

Prognostic Value of Bone Formation and Resorption Proteins in Heterotopic Ossification in Critically-Ill Patients. A Single-Centre Study

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ABSTRACT

Introduction: A potential complication in critically ill patients is the formation of bone in soft tissues, termed heterotopic ossification. The exact pathogenetic mechanisms are still undetermined. Bone morphogenetic proteins induce bone formation, while signalling through the receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL), regulates osteoclast formation, activation, and survival in normal bone modelling and remodelling. Osteoprotegerin protects bone from excessive bone loss by blocking RANKL from binding to RANK. **Aim:** The study aimed to investigate these molecules as potential prognostic biomarkers of heterotopic ossification development in critically ill patients. **Materials and Methods:** In this prospective observational study, BMP-2, RANKL, and osteoprotegerin were measured by ELISA in twenty-eight critically-ill, initially non-septic patients, on admission to an ICU, seven days post-admission, and thirty days after ICU discharge. **Results:** In the critically-ill cohort, nine of the twenty-eight patients developed heterotopic ossification up to the 30-day follow-up time-point. The patients who developed heterotopic ossification exhibited significantly reduced BMP-2 and RANKL levels on ICU admission, compared to patients who did not; Osteoprotegerin readings were similar in both groups. **Conclusions:** Critically-ill patients who will subsequently develop heterotopic ossification, have significantly lower BMP-2 and RANKL levels at the time of ICU admission, suggesting that these proteins may be useful as prognostic markers for this debilitating condition.

Keywords: bone morphogenetic proteins, critically-ill, heterotopic ossification, osteoprotegerin, receptor activator of nuclear factor kappa-B ligand

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INTRODUCTION

Heterotopic ossification is defined as the development of mature lamellar bone in soft tissues [1]. It is a condition affecting a substantial minority of critically-ill patients even after discharge from hospital. The cause of heterotopic ossification has not yet been entirely determined. Bone morphogenetic protein (BMP) signalling is thought to play a central part in the heterotopic ossification process. BMPs are members of the transforming growth factor- β (TGF- β) family that regulate proliferation, differentiation, apoptosis, and motility of various cell types in the course of development and tissue homeostasis, including regulation of bone homeostasis [2, 3].

BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 have been shown to induce adipogenic and osteogenic differentiation in vitro and in vivo [4, 5] while a central role for BMP signalling has been demonstrated in trauma-induced heterotopic ossification [6]. Likewise, BMP-2 has been demonstrated to cause heterotopic ossification in a murine model studying spinal cord injury (SCI) [7]. Thus, it has been suggested that the BMP signalling pathway may be used as a target in the treatment of bone diseases [2]. On the other hand, signalling through the receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL) regulates osteoclast formation, activation, and survival in normal bone modelling and remodelling [8, 9]. Osteoprote-

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gerin prevents RANKL from binding to RANK, thus preventing excessive bone loss [10].

In critically-ill patients, the timely diagnosis of heterotopic ossification has been a challenge. Age, sex, and previous trauma and surgery have been proposed as predisposing factors. Laboratory data, such as increased serum alkaline phosphatase are considered non-specific, while elevated serum creatine kinase has been suggested to be associated with a more aggressive course of heterotopic ossification [11]. Until now, there is no way to identify critically-ill patients who will develop this condition.

The present study aimed to investigate serum biomarkers as possible indicators of heterotopic ossification development in a cohort of ICU patients.

■ MATERIALS AND METHODS

The study was approved by the Hospital's Ethics Committee (study approval number 80 – 1/2/2010). All procedures conformed with the Helsinki Declaration. Informed written consent was obtained from all patients' next-of-kin before any study procedure.

Study Design

A prospective, observational study of critically ill patients treated in one academic, multidisciplinary ICU.

Study Population

All consecutive admissions to the intensive care unit of the “Evangelismos” General Hospital, Athens, Greece, were screened for eligibility over fifteen months from the 16th of June 2012 to the 27th of September 2014.

