

# Serum creatine kinase isoenzymes in children with osteogenesis imperfecta

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## Abstract

**Summary** This study evaluates serum creatine kinase isoenzyme activity in children with osteogenesis imperfecta to determine its usefulness as a biochemical marker during treatment with bisphosphonate. The changes of creatine kinase (CK) isoenzyme activity during and after discontinuation therapy were observed. These results could be useful in addressing over-treatment risk prevention.

**Introduction** The brain isoenzyme of creatine kinase (CKbb) is highly expressed in mature osteoclasts during osteoclastogenesis, thus plays an important role in bone resorption. We previously identified high serum CKbb levels in 18 children with osteogenesis imperfect (OI) type 1 treated for 1 year with bisphosphonate (neridronate). In the present study, serum CK isoenzymes were evaluated in the same children with continuous versus discontinued neridronate treatment over a further 2-year follow-up period.

**Methods** This study included 18 children with OI type 1, 12 with continued (group A) and 6 with ceased (group B) neridronate treatment. Auxological data, serum biochemical markers of bone metabolism, bone mineral density z-score, and serum total CK and isoenzyme activities were determined in both groups.

**Results** Serum CKbb was progressively and significantly increased in group A ( $p < 0.004$ ) but rapidly decreased to undetectable levels in group B. In both groups, the cardiac muscle

creatinase isoenzyme (CKmb) showed a marked decrease, while serum C-terminal telopeptide (CTX) levels were almost unchanged. **Conclusions** This study provides evidence of the cumulative effect of neridronate administration in increasing serum CKbb levels and the reversible effect after its discontinuation. This approach could be employed for verifying the usefulness of serum CKbb as a biochemical marker in patients receiving prolonged bisphosphonate treatment. Moreover, the decreased serum CKmb levels suggest a systemic effect of these drugs.

**Keywords** Bisphosphonates · Bone metabolism · Creatine kinase isoenzymes · Osteoclast · Osteogenesis imperfecta

## Introduction

Creatine kinase (CK) is a dimeric enzyme that catalyzes the reversible reaction of creatine and adenosine triphosphate (ATP) to form phosphocreatine and adenosine diphosphate (ADP), a crucial reaction for cellular energy generation and metabolism [1]. The three known cytosolic CK isoenzymes include brain (CKbb), sarcomeric muscle (CKmm), and cardiac muscle (CKmb) types [2]. CKbb is present in a range of tissues including the brain, retina, uterus, testes, and bone, in which it executes the function of energy maintenance and regulation [1].

It has been reported that the CKbb gene is highly expressed in rabbit osteoclasts [3] and that receptor activator of nuclear factor kappa-B ligand (RANKL) up-regulates CKbb during osteoclastogenesis [4]. Moreover, the decrease of CKbb expression by RNA interference suppresses bone resorption by osteoclasts grown in vitro, showing that CKbb has a crucial role in bone remodeling [5].

In humans, elevation of serum levels of CKbb has been found in some types of osteopetrosis (OPT), a genetic disease in which osteoclasts fail to resorb bone; therefore, CKbb is proposed as a biochemical marker to distinguish true OPT from other sclerosing bone disorders [6–10]. Interestingly, serum CKbb has been reported to increase in patients affected by OPT as a consequence of bisphosphonate (BP) therapy [11, 12].

Osteogenesis imperfecta (OI) is a heritable, heterogeneous connective tissue disorder, causing bone fragility and several other connective tissue abnormalities with severities ranging from asymptomatic individuals to perinatal death. Osteoblasts in OI patients are unresponsive to mechanical load; therefore, they produce an abnormal bone matrix. To compensate for this, the osteoblast population increases alongside increased osteoclast activity, leading to a high bone turnover rate [13]. Other than bone fragility, OI is characterized by low bone mineral density (BMD), increased fracture rate, short stature, skeletal deformities, blue sclerae, dentinogenesis imperfecta, joint laxity, and deafness later in life [14]. On the basis of clinical, radiological, and genetic features, several OI types have been described [15], with type 1 being the mildest [16].

BPs are considered the treatment of choice in children with OI [17, 18]. These synthetic analogs of inorganic pyrophosphate strongly inhibit bone resorption by suppressing the activity of osteoclasts and shortening their life span [19].

In 2004, we reported an increased serum CKbb level in 18 children with OI type 1 during the first year of treatment with neridronate [20]. Recently, BP treatment was reported to result in increased CK release from rabbit osteoclasts in vitro [21]. This result explains a possible mechanism for increased serum CK in patients treated with BPs [11, 20].

