Intact or N-MID osteocalcin assays for assessment of bone formation in hemodialysis patients?

Intaktna ali N-MID-OC-osteokalcin metoda za oceno tvorbe kosti pri hemodializnih bolnikih?

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Ključne besede:
hemodializni bolniki, zdravljenje s kalcitriolom, imunoanalize za osteokalcin

Izvleček

Izhodišče: Osteoklacin (OC) je nekolageni protein kostnega matriksa, ki se diagnostično uporablja kot neinvazivni kazalec tvorjenja kosti. V krvnem obtoku se nahaja v obliki intaktne molekule in manjših fragmentov. Za določanje njegove koncentracije v serumu uporabljamo dve metodi, ki se razlikujeta po protitelesih, ki lahko zaznajo intaktno molekulo OC ali pa samo njen N-terminalni fragment (N-MID- OC). V raziskavi smo želeli ugotoviti, ali omenjeni metodi dajeta primerljive rezultate.

Metode: V raziskavo smo vključili serumske vzorce 102 bolnikov, ki so se zdravili s hemodializo (HD). 26 bolnikov se je zdravilo s kalcitriolom, ostalih 76 pa ne. Povprečna starost bolnikov je bila 60 let, vzroki za kronično ledvično odpoved so bili: diabetična nefropatija (27), kronični glomerulonefritis (31), policistične ledvice (26) in drugo (18). V serumskih vzorcih HD bolnikov smo izmerili koncentracije OC, prečnih povezovalcev kolagena (CTx) in paratiroidnega hormona (PTH). Koncentracije OC smo določali z metodama intact-OC in N-MID- OC. Primerjali smo rezultate obeh metod in ocenili povezanost OC s CTx in PTH. Za statistično analizo smo uporabili statistični program SPSS za okolje Windows.

Rezultati: Pri celotni skupini HD bolnikov so bile serumske koncentracije N-MID-OC 10,6-krat višje od intact-OC. Koncentracije N-MID--OC in inact-OC se niso značilno razlikovale med bolniki, ki so prejemali kalcitriol in tistimi, ki ga niso. Korelacija med rezultati obeh metod je bila visoka (r=0,702, p< 0,001).

Zaključki: Razlike med koncentracijami OC, izmerjenimi z metodoma intact-OC in N-MID--OC, so bile statistično značilne, vendar so dobro korelirale, zato ocenjujemo, da je njuna klinična uporabnost primerljiva.

Abstract

Background: Osteocalcin (OC) is a non-collagen bone matrix protein that is used as a non-invasive marker of bone formation. It is present in the circulation as an intact molecule and as fragments. The two known methods for OC determination in serum samples differ in the detection antibodies for an intact OC molecule and its N-terminal fragment. The aim of our study was to find out if these two methods give comparable results.

Methods: 102 serum samples of hemodialysis patients were analyzed. While 26 patients were receiving calcitriol treatment, the remaining 76 patients were not. The average age of patients was 60 years. The causes of chronic renal failure were: diabetic nephropathy (27), chronic glomerulonephritis (31), polycystic kidney (26), and other (18). OC levels, β-CrossLaps (CTx) and parathyroid hormone (PTH) were measured. Serum levels of OC were determined by intact-OC and N-MID-OC methods. Following the comparison of results, we assessed the relationships between OC, CTx, and PTH. SPSS 12.1 for Windows was used for statistical analysis.

Key words:
hemodialysis patients, calcitriol therapy, osteocalcin immunoassays

Citirajte kot/Cite as:
Zdrav Vestn 2011; 80: 11–7

Prispelo: 24. maj 2010,
Sprejeto: 14. jul. 2010

Results: Serum levels of N-MID-OC were 10.6-fold higher than intact-OC. N-MID-OC and intact-OC levels did not differ between patients with and without calcitriol therapy. The results of both methods correlated well ($r = 0.702$, $p < 0.001$).

Conclusions: The differences between osteocalcin serum levels assessed by intact-OC and N-MID-OC methods were statistically significant. However, their correlation was good, so we can conclude that their clinical application is comparable.

