusabio (Human Osteocalcin/Bone gla protein, OT/BGP ELISA kit; Cusabio, Houston, USA)	ELISA	Human OC	31.25–2000 pg/mL	31.25 pg/mL	Intra-assay: >8% % Inter-assay: >10 %	/	/
Rahimi *et al.* 2016[45]	Competitive ELISA	Human tOC	/	0.1 ug/ mL	/	/	89 %
Kim *et al.* 2018[46]	Membrane-Based Microwave-Mediated Electrochemical Immunoassay	Intact OC	0.5–300 ng/ mL	100 pg/ mL	Intra-assay: 2.63–7.67 % Inter-assay: 1.20–6.59 %	Cross reactivity with CTX: 13.1 % P1NP: 28.5 % PTH: 17.1 %	/
Bhadricha *et al.* 2019[39]	Sandwich ELISA	Human tOC	/	1 ng/mL	Intra-assay: 8.6 % Inter-assay: 9.4 %	/	92.38–105.96 %
Eick *et al.* 2020[40]	ELISA on dried blood spots	tOC (1–49)	0.2 to 10 ng/ mL	0.34 ng/ mL	Intra-assay: 8.3 % Inter-assay: 14.8 %	tOC	96–102 %
Lacombe *et al.* 2020[53]	Sandwich ELISA	unOC	0.0375–1.8 ng/mL	0.0147 ng/mL	Intra-assay: 3.6 % Inter-assay: 7.6 %	Cross-reactivity toward partially or fully carboxylated OC (5 %)	94–111 %

splenocytes and screened to identify clones producing antibodies that recognize the full-length unOC or the C-terminal region. Various monoclonal antibodies were selected, and their specificity was validated through dot blots using serial dilutions. Following the development of antibodies, the next step involved pairing them for the creation of an immunoassay. Multiple pairs were evaluated, and the capture/detection pairs 4C5/4B and 8H4/4B6 exhibited the highest efficacy in detecting unOC with minimal cross-reactivity. The immunoassay underwent testing on diverse cohorts, including overweight and obese women without diabetes, as well as severely obese men and women with and without type 2 diabetes, to evaluate the performance parameters of the assay kit [53].

The ELISA kit detects unOC in the range of 0.0375 to 1.8 ng/mL with low cross-reactivity to cOC (<4%) or ucOC (<5.2 %). The intra-assay and inter-assay CVs were 3.6 % and 7.6 %, respectively. The detection limit was 0.0147 ± 0.005 ng/mL, and the linearity with serum and plasma was excellent (94–111 %). The results remained unchanged following two freeze–thaw cycles [53]. While these results seem promising, they remain unvalidated by individuals outside those cited in the article. Consequently, caution is warranted in their interpretation due to the absence of independent verification and the ongoing absence of retrospective analysis.

Before commercially available kits were introduced, two separate assays had to be run simultaneously, measuring the ratio of Gla17-OC (OC carboxylated only on the glutamine 17) to tOC, providing an estimation of the proportion of inactive OC in circulation. However, this method has limitations: 1) it requires running two separate assays for Gla17 and tOC, and 2) it does not directly measure unOC. In addition to the kit for unOC, Takara Shuzo Co. also developed a kit recognizing the Gla17-OC, with a detection limit of 0.5 ng/mL and intra-assay and inter-assay CVs of 3.3 % and 1.0 %, respectively. A summary table is present in Table 6.

Before the availability of these two immunoassays that directly recognized unOC, it had to be calculated using the formula ucOC = tOC – cOC. Serum tOC and cOC were measured using two-site immunoassays based on monoclonal antibodies, with intra-assay and inter-assay CVs of < 5 % and < 8 %, respectively. The difference between tOC and cOC indirectly indicates the relative amount of ucOC [54]. Moreover, besides

Table 6
Comparison of the parameters of the Biolegend kit with those of the Takara kit.

Authors	Immunoassays	Targeted OC	Linear range	LOD	%CV	Specifictiy	Accuracy/ recovery
Lacombe *et al.* 2020[53]	Sandwich ELISA	unOC	0.0375–1.8 ng/ mL	0.0147 ng/ mL	Intra-assay: 3.6 % Inter-assay: 7.6 %	Cross-reactivity toward partially or fully carboxylated OC (5 %)	94–111 %
Takara (Glu-OC MK-118; Takara Bio Inc., Otsu, Shiga, Japan)	ELISA	unOC	0.25—–8 ng/ mL	0.25 ng/ mL	Intra-assay: 4.6 % Inter-assay: 5.7 %	Cross reactivity to cOC (5 %) bOC (1.7 %)	71–124 %

the two aforementioned immunoassays, the others were testing ucOC but not unOC.