Exclusion criteria were:

- sepsis within the first 24 hours of ICU admission [12]
- BMI greater than 35 kg/m²
- age less than 18 years
- pregnancy
- total ICU stay less than three days
- end-stage cancer or brain death
- no need for mechanical ventilation
- re-admission or transfer from another ICU
- contagious diseases (human immunodeficiency virus, hepatitis)
- oral intake of corticosteroids at an equivalent dosage of ≥ 1 mg/kg prednisone per day for more than one month

Out of sixty-four patients screened for eligibility, thirty-six patients were initially included in the study based on the above criteria. The study number was also reduced by patients refusing consent. Finally, twenty-eight critically-ill patients, twenty-one male and seven female, completed the study, as an additional eight patients were lost to follow-up (Figure 1).

The patients enrolled in the study were mainly neurosurgical and trauma patients. Clinical and laboratory data were recorded for all enrolled patients.

ICU admission anthropometric data, severity scores and medical history were noted.

Blood samples were drawn at three-time points:

- on ICU admission (baseline)
- Seven days following admission
- Thirty days after ICU discharge, approximately two months post-admission to the ICU.

The demographics and clinical characteristics of the patients are listed in Table 1.

Patients were followed-up for 30 days following discharge from the ICU.

Patients were allocated to two groups based on the development of heterotopic ossification at any point during their ICU stay or during the 30-day follow-up period, as verified by X-rays.

This resulted in two groups:

- Group 1. heterotopic ossification -positive, N= 9
- Group 2. heterotopic ossification -negative, N= 19

None of the patients had heterotopic ossification on admission.

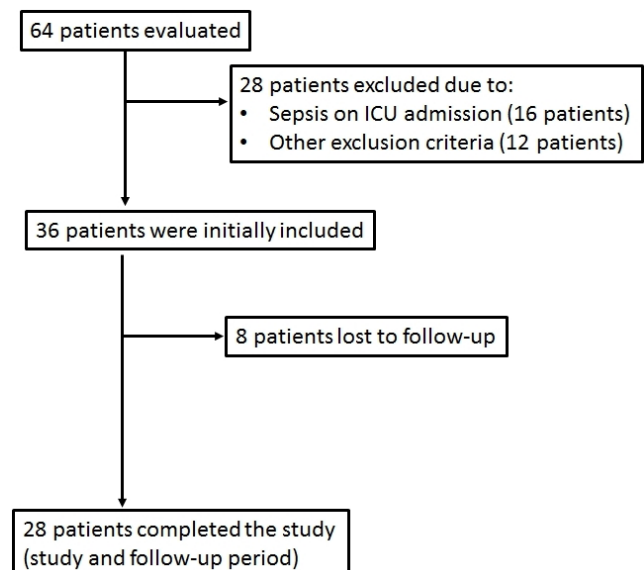


Fig. 1. Study enrolment flow chart

Table 1. Demographics on ICU admission and important outcomes of the patients who developed heterotopic ossification (HO) (HO-positive) versus those who did not (HO-negative).

Demographics/Outcomes	HO-positive patients	HO-negative patients	p-value
Number of patients (N)	9	19	
Age	51(19)	51(14)	ns
Sex			ns
Male	7	14	
Female	2	5	
APACHE II, mean (SD)	16(6)	17(5)	ns
SOFA, mean (SD)	7(1)	7(2)	ns
CRP (mg/dl), (median, IQR)	5.1 (1.6-12.0)	5.5 (1.1-8.8)	ns
PCT (ng/ml), (median, IQR)	1.1 (0.2-2.5)	0.1 (0.1-0.8)	ns
Creatinine (mg/dl), mean (SD)	0.7(0.2)	0.9(0.3)	ns
Vitamin D (ng/ml), (median, IQR)	6.8 (4.2-13.6)	12.7 (6.5-16.8)	ns
Diagnosis			ns
Medical	0%	15.8%	
Surgery/Trauma	100%	84.2%	
Medical	0	3	
Cerebral abscess	-	1	
Subarachnoid haemorrhage	-	1	
Cerebral haemorrhage	-	1	
Surgical Emergency	5	8	
Thoracic	0	0	
Neurosurgery	5	8	
Abdominal	0	0	
Trauma	4	8	
Comorbidities	3	7	ns
CAD	1		
Arterial Hypertension	1	6	
Hypothyroidism	1	2	
Mechanical ventilation (days)	20 (17-25)	22 (16-30)	ns
ICU Length of Stay (days)	29 (21-35)	30 (22-40)	ns
Sepsis	66.7%	84.2%	ns
Day of sepsis	10 (7-12)	8 (5-10)	ns