Introduction

Osteocalcin (OC) is a noncollagenous protein secreted by osteoblasts, osteocytes, and odontoblasts[1] and thus reflects osteoblastic function[2]. Serum OC has demonstrated clinical utility as a biomarker of bone formation. In a review by Camachio PM and Lopez NA[3], the measurement of bone turnover markers was recommended in the management of postmenopausal osteoporosis. The authors implied that turnover markers are a potential non-invasive, but reliable way of assessing skeletal activity after antiresorptive or anabolic therapy. Other authors[4-6] proved OC to be a valuable non-invasive index of metabolic bone disease in hemodialysis (HD) and/or in continuous ambulatory peritoneal dialysis patients. However, there are also limitations to the clinical usefulness of OC, such as short-term and long-term analyte instability, in addition to non-specificity. It is also worth mentioning that osteoblasts synthesize only intact OC which is secreted as a mature protein and stored mostly in the extracellular bone matrix. Only a small amount of OC is released into the blood stream. It has been confirmed that circulating OC is linked to changes in bone turnover rate during metabolic disorders such as osteoporosis, Paget's disease, and renal osteodystrophy. Despite the proteolytic degradation, both intact and fragmented OC can be identified in the patients' serum (Figure 1). One third of OC forms are present as intact OC molecule (aminoacids 1–49), one third as the N-terminal – MID fragment (aminoacids 1–43) and the last third as the C-terminal fragment (aminoacids 44–49). Various smaller fragments (aminoacids 1–19, 20–43) can also be present. OC fragments produced in various bone diseases may interfere with many existing assays. Because a variety of OC fragments are present in HD patients[7], we designed a short study of two OC assays that may yield different results. The study was carried out using sera of 102 HD patients.

The complex pathophysiology of bone and mineral metabolism in HD patients is fully influenced by oral calcitriol therapy for lowering PTH levels (K/DOQI Guideline 8B, 1a, p.593). The results of *in vitro* studies showed that the amount of osteocalcin is increased in the presence of small doses of calcitriol[8] and parathyroid hormone[9]. In HD patients the coexistence of both conditions, i.e. PTH secretion disturbances and

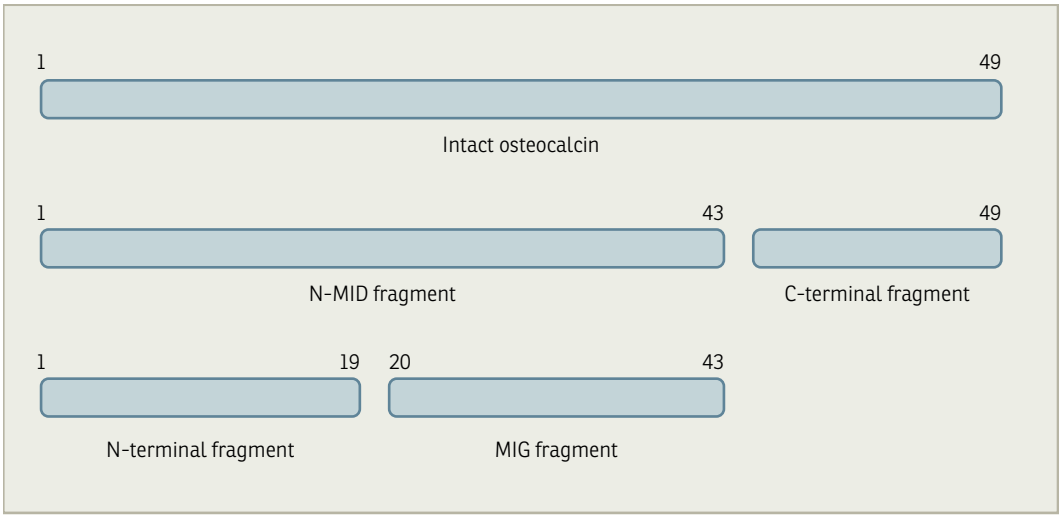

Figure 1: Intact osteocalcin and its main fragments in serum.