4. Mass spectrometry

The analysis of OC by mass spectrometry has been conducted in two different phases. The first phase occurred around the 2000 s, where studies primarily focused on measuring cOC. These studies mainly used bOC or synthesized OC, with hOC in serum not being a priority during this phase.

Various techniques have been tested to assess OC levels using mass spectrometry, but a gold standard method has not yet been approved. In 1981, Carr *et al.* conducted the first mass spectrometry analysis on OC derived from chicken bone. Decarboxylation of the intact protein was performed under low-pressure atmospheric and hydrolysis, followed by pre-treatment of the samples with high-performance liquid chromatography. Identification was realised using gas chromatography coupled to mass spectrometry [55]. Vahatalo *et al. (1999)* were the first to analyse hOC using tryptic digestion, which cleaved the molecule into three different parts observables in the mass spectrum. They also examined the degradation profile of the molecule using synthesized cOC [56].

The first Fourier Transform Ion Cyclotron Resonance (FTICR) analysis of OC was conducted by Nousiainen in 2002 on bOC. This study provided mass spectrometry evidence that cOC binds to metal ions such as Ca^{2+}, Mg^{2+}, or La^{3+}. FTICR was used because of its higher resolution, necessary for the identification of complexes containing multiple components [57].

The first analysis of human urine samples using high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was conducted by Ivaska's team. Immunoaffinity chromatography was performed to isolate OC, which was then analysed using Matrix-Assisted Laser Desorption Ionization Time of Flight mass spectrometry (MALDI-TOF). Human urine served as the matrix, and MALDI-TOF was used to characterise the different proteolytic forms of urine OC. This study was the first to report the relationship between bone mass and urine hOC [58].

OC levels were also measured in bovine and porcine animal feed. This study explored the application of high-resolution Q/TOF mass spectrometry to analyse OC as a genus-specific marker in meat and bone meal (MBM), given that immunoassays lack sufficient sensitivity. The high sensitivity of this MS-based technique enables the detection of trace amounts of OC in highly processed samples, facilitating reliable differentiation between major species, such as cattle and pigs. [59].

In the first phase of OC analysis by mass spectrometry, studies were also conducted on different animals, including fish, Neanderthals and modern primates. OC sequences from bones were tested using mass spectrometry and Edman sequencing [60].

The second phase occurred later, from approximately 2015. In this phase, the focus shifted towards clinical measurements, with mass spectrometry being applied to serum samples. The aim in this phase was primarily the detection of OC, but attention was given to the carboxylation status and fragmentation of the molecule, as these are major challenges in immunoassays [61].

Several articles have confirmed these developments. In 2015, Rehder et al. developed a MALDI-TOF method to assess various OC fragments present in human serum. This approach uniquely focused on evaluating the carboxylation state of these fragments, marking them as the first to analyse distinct OC degradation fragments in human serum. Initially, they used a sandwich immunoassay using tips coated with anti-human OC antibodies to isolate all forms of OC, followed by gradient chromatography to separate these forms. Finally, they analysed OC fragments via mass spectrometry [6263].

This technique has been used in research, notably in 2021 in a study on chronic kidney disease, where the carboxylation state plays a crucial role in fully understanding disease mechanisms and function [64]. Carboxylation has also been assessed on orbitraps using bOC [61]. In addition to carboxylation, other post-translational modifications have been tested using mass spectrometry, such as glycation [65].

From a clinical perspective, research has investigated OC variations across the human lifespan to aid in the age and sex identification of archaeological skeletons. Initial analyses were conducted using immunoassays, specifically ELISA, to assess its effectiveness in detecting OC in ancient bone samples. To validate ELISA's reliability, four samples underwent further analysis via LC-MS/MS, with quantification achieved through MaxQuant label-free quantification (LFQ). This study is the first to employ Data Independent Acquisition (DIA) with OC to enhance data accuracy. [22].