ns; p-value > 0.05. Number of patients (N) and percentages of totals (%), mean \pm SD, or median (Q1-Q3) are given. The Student's t-test or the non-parametric Mann-Whitney test were used for quantitative variables, accordingly, and the chi-square test or the Fisher's exact for qualitative variables. APACHE II, SOFA score, circulating PCT, CRP, creatinine, and vitamin D levels were estimated on ICU admission (within 24 hours). Day of sepsis denotes the day on which sepsis occurred. APACHE= Acute physiology and chronic health evaluation; CAD= Coronary artery disease; CRP= C-reactive protein; ICU= Intensive care unit; PCT= Procalcitonin; SOFA= Sequential organ failure assessment.

Measurement of Biomarkers

BMP-2, 4, 6, 7, RANKL, and OPG were measured in plasma samples by enzyme-linked immunosorbent assay (ELISA), according to the manufacturers' instructions. BMP-2, 4, 7, RANKL, and Osteoprotegerin was purchased from Elabscience [Elabscience, Houston, USA] and BMP-6 from Sigma-Aldrich [Sigma-Aldrich, Saint Louis, USA].

On Day 7 post-admission to the ICU, and Day 30 of the follow-up period, X-rays of the large joints, knee, elbow, shoulder, pelvis, and hip, were obtained from all patients to verify the presence of heterotopic ossification.

Measurements were performed using the Brooker classification for the hip and the Graham classification for the elbow, by a specialist orthopedist who was blinded to the biochemical marker data.

Statistical Analysis

Individual values, mean (SD) for normally distributed variables, or median with interquartile range (IQR) for variables with skewed distribution, are given.

The Student's t-test, the non-parametric Mann-Whitney test, one-way ANOVA for repeated measures or Kruskal-Wallis ANOVA followed by Dunn's post hoc test were used, accordingly.

The chi-square test or Fisher's exact were used to examine associations between qualitative variables. Receiver operating characteristic (ROC) curves were plotted after that using HO development as the classification variable and BMP-2 and RANKL levels on ICU admission as prognostic variables. The optimal cut-off value for predicting heterotopic ossification was calculated as the point with the greatest sensitivity and specificity.

The power of the study was calculated post-hoc.

GraphPad Prism 6 (GraphPad Software, Inc) was used for the analyses.

The significance level was set at $\alpha = 0.05$.

■ RESULTS

Among the 28 critically-ill adult patients who completed the study, 75% were male, and 25% were female. The mean (SD) age of the population was 51 (16) years old.

The mean admission acute physiology and chronic health evaluation (APACHE) II score was 17 (5) and of the sequential organ failure assessment (SOFA) score 7 (2). The overall ICU mortality rate amongst our study patients was 7%. Approximately 90% of the patients were trauma patients (43%) and surgical (46%) patients. The remainder had diagnosed medical conditions.

About 80% of the patients developed sepsis during their ICU stay. Demographics, severity scores, and outcomes of the two patient groups are listed in Table 1.

X-rays showed that during the 30-day follow-up period, nine patients developed heterotopic ossification. Seven patients developed mild bilateral heterotopic ossification (Brooker grade 1 or 2), while two developed mild unilateral heterotopic ossification (Brooker grade 1). Four of these patients also developed Graham Class I heterotopic ossification in one elbow.

Human BMP-2, 4, 6, 7, RANKL, and OPG were measured simultaneously by enzyme-linked immunoassay (ELISA) in the three time-point samples of all patients.