In the present study, we evaluated serum CK isoenzymes in the same 18 children used in our previous study during a further 2-year follow-up period [20]. The study included two groups: subjects who continued with neridronate treatment and subjects who discontinued neridronate treatment. The aim of this study therefore was to evaluate the effect of BP treatment on serum CK isoenzyme activity to determine its usefulness as a biochemical marker during the treatment of OI patients.

## Subjects and methods

Written informed consent was obtained from all patient parents prior to patient inclusion in the study. The study protocol was approved by the ethics committee of the Department of Pediatrics, Sapienza University of Rome.

This study included 18 children affected by OI type 1 who received 1 year neridronate treatment as described in our previous report [20]. Twelve children, five boys, and

seven girls (mean age [years]  $\pm$  standard deviation [SD]:  $6.7 \pm 1.5$ ) continued neridronate treatment throughout the 2-year follow-up period (group A). Six children, four boys, and two girls (mean age [years]  $\pm$  SD:  $5.2 \pm 3$ ) discontinued neridronate treatment (group B) after reaching lumbar spine BMD (z-score) values of  $\pm 0.1$ , in accordance with our protocol treatment of OI. All children were followed up for 2 years as outpatients in the Department of Pediatrics, Sapienza University of Rome. Exclusion criteria were the occurrence of fractures during the study, assumption of drugs, and/or acute illness interfering in bone metabolism. Neridronate (sodium neridronate, ABIOTEC PHARMA SpA, Pisa, Italy) was administered as a single infusion once every 3 months. Each infusion consisted of 2-mg/kg body weight, diluted in 100-mL isotonic saline.

Baseline clinical and biochemical data of group A and group B are reported in Tables 1 and 2, respectively. Auxological data and serum biochemical markers of bone metabolism, including calcium, phosphate, intact parathyroid hormone (iPTH), 25 (OH) vitamin D (25(OH)D), basal alkaline phosphatase (ALP), C-terminal telopeptide (CTx), lumbar spine radiographs, and BMD z-score, were determined in both groups at baseline and at 1 year in the previous study [20] and at 2 and 3 years during the follow-up period of the current study. Total serum CK and isoenzymes were analyzed in the same samples.

### Serum biochemical studies

Total serum CK activity was measured in fasting blood samples using the kinetic UV method (Instrumentation Laboratory SpA, Milano, Italy) optimized according to the Federation of Clinical Chemistry [22]. CK isoenzymes (mm, mb, and bb) were separated by electrophoresis on agarose gels. After electrophoresis, the sample strips were incubated for 1 h at 37 °C with the reaction mixture. Catalytic activity was determined by spectrophotometric measurements as described previously [23].

Isoenzyme activity was expressed as international units per liter (U/L) (Helena Biosciences Europe, Sunderland, UK). Serum calcium, phosphate, ALP, iPTH, CTx, and 25(OH)D levels were measured using chemiluminescence and an automatic analyzer (Roche diagnostic SpS, Monza, Italy).

### Radiological studies

BMD z-score was measured in the lumbar spine (L1–L4) by dual-energy x-ray absorptiometry

using a Hologic QDR 4500° system with reference values provided by the manufacturer (Hologic, Bedford, MA) [24].

## Statistics

Statistical differences in demographic, densitometric, and biochemical parameters were determined within groups. Comparisons between two time points in the same groups were carried out using the paired t test. Because the data were approximately log-normally distributed, they were log-transformed before statistical analysis. Comparisons between more than two time points were performed using analysis of variance (ANOVA) for repeated measures. Results were expressed as means  $\pm$  SD. Significant deviations from the null hypothesis were stated when  $p < 0.05$ .

## Results

Total serum CK and isoenzyme levels are reported in Tables 3 and 4 for continued (group A) and discontinued (group B) neridronate treatment, respectively. The data include the first year of treatment (0 to 1 year) published in our previous study [20] and 2-year observation time of the present study (1 to

3 years). Baseline data were determined before the first cycle of treatment and represent the pre-treatment values.

In group A, serum CKbb showed a progressive increase during the 2-year follow-up that became statistically significant at the end of follow-up (3 vs 1 year;  $p < 0.004$ ) with a mean increase rate of 4.4 U/L/yr. This change was less evident compared with the rapid increase observed in the previous study during the first year of neridronate treatment (8.33 U/L/yr) (Fig. 1).