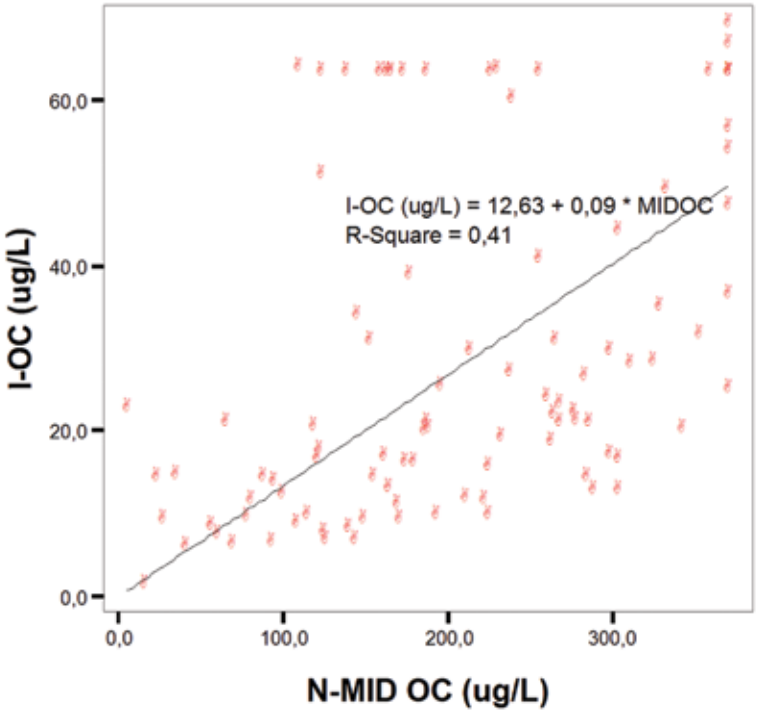

Figure 2: Correlation of intact and N-MID-OC in HD patients (r = 0.702, p< 0.05).

calcitriol substitution, influence OC-production and consequently its serum levels. Therefore, the aim of our study was to ascertain if intact OC (i-OC) and N-MID-fragment (N-MID-OC) assays give comparable results, and to elucidate the differences in serum levels of OC determined by both assays in HD patients. Because calcitriol influences OC serum levels, we formed two groups of patients: those receiving calcitriol therapy, and those without treatment.

Materials and methods

Patients

102 HD patients (age 60.1 ± 12.0 years) were enrolled in the study. Terminal chronic renal failure was caused by various kidney diseases: diabetic nephropathy (27 patients), chronic glomerulonephritis (31 patients), polycystic kidney disease (26 patients), and unknown disorders (18 patients). The patients had been treated with bicarbonate-containig dialysis solution, calcium and glucose concentrations being adjusted to the needs of dialysis. All patients were receiving phosphate-binding agents. In addition to that, 26 patients were receiving calcitriol treatment (subgroup 2) while the remaining 76 patients were not (subgroup 1). Residual renal function was present in 32 patients, its mean rate was 0.05 ± 0.03 ml/s/1.73 m^2. The study was approved by the Slovenian Ethics Committee for Research in Medicine. Informed consent was obtained from all participants.

Sample collection

Patients underwent HD sessions three times a week: on Mondays, Wednesdays, and Fridays. Blood sampling was performed shortly prior to a dialysis session, exactly 72 h after the previous session. Venous blood was drawn into Vacutainer tubes (Becton Dickinson, Rutherford, USA). The collected samples were left to clot for 30 min at room temperature. Aterwards they were centrifuged, aliquoted, and stored at -70°C for 3 months until assay.

Table 1: General charasteristics of HD patients.

	HD patients	Subgroup 1	Subgroup 2
Number	102	76	26
Age (years)	60.1 ± 12.0	59.7 ± 13.3	60.1 ± 12.9
Males/Females	52/50	36/40	16/10
HD (months)	38 (4–384)	35 (6–240)	42.5 (4–384)
CaxP (mmol^2/l^2)	3.99 ± 0.34	3.96 ± 0.36	4.02 ± 0.21

Subgroups 1 and 2: HD patients without and with calcitriol therapy.