Nine of the twenty-eight patients developed heterotopic ossification during the follow-up period. The results revealed that the patients who developed heterotopic ossification had significantly reduced ICU admission BMP-2 levels compared to patients who did not [566.7 (216.7-883.3) pg/ml vs 1300 (566.7-2817) pg/ml, respectively; $p = 0.037$] (Figure 2a).

ICU admission RANKL levels were also lower in the patients who subsequently developed heterotopic ossification [1.495 (0.285-2.465) ng/ml vs. 2.465 (1.535-6.67) ng/ml; $p = 0.048$] (Figure 2b).

The OPG admission levels and the RANKL: OPG ratio were similar in both groups (Figure 2c and d).

In the patients who developed heterotopic ossification, the levels of BMP-2 were increased by Day 7, whereas by Day 30 of the follow-up period, they tended to increase ($p = 0.064$) (Figure 3a).

The patients who did not develop heterotopic ossification had stably elevated BMP-2 levels during the entire study period (Figure 4a).

A small rise was observed in RANKL levels in the heterotopic ossification patients on Day 30 of the follow-up period (Figure 3b). OPG levels and the RANKL: OPG ratio remained unaltered during the entire study period in both groups (Figure 3c and d and Figure 4c and d).

BMP-4, 6, and 7 were only detectable in a small percent of the patients. This precluded any further statistical analysis.

A ROC curve was generated to determine the prognostic accuracy of BMP-2 and RANKL admission levels.

The area under the ROC curve (AUC) and 95% confidence intervals (CI) for detecting the main outcome, i.e. heterotopic ossification development, was estimated for BMP-2 at 0.823 (0.623-1.023, $p = 0.038$) and for RANKL at 0.767 (0.557-0.977, $p = 0.048$) (Figure 5). BMP-2 at 833 pg/ml showed a sensitivity of 80% and a specificity of 69.2%, while RANKL at 1.52 ng/ml showed a sensitivity of 71.4% and a specificity of 80%.

Additional parameters analysed in the cohort with regards to their association with heterotopic ossification development, included age, sex, diagnostic category, APACHE II, SOFA score, procalcitonin (PCT), C-reactive protein (CRP), creatinine, and vitamin D levels, all recorded on ICU admission (Table 1).

There was no significant difference between the two groups concerning the quantitative variables (Student's t-test or non-parametric Mann-Whitney test ($p > 0.05$)) or the qualitative variables (chi-square test or the Fisher's exact ($p > 0.05$)) (Table 1).

■ DISCUSSION

In the present study, we demonstrated that in a critically-ill cohort, patients who will develop heterotopic