In Group B, serum CKbb exhibited a mild increase in all patients in the first year after discontinuing neridronate treatment, followed by a rapid decrease to undetectable levels at the end of the 2-year follow-up (3 vs 1 year;  $p < 0.001$ ). Furthermore, the values were lower than those observed at baseline (3 vs 0 year;  $p < 0.001$ ).

Regarding serum CKmb, group A showed a slight increase after 1 year of observation time followed by a marked decrease (3 vs 2 years;  $p < 0.005$ ) and values were significantly lower compared with baseline (3 vs 0 year;  $p < 0.002$ ) (Table 3, Fig. 1). In group B, serum CKmb tended to decrease from 1 year after discontinuation of neridronate treatment and was reduced to

undetectable level after the second year (3 vs 1 year;  $p < 0.002$ ) (Table 4, Fig. 1). In both groups, no significant changes were observed in the levels of CKmm isoenzyme (Tables 3 and 4).

Total serum CK showed a significant increase after 3 years of treatment compared with pre-treatment but only in group A (3 vs 0 year;  $p < 0.05$ ). In both groups, no significant changes in

total serum CK level were observed during follow-up in the present study.

Demographic, biochemical, and densitometric characteristics of group A and group B are reported in Tables 1 and 2, respectively. In group A, BMD z-score increased progressively, while in group B there was a slight increase 1 year after discontinuing neridronate treatment, with a decrease at the end of follow-up.

CTx values in both groups remained almost unchanged during the 2-year follow-up time in the present study, with lower values in comparison with pre-treatment, which were comparable with normal range (Tables 1 and 2; Fig. 2). In both groups, lumbar spine radiographs showed no sign of osteosclerosis at the end of the evaluation period.

## Discussion

In our previous study, we identified a significant increase in serum CKbb after 1 year of neridronate treatment in children affected by OI [20]. Because osteoclasts are considered a major target of the pharmacologic action of BPs, and are rich of cytosolic soluble CKbb, we postulated that this finding could reflect an osteoclast dysfunction inducing CKbb release. Recently, an in vitro study using BP-stimulated rabbit osteoclasts supported that osteoclasts are the main source of CK release from the bone, and that this phenomenon is an osteoclast apoptosis-related event [21]. These observations support the hypothesis that BP-induced osteoclast damage could account for serum Ckbb increase in our OI-treated patients, sustaining the role of serum Ckbb as a marker of osteoclast dysfunction [6–12].

In the present study, a significant serum CKbb increase was observed in the 12 children receiving continuous neridronate therapy after the 2-year follow-up (group A; 3 vs 0 year). However, the rate of this increase (1.5 U/L/year) was lower than that observed during the first year of treatment (8.33 U/L/year). In vitro, CK release from osteoclasts exposed to BPs has been shown to be dose-dependent [21]. We therefore expected a linear increase in serum CKbb over the study time due to the pharmacological effect of neridronate accumulation in the bone matrix. However, the aforementioned in vitro study also demonstrated that reduction of the osteoclast population occurs when CK release is increased 2.6 times the basal value. Therefore, the reduced rate of serum CKbb increase observed in the present study could reflect reduction of the osteoclast population.

In group B, the level of serum Ckbb showed a mild increase after the first year of discontinued neridronate treatment, presumably reflecting the action of neridronate accumulation in the bone matrix during treatment. The rapid decrease to undetectable levels observed at the end of follow-up was surprising. In fact, compared with controls, untreated OI patients showed slightly elevated serum CKbb levels, likely related to increased bone turnover [20]. Therefore, the undetectable CKbb levels observed 2 years after treatment discontinuation seem to reflect the full suppression of osteoclast

apoptosis below pre-treatment level. This finding could represent a mechanism finalized to a rapid recovery of the osteoclast population.

Regarding serum CTx, which reflects osteolytic activity by osteoclasts, we observed persistently lower levels in both groups in comparison with pre-treatment. To further evaluate these results, we compared them to the serum CKbb levels. In group A, the CTx values indicated that residual resorption activity was maintained after prolonged neridronate osteoclast exposure, even when a high apoptotic rate occurred, as indicated by elevated serum CKbb levels. Conversely, in group B, the CTx levels showed that neridronate continued to inhibit bone resorption 2 years after discontinuing treatment. This finding is in contrast with the undetectable serum CKbb levels observed at this time that reflect the recovery of the anti-resorptive activity of osteoclasts. However, the aforementioned *in vitro* study shows that CK release begins to increase following BP exposure resulting in 60 % inhibition of CTx release [21]. Therefore, low-level BP exposure may occur, resulting in anti-resorptive activity not mediated by osteoclast

apoptosis. A similar situation could occur *in vivo* after discontinuation of treatment as a consequence of the continuous release of BPs accumulated in the bone matrix. This hypothesis is reasonable considering the two independent signaling pathways by which BPs exert their anti-resorptive and pro-apoptotic effects on osteoclasts [25, 26].