Mass spectrometry has been employed to enhance our understanding of the protein's chemical structure, particularly in relation to OC variants. Notably, Ami et al. conducted an experimental and computational investigation into the structural properties and calcium-binding activity of all OC variants. Their findings reveal that carboxylation sites differ in their impact on the OC structure and its interaction with calcium [21]. Further research, including doctoral theses, has focused on developing mass spectrometry methods to quantify the various OC forms and identify distinct fragments [23,66].

Mass spectrometry allowed the detection of OC, its fragments, and post-translational modifications, although their quantification remains pending. A reference method is required to quantify OC in clinical routine analysis [67].

Further developments are necessary to establish a reliable analytical method with appropriate limits of quantification or detection. The development of such a technique could be highly beneficial for assessing the concentration of unOC and the presence of different fragments in newly designed clinical studies, with greater specificity than immunoassays. Mass spectrometry has the potential to play a crucial role in future clinical studies due to its enhanced specificity and selectivity compared to other analytical methods. By utilizing mass spectrometry, the concentration of analytes can be assessed with improved accuracy and reproducibility, resulting in more reliable and precise results.

5. Conclusion

The field of immunoassays for the analysis of OC has undergone significant evolution, incorporating diverse techniques and strategies to enhance both sensitivity and specificity. This array of methods encompasses RIA, EIA, ECLIA, IRMA, and ELISA. Over time, immunoassays have progressed from scrutinizing bone Gla-Proteins through techniques like acrylamide electrophoresis and isoelectric focusing to more sophisticated approaches such as RIA. Despite their heightened sensitivity, RIA exhibited limitations, particularly in terms of cross-reactivity among OC variants. In subsequent phases, IRMA methods and ELISA/Immunofluorometric Assays emerged, aiming to refine sensitivity and specificity. However, ELISA faced challenges in distinguishing between carboxylated and undercarboxylated forms.

To address this issue, mass spectrometry has emerged as a valuable tool, offering enhanced specificity and enabling precise quantification of OC carboxylation and fragmentation. However, mass spectrometry is not without limitations. The standardization of protocols remains challenging, equipment costs are high, and the technique requires specialized training for operators.

Still, the emergence of LC-MS/MS methods facilitated the identification and characterization of various OC fragments. However, the current focus remains on characterization rather than quantification, with no established limit of quantification (LOQ) thus far.

In a prospective context, the development of an LC-MS/MS method for OC quantification, encompassing its carboxylation status and fragment composition, would be advantageous. The formulation of official guidelines in this regard could substantially enhance measurement specificity. The establishment of a mass spectrometry-based quantification approach would not only prove valuable for routine analyses but also hold the potential to elevate measurement accuracy in clinical

studies.

From a clinical perspective, standardized OC measurement would facilitate a clearer understanding of its role in glucose metabolism. Currently, conflicting findings surround osteocalcin's involvement in conditions such as Type 2 diabetes, partly due to variations across different immunoassay techniques.

Evidence suggests that unOC may also impact central nervous system function; however, results remain inconclusive. A standardized approach to measurement could resolve these discrepancies, providing a more accurate perspective on these associations. The discrepancies in results may also be attributed to insufficiently heterogeneous study populations. Future research could focus on examining these relationships in larger and more diverse populations.

Additionally, the bone-liver axis, while not fully understood, appears to be relevant in conditions like osteoporosis and non-alcoholic fatty liver disease (NAFLD), with OC and other bone markers implicated in this connection. Standardized measurement could therefore deepen our understanding of these diseases, potentially leading to improved treatments and outcomes.

The utilization of mass spectrometry in clinical studies holds great promise for improving the accuracy, reproducibility, and overall quality of analytical measurements. It has the potential to advance our understanding of diseases, facilitate personalized medicine approaches, and ultimately contribute to better patient outcomes.

CRediT authorship contribution statement

Determe William: Writing – original draft. **Hauge Sabina Chaudhary:** Investigation. **Demeuse Justine:** Writing – review & editing. **Massonnet Philippe:** Writing – review & editing. **Grifnée Elodie:** Writing – review & editing. **Huyghebaert Loreen:** Writing – review & editing. **Dubrowski Thomas:** Writing – review & editing. **Schoumacher Matthieu:** Writing – review & editing. **Peeters Stéphanie:** Writing – review & editing. **Le Goff Caroline:** Writing – review & editing. **Evenepoel Pieter:** Writing – review & editing. **Hansen Ditte:** Writing – review & editing. **Cavalier Etienne:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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