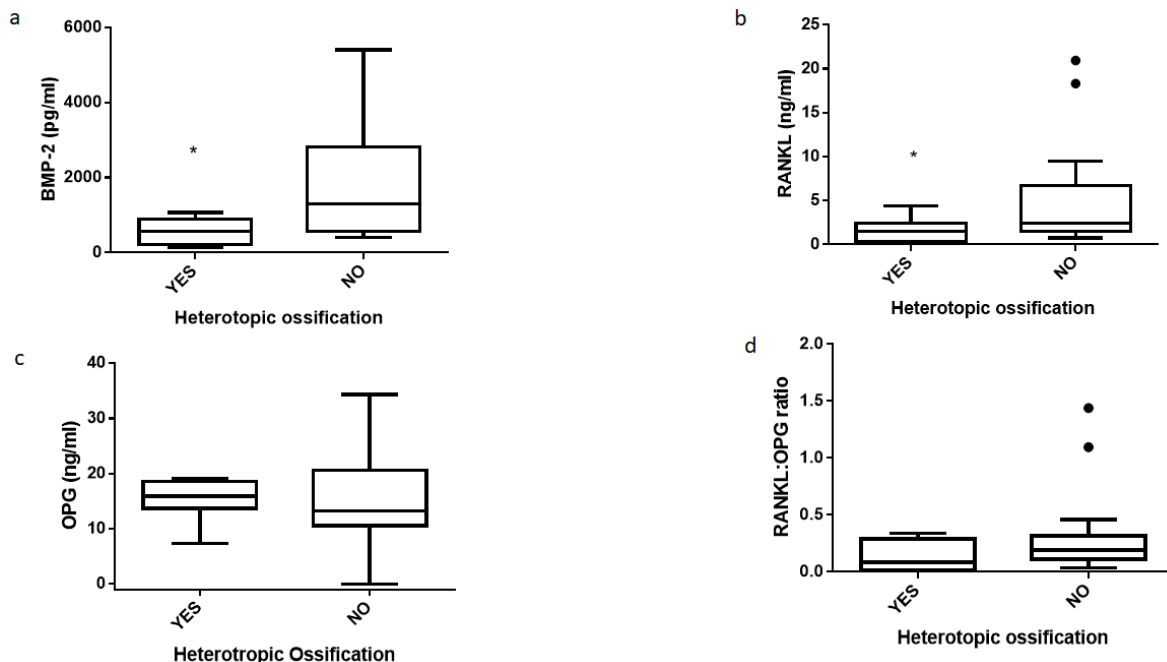


Fig. 2. BMP-2, RANKL and Osteoprotegerin levels on admission. a) BMP-2, b) RANKL, c) Osteoprotegerin and d) RANKL: Osteoprotegerin ratio were measured in 28 critically ill, initially non-septic patients on ICU admission. Patients were subsequently categorised as heterotopic ossification-positive (those who subsequently developed heterotopic ossification, yes, N= 9) and heterotopic ossification -negative (those who did not develop heterotopic ossification, no, N= 19). Two-group comparisons were performed with the non-parametric Mann-Whitney test, * p< 0.05. Data are presented as box plots. Line in the box, median value; box edges, 25th to 75th centiles; whiskers, range of values; bullet points, outliers. BMP-2= bone-morphogenetic protein 2, RANKL= receptor activator of nuclear factor kappa-B ligand, OPG= osteoprotegerin.