Taken together, these data show that neridronate exerts a high and constant inhibitory effect on osteoclast activity over a wide exposure-time range. Maximum anti-resorptive activity is obtained after the first year of treatment when CKbb release is augmented threefold. Furthermore, basal resorption activity is not abolished after long-term exposure when serum CKbb is strongly augmented. Moreover, neridronate continues to exert its maximal inhibition on bone resorption up to 2 years after discontinuation of treatment when serum CKbb is undetectable. It is likely that serum CKbb has a different indication in relation to therapy. For example, in patients who have undergone 1 year of treatment, a threefold increase seems to indicate the maximum inhibition of osteolytic activity, whereas in patients who discontinued treatment, reduction of serum CKbb

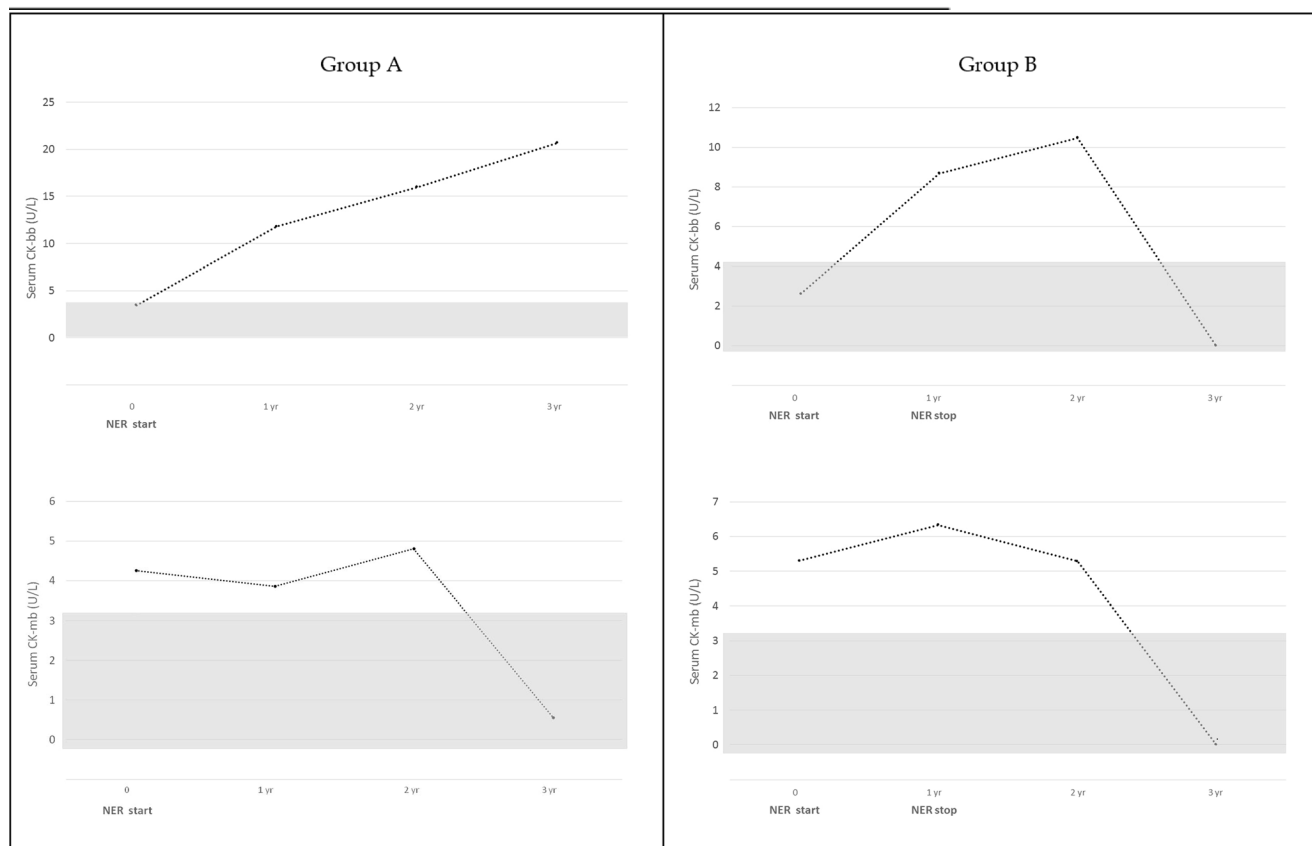
L/year) and lower increase in the further 2-year treatment (4.4 U/L/year) without evidence of plateau. Vertical bars represent SD. NER start start of neridronate treatment, NER stop stop of neridronate treatment. The shadow area shows the range of serum CKbb and CKmb values obtained from 20 healthy children matched for sex and age [20]