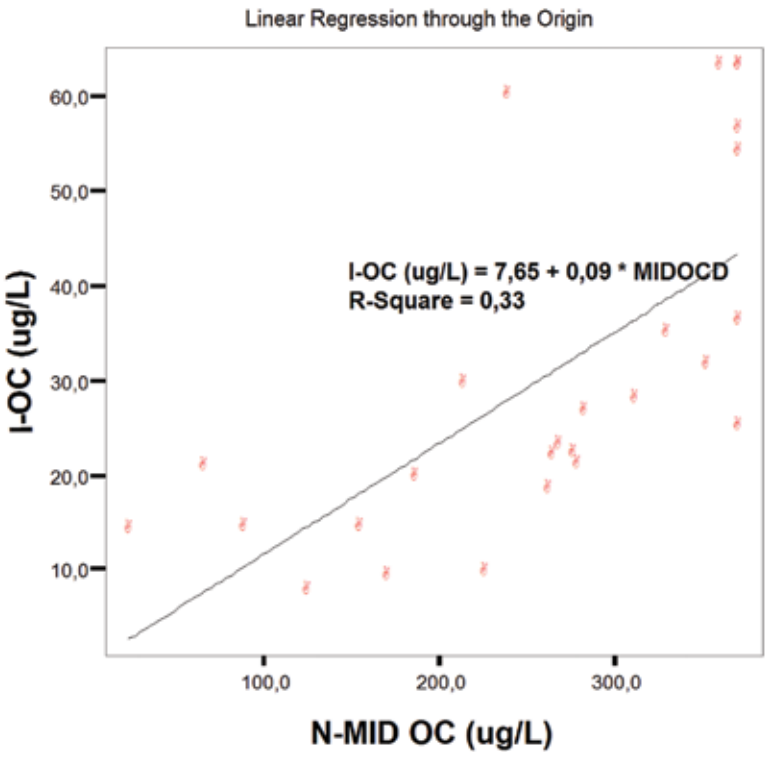

Figure 3: Correlation of intact and N-MID-OC in Subgroup 2 (r = 0.817, p< 0.001).

Measurement of biochemical markers

The serum levels of intact OC and N--MID-OC, CTx, and intact PTH were measured.

Intact OC

Serum levels of iOC were measured using a two site chemiluminiscent immunometric assay (Immulite 1000 Osteocalcin, DPC, Los Angeles, CA, USA). This assay employs rat monoclonal antibodies against the C-terminus of the OC molecule and conjugated goat polyclonal antibodies against the N-terminus of the OC molecule. The intra- and inter-assay CVs were 3.9 % and 5.9 %, respectively. The expected values for healthy adults were in the range of 3.1 to 13.7 µg/l.

N-MID-OC

Serum levels of N-MID–OC were determined using the Elecysis N-MID-OC assay kit (Roche Diagnostics GmbH, Mannheim, Germany), which employs two monoclonal antibodies directed against epitopes on the N-midfragment and the N-terminal fragment of the OC molecule. The test does not depend on the unstable C-terminal fragment (amino acids 43–49) of the OC molecule, which ensures easy sample handling. Briefly, 20 microliters of serum sample was added to a biotinylated monoclonal OC--specific antibody and then to a monoclonal OC antibody labelled with ruthenium complex. The reaction mixture was added to streptavidin-coated microparticles and aspirated into the measuring cell, where the microparticles were magnetically captured onto the surface of the electrode. After unbound substances had been removed with ProCell, a voltage was applied to electrode to induce chemiluminiscent emission. The luminescence was captured by photomultiplier and the results quantified on the basis of the calibration curve. The intra- and inter--assay CVs were 3.0 % and 4 %, respectively. The expected median value for healthy men aged from 50 to 70 years was 24 µg/l, range: 14.0–46.0 µg/l.

β-crosslaps (CTx)

The bone resorption marker serum β-CrossLaps was measured using the Elecysis 2010 analyzer (Roche Diagnostics GmbH, Manheim, Germany) with an intra- and inter-assay CVs of 4.6 % and 4.7 %, respectively. The expected values for healthy men aged from 50 to 70 years were 0.104 to 0.504 µg/l.

Table 2: Serum concentrations of bone markers and PTH in HD patients.

Bone marker	HD patients	Subgroup 1	Subgroup 2
N-MID-OC (µg/l)	224.8 (8.3 – 371,6)	193.7 (8.3 – 362.6)	271.7 (22.6 – 371.6)
i-OC (µg/l)	21.2 (2.7 – 66.3)	20.2 (2.7 – 64.4)	23.8 (15.3 – 66.3)
CTx (µg/l)	1.845 (1.107 – 3.015)	1.805 (1.103 – 3.078)	1.905 (1.128 – 2.858)
PTH (pmol/l)	22.3 (11.1 – 39.4)	21.8 (9.25 – 39.5)	24.7 (17.9 – 39.2)

Data are presented as median and range.