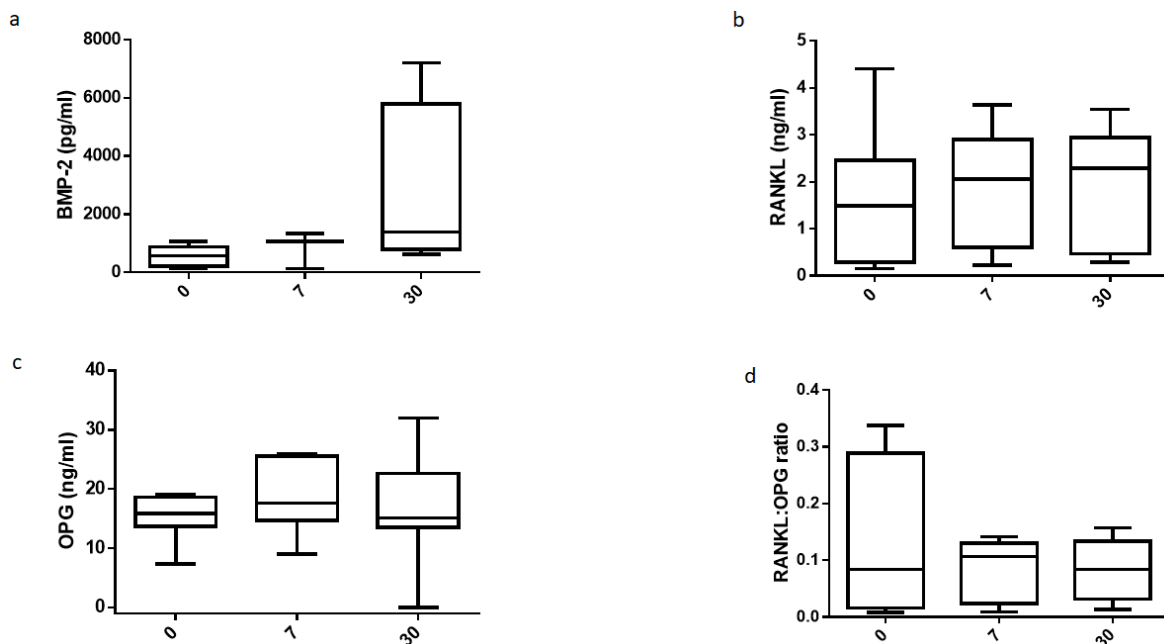


Fig.3. Time course of BMP-2, RANKL and OPG levels during ICU stay and the follow-up period in the patients who developed heterotopic ossification. a) BMP-2, b) RANKL, c) OPG and d) RANKL: OPG ratio were measured in 9 patients who developed heterotopic ossification on ICU admission (0), and thereafter seven days post-admission (7) and 30 days following ICU discharge (30). Data are presented with box plots. Line in the box, median value; box edges, 25th to 75th centiles; whiskers, range of values. Comparisons were performed using one-way ANOVA followed by Dunn’s multiple comparisons. BMP-2= bone-morphogenetic protein 2, RANKL= receptor activator of nuclear factor kappa-B ligand, OPG= osteoprotegerin.

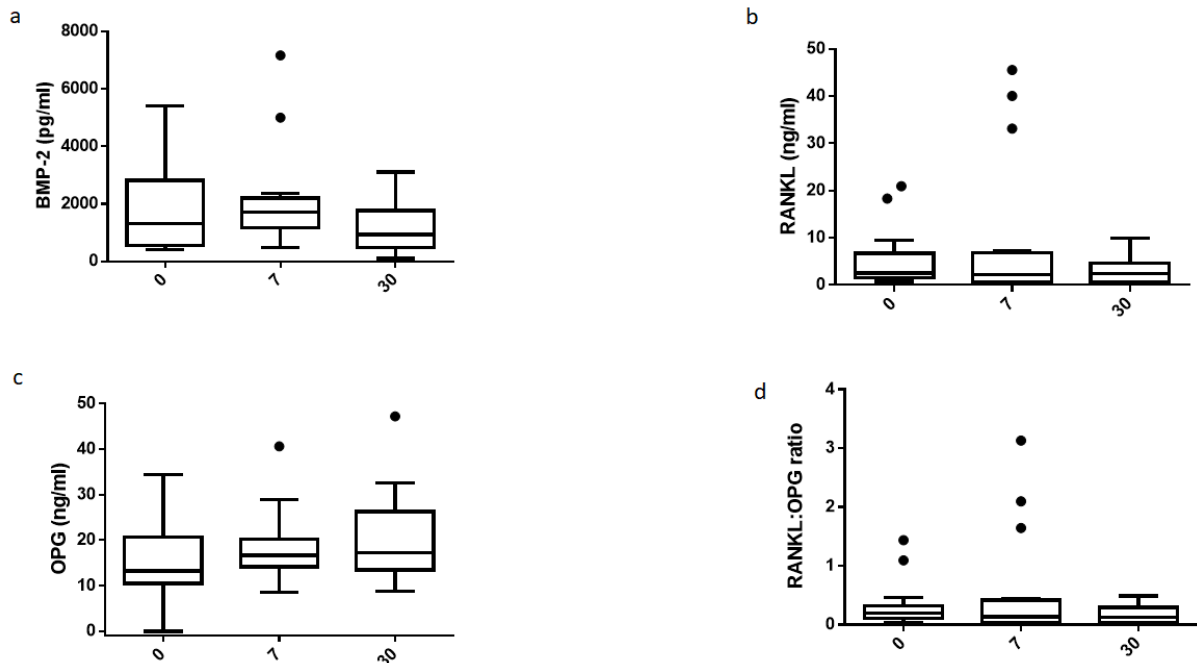


Fig. 4. Time course of BMP-2, RANKL and OPG levels during ICU stay and the follow-up period in the patients who did not develop heterotopic ossification. a) BMP-2, b) RANKL, c) OPG and d) RANKL: OPG ratio were measured in 19 patients who did not develop heterotopic ossification on ICU admission (0), and thereafter seven days post-admission (7) and 30 days following ICU discharge (30). Data are presented with box plots. Line in the box, median value; box edges, 25th to 75th centiles; whiskers, range of values; bullet points, outliers. Comparisons were performed using one-way ANOVA followed by Dunn's multiple comparisons. BMP-2= bone-morphogenetic protein 2, RANKL= receptor activator of nuclear factor kappa-B ligand, OPG= osteoprotegerin.

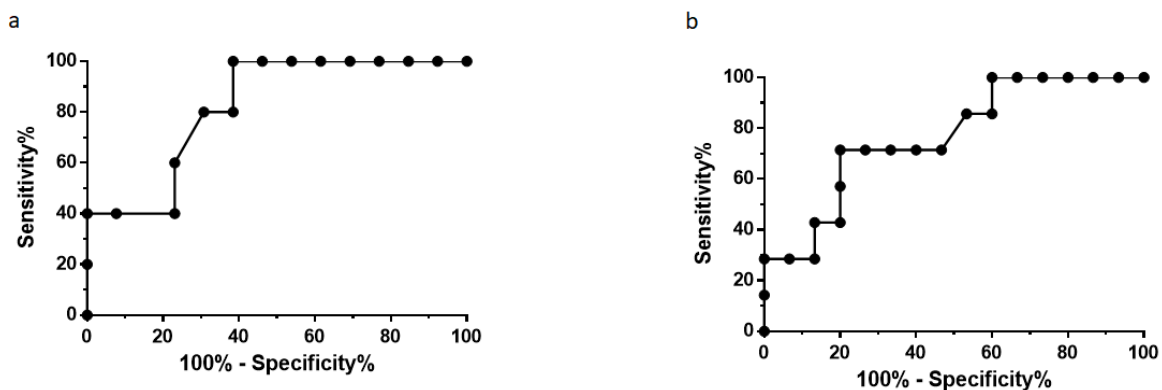


Fig. 5. Receiver operating characteristic (ROC) curve analysis. ROC curves were generated to determine the prognostic accuracy of a) BMP-2 and b) RANKL; the corresponding areas under the curve (AUC) and 95% confidence intervals (CI) were estimated as follows: BMP-2 at 0.823 (0.623-1.023, $p = 0.038$); RANKL at 0.767 (0.557-0.977, $p = 0.048$). The levels of both molecules were estimated on ICU admission. BMP-2= bone-morphogenetic protein 2, RANKL= receptor activator of nuclear factor kappa-B ligand.

ossification have lower BMP-2 and RANKL levels on ICU admission compared to patients who do not develop heterotopic ossification. This indicates that these proteins might be useful as prognostic markers for this debilitating condition.

The results support the notion that a small but not negligible minority of ICU patients will develop heterotopic ossification during ICU stay or after discharge;

no decisive way to identify these patients exists at present.

Furthermore, clinical diagnosis of heterotopic ossification is frequently impeded due to sedation and immobilisation of the patients. The X-ray confirmation of heterotopic ossification may be only evident a few weeks after the initial diagnosis is clinically speculated [13]. Thus, early detection of critically-ill patients who

will develop heterotopic ossification constitutes a clinical challenge. In the present study, molecules involved in bone modelling and remodelling as potential indicators of heterotopic ossification development in ICU patients were investigated. The results indicated that low levels of BMP-2 and RANKL on ICU admission might serve as serum prognostic markers of heterotopic ossification in critically ill patients.

Most studies have focused on diagnostic indicators and treatments, and more specifically on the role of BMP-2 in inducing bone formation. Injection or the surgical implantation of BMPs and progenitor cells, expressing mainly BMP-2, can be used to induce heterotopic ossification formation [14-16].