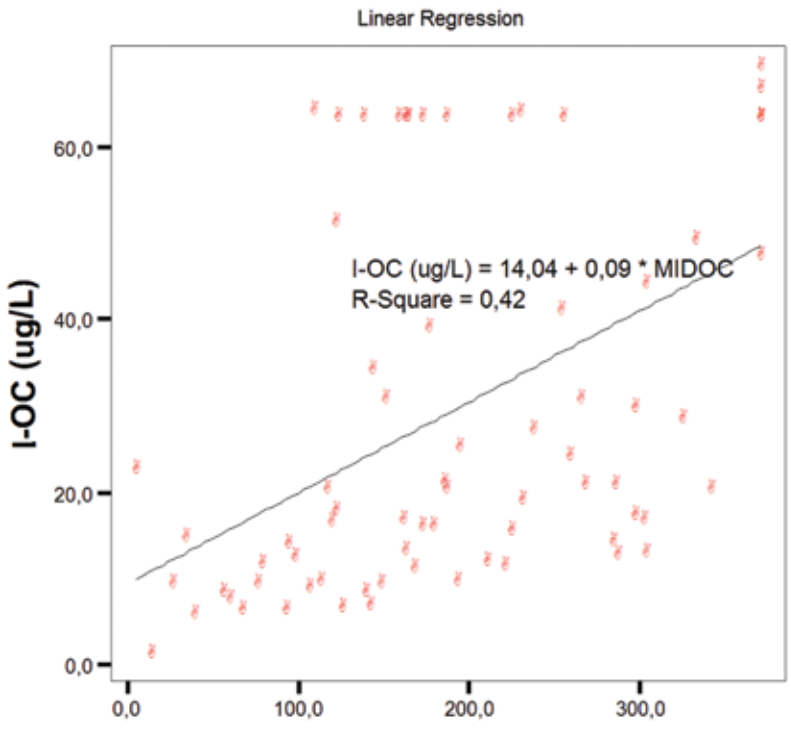

Figure 4: Correlation of intact and N-MID-OC in Subgroup 1 (r = 0.695, p< 0.001).

Intact PTH

Intact PTH was determined using Immulite DPC assay kit (Los Angeles, USA). This assay recognizes intact PTH and very large degradation fragments of PTH. CVs for intra- and inter-assay were less than 8 %. The expected values for healthy adults are in the range of 1.68 to 9.16 pmol/l.

Statistical analysis

All data are presented as medians and ranges, unless otherwise indicated. Since all serum markers were not normally distributed, the differences between groups were analyzed by Kruscal Wallis test and relationships between markers by Spearman's correlation method. The value of $p < 0.05$ was considered statistically significant. All statystical analyses were performed using statystical software package SPSS 10.1. for Windows (SPSS Inc. Chicago, USA).

Results

Comparison of OC levels determined by i-OC and N-MID-OC assays

Key characteristics of HD patients are given in Table 1. There were no statistically significant differences in age, gender distribution, and duration of hemodialysis between calcitriol-treated and non-treated HD patients. Biochemical parameters including serum i-OC, N-MID-OC, CTx, and PTH levels are reported in Table 2. Median levels of N-MID-OC were 10.6-fold higher than i-OC levels. Serum CTx and plasma PTH were both elevated above the normal range. No significant changes in bone markers between both groups were found.

Correlation studies

Linear correlation between i-OC and N-MID-OC is shown in Fig.2. This comparison yields a regression equation of $y = 12.63 + 0.09x$. The correlation is significant ($r = 0.702$; $p < 0.0001$). In the subgroups of HD patients the correlation factor between both assays was found to be higher in the calcitriol-treated subgroup 2 (r=0.817) in comparison to the non-treated subgroup 1 (Figures 3 and 4).

For further investigation of both assays, we assessed the correlation of OC with CTx and PTH. The results are shown in Table 3. Intact OC levels and N-MID-OC correlated significantly with both markers. In the subgroup 1 we also found a significant corre-

Table 3: Spearman's correlation results for the whole group of HD patients.

HD patients (N = 102)	N-MID-OC (µg/l)	i-OC (µg/l)	CTx (µg/l)
N-MID-OC (µg/l)			
i-OC (µg/l)	0.702 *		
CTx (µg/l)	0.720 *	0.779 *	
PTH (pmol/l)	0.558 *	0.589 *	0.586 *

$p < 0.05$

Table 4: Spearman's correlation results in the subgroups of HD patients

Subgroup 1	N-MID-OC (µg/l)	i-OC (µg/l)	CTx (µg/l)
N-MID-OC (µg/l)			
i-OC (µg/l)	0.695 *		
CTx (µg/l)	0.739 *	0.791 *	
PTH (pmol/l)	0.600 *	0.622 *	0.605 *
Subgroup 2			
N-MID-OC (µg/L)			
i-OC (µg/L)	0.817 *		
CTx (µg/L)	0.672 *	0.732 *	
PTH (µg/L)	0.310	0.357	0.443 **

** p< 0.001, **p< 0.05*

lation between all markers. In the calcitriol treated subgroup 2, there was a significant correlation with CTx, but not with PTH. Intact OC correlated with CTX even better (r=0.732) than N-MID-OC (r=0.672). Correlation results of subgroups are shown in table 4.