The BMP-2 gene is expressed in the spinal ligaments of patients with ossification of the spinal ligament (OSL), and exogenous BMP-2 seems to stimulate osteogenic differentiation of spinal ligament cells [17]. It has also been proposed that BMP-2 may be implicated in the pathogenesis of osteoarthritic alterations or the repair process of temporomandibular joint internal derangement [18]. In foetal limb cultures, BMP-2 promoted chondrocyte hypertrophy and endochondral ossification [19], while two novel BMP-2 variants were identified in patients with thoracic ossification of the ligamentum flavum and functional assays revealed increased BMP-2 expression, elevated osteogenic marker expression, and augmented osteogenic differentiation [20]. Disrupting the BMP-signalling pathway has been proposed as a treatment strategy. Inhibition of BMP type I receptor (BMPRI) activity has been suggested in treating fibrodysplasia ossificans progressiva (FOP) and other heterotopic ossification syndromes associated with excessive BMP signalling [21], as well as blocking TGF- β signalling to decrease the osteogenic trans-differentiation of FOP fibroblasts [22]. Others have demonstrated that the targeting of multiple BMP receptors averts trauma-induced heterotopic ossification [23]. The discovery of an mTOR-BMP signalling pathway has also been proposed as a possible molecular mechanism of heterotopic ossification that may constitute a novel therapeutic target for heterotopic ossification [24].

RANK, RANKL, and OPG recently appear as central regulators of bone metabolism [8]. Studies focusing on this pathway have shown the expression of RANKL and OPG in heterotopic vascular ossification [25], while OPG-deficient mice exhibit early-onset osteoporosis and arterial calcification [26].

The only study measuring factors belonging to these two pathways before developing heterotopic ossification demonstrated transient increases in osteoblast and osteoclast activity after total hip arthroplasty [27]. In this study development of heterotopic ossification was associated with an increase in the osteoblast markers N-terminal pro-peptide of type-I procollagen (PINP), bone alkaline phosphatase (ALP) and osteocalcin (OC). However, the results showed that the osteoclast marker C-telopeptide of type-I collagen (CTX-I) was increased during the early phase of heterotopic ossification. Since this rise preceded heterotopic ossification development, the authors proposed that the pathogenesis of heterotopic ossification formation also involves early changes in osteoclast activity, rather than merely inappropriate new bone formation.

Normal bone remodelling involves osteoclasts removing the trenches and tunnels from the trabecular and cortical bones, respectively [10]. Subsequently, osteoblasts fill in these tunnels with new bone matrix. During normal bone remodelling, bone formation equals resorption [10]. In our critically-ill patients who will develop heterotopic ossification during their ICU stay or during the 30-day follow-up period, both osteoclast and osteoblast activity is reduced on ICU admission.

More specifically, our results showed that in the nine neurosurgical and trauma patients who were admitted to the ICU and developed heterotopic ossification, levels of BMP-2 were low, indicating that BMP-2 is probably not accumulated, and hence no new bone formation seems to occur. Furthermore, osteoclasts are not activated, as indicated by the low RANKL levels, hence old bone is not resorbed. In these patients, it becomes apparent that normal bone remodelling does not occur. Once heterotopic ossification is established, as verified by X-rays, both BMP-2 and RANKL return to levels comparable to the patients who did not develop heterotopic ossification.

The power of the study was calculated post-hoc, and the observed power was 60%. The clinical assessment of heterotopic ossification was performed with X-rays, and not using MRI, which is considered a more sensitive method [13, 28]. Also, variation between patients may limit the use of markers as individual prognostic factors, since bone turnover marker levels change at different time points in men and women and in metabolic bone disease [29, 30]. Moreover, previous treatment with bisphosphonates, which may alter or inhibit

osteogenesis in a chronic way, were not recorded, nor were the levels of the biomarkers before ICU admission.

■ CONCLUSION

In a critically-ill cohort, lower levels of both bone formation and resorption markers were observed on ICU admission in patients who eventually developed heterotopic ossification. The identification of reliable biomarkers might provide further insight into the pathogenesis of heterotopic ossification and may serve as an early prognostic tool for heterotopic ossification, especially given the absence of effective treatments.

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■ CONFLICT OF INTEREST

None to declare.

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