Discussion

The present study demonstrates that the results of i-OC and N-MID-OC assays are statististicaly different, but comparable enough to be used in the assessment of metabolic bone disorders in HD-patients with relation to CTx and PTH concentrations.

Many immunometric assays have been used to assess OC-levels in various patients.[10-15] These assays have provided different results because of assay limitations and various circulating fragments, which are present in extremely high amounts in HD--patients who have very low renal clearance. Assay limitations depend on their non-specificity and molecular instability. The use of an appropriate OC assay might improve patient care. We measured OC serum levels in a group of HD-patients by i-OC and N--MID-OC assays. Both of them have their own advantages and limitations, which are associated with detection antibodies that can recognize different epitopes on the OC molecule and/or OC degradation products. The i-OC assay recognizes the whole OC molecule, while the N-MID assay detects the N-MID fragment (aminoacids 1–43). Careful handling of samples after collection is of utmost importance for both assays and was given special attention in our study.

The levels of N-MID-OC were 10.6-fold higher than i-OC levels because a large part of intact OC is degraded by proteolytic enzymes in the blood. Our results are consistent with those of Nagasue *et al*[6].This difference can also be explained by different stability of the two forms of OC (i-OC, N-MID-OC) *in vivo*. Nagasue *et al*[6] have established that the intact molecule of OC is very unstable in the human serum. They had determined experimentally that serum N-MID-OC concentrations did not decrease significantly during 12h incubation period at room temperature, whereas serum i-OC decreased significantly after only 1 hour of incubation. By ensuring equal conditions for sample collection and storage for both assays, we eliminated the influence of this factor. The differences in measured values must therefore be attributed to other factors.

For further investigation we examined correlations between i-OC and N-MID-OC, CTx and PTH in relation to calcitriol therapy. Intact OC correlated well with serum N-MID-OC levels (r=0.817; p< 0.001), so we concluded that clinical applications of these two assays are comparable in HD patients. As osteocalcin gene is one of the target genes of vitamin D receptor, calcitriol directly regu-

lates the osteocalcin synthesis at DNA level. Carvallo *et al*[16] showed that *in vivo* synthesised calcitriol stimulates OC-gene expression and consequently increases endogenous synthesis of OC in osteoblasts. Miki *et al*[17] showed that small doses of calcitriol also stimulate the synthesis of OC. In our HD patients treated with calcitriol, the concentrations of i-OC and N-MID-OC were greater than in the non-treated group. N-MID-OC is increased more than i-OC (40 % vs. 17 %, respectively). This could mean that N-MID--OC assay is more sensitive for the detection of increased OC levels due to calcitriol intake. The levels of CTx and PTH did not show a significant decrease associated with calcitriol therapy. PTH is known as a stimulator of bone resorption in uraemic patients and a potential stimulator of OC synthesis. Our results have confirmed those of Samadfam *et al* [18]. In calcitiol treated group we did not find a correlation between OC and PTH. The effect of both, calcitriol and PTH on OC synthesis is not synergistic; calcitriol diminishes PTH *in vivo* and consequently decreases OC synthesis. So OC levels might be an indicator of calcitriol therapy effectiveness. If OC levels are not increased significantly, the treatment with calcitriol might be less successful.

In conclusion, although we found that serum OC concentrations measured by i-OC assay and N-MID-OC assay differed significantly, their clinical applications are comparable. Furthermore, our results suggest that calcitriol treatment increases OC synthesis.

References

1. Price PA, Otsuka AS, Poser JW, Kristaponis I, Raman N. Characterization of a gamma-carboxyglutamic acid-containing protein from bone. Proc Natl Acad Sci USA 1976; 73: 1447-51.
2. Lian JB, Gundberg CM. Osteocalcin. Biochemical considerations and clinical applications. Clin Orthop Relat Res 1988; 226: 267-91.
3. Camacho PM, Lopez NA. Use of biochemical markers of bone turnover in the management of postmenopausal osteoporosis. Clin Chem Lab Med 2008; 46: 1345-57.
4. Joffe P, Heaf JG, Hyldstrup L. Osteocalcin: a non--invasive index of metabolic bone disease in patients treated by CAPD. Kidney Int 1994; 46: 838-46.
5. Morishita T, Nomura M, Hanaoka M, Saruta T, Matsuo T, Tsukamoto Y. A new assay method that detects only intact osteocalcin. Two-step non-invasive diagnosis to predict adynamic bone disease in haemodialysed patients. Nephrol Dial Transplant 2000; 15: 659-67.
6. Nagasue K, Inaba M, Okuno S, Kitatani K, Imanishi Y, Ishimura E, et al. Serum N-terminal midfragment vs. intact osteocalcin immunoradiometric assay as markers for bone turnover and bone loss in hemodialysis patients. Biomed Pharmacother 2003; 57: 98-104.
7. Gundberg CM, Weinstein RS. Multiple immunoreactive forms of osteocalcin in uremic serum. J Clin Invest 1986; 77: 1762-7.
8. Beresford JN, Gallagher JA, Poser JW, Russell RG. Production of osteocalcin by human bone cells in vitro. Effects of 1,25(OH)2D3, 24,25(OH)2D3, parathyroid hormone, and glucocorticoids. Metab Bone Dis Relat Res 1984; 5: 229-34.
9. Ivaska KK, Hentunen TA, Vääräniemi J, Ylipahkala H, Pettersson K, Väänänen HK. Release of intact and fragmented osteocalcin molecules from bone matrix during bone resorption in vitro. J Biol Chem 2004; 279: 18361-69.
10. Nielsen H, Charles P, Mosekilde L. The effect of single oral doses of prednisone on the circadian rhythm of serum osteocalcin in normal subjects. J Clin Endocrinol Metab 1988; 67: 1025-30.
11. Woitge HW, Oberwittler H, Heichel S, Grauer A, Ziegler R, Seibel MJ. Short-and long-term effects of ibandronate treatment on bone turnover in Paget disease of bone. Clin Chem 2000; 46: 684-90.
12. Rosenbrock H, Seifert-Klauss V, Kaspar S, Busch R, Luppa PB. Changes of biochemical bone markers during the menopausal transition. Clin Chem Lab Med 2002; 40: 143-51.
13. Buargub MA, Nabulsi MF, Shafeh TA. Prevalence and pattern of renal osteodystrophy in cronic hemodialysis patients: a cross sectional study of 103 patients. Saudi J Kidney Dis Transpl 2006; 17: 401-7.
14. Kokuho T, Toya Y, Kawaguchi Y, Tamura K, Iwatsubo K, Dobashi Y, et al. Sevelamer hydrochloride improves hyperphosphatemia in hemodialysis patients with low bone turnover rate and low intact parathyroid hormone levels. Ther Apher Dial 2007; 11: 442-8.
15. Herrmann M, Umanskaya N, Traber L, Schmidt--Gayk H, Menke W, Lanzer G, et al. The effect of B-vitamins on biochemical bone turnover markers and bone mineral density in osteoporotic patients: a 1-year double blind placebo controlled trial. Clin Chem Lab Med 2007; 45: 1785-92.
16. Carvallo L, Henriquez B, Paredes R, Olate J, Onate S, van Wijnen AJ, et al. 1alpha,25-dihydroxy vitamin D3-enhanced expression of the osteocalcin gene involves increased promoter occupancy of basal transcription regulators and gradual recruitment of the 1alpha,25-dihydroxy vitamin D3 receptor-SRC-1 coactivator complex. J Cell Physiol 2008; 214: 740-9.
17. Miki T, Nakatsuka K, Nishizawa Y, Emoto M, Morita A, Tabata T, et al. Effect of intermittent oral 1,25(OH)2D3 therapy on bone Gla protein in dialysis patients. Endocrinol Jpn 1991; 38: 479-83.
18. Samadfam R, Xia Q, Miao D, Hendy GN, Goltzman D. Exogenous PTH and endogenous 1,25-dihydroxy vitamin D are complementary in inducing an anabolic effect on bone. J Bone Miner Res 2008; 23: 257-66.