does not predict the recovery to pre-treatment levels of osteolytic activity. These concepts are only an inference of our results in the light of evidence emerging following *in vitro* studies. Further studies are needed to elucidate if they are useful for clinical purpose.

Another interesting finding from our study regards serum CKmb, the specific cytosolic cardiac isoform [2]. It is well known that BPs exert their specific action on bone resorption because they accumulate by affinity in the bone matrix and are solubilized prior to ingestion by osteoclasts during osteolytic activity [25, 26]. In the previous report, we found that the pre-treatment serum CKmb level was significantly higher compared with the control, although cardiovascular disease was excluded in the study population [20]. In the present study, we observed an intriguing reduction in serum CKmb in both groups during the second year of

follow-up. These results suggest a possible systemic effect of BPs that implicates a different mechanism of entry through the plasma membrane in non-bone tissue cells. Moreover, it seems that neridronate, even after discontinuation, continues to exert an effect on

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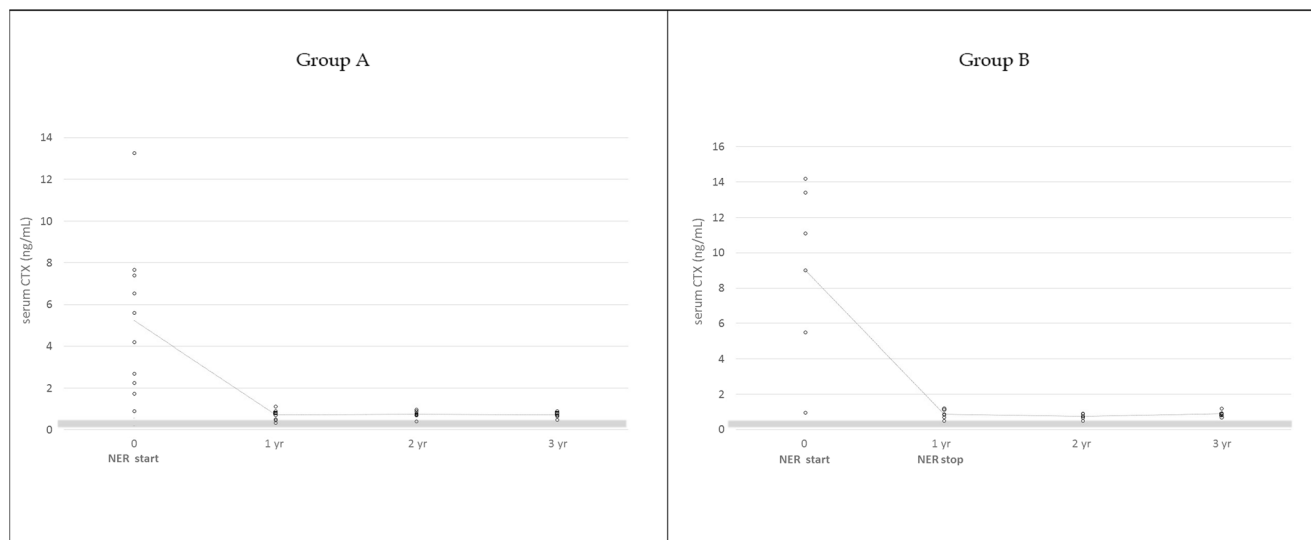


Fig. 2 Changes of serum CTX in 12 children who continued neridronate treatment (group A) and in 6 children who stopped neridronate treatment after 1 year (group B). The data include the first year of treatment (0 to 1 year) published in our previous study [20] and 2-year observation time

CKmb release by cardiomyocytes. Further studies are needed to elucidate if this observation is merely an epiphenomenon or could correlate with clinical cardiological parameters in these patients.

Our study has a number of limitations. First, the relatively small sample size limited the power of our statistical analysis, restricting statistically significant correlations to be obtained with clinical parameters. Studies in larger cohorts (multicentric studies) are therefore required to better elucidate the usefulness of our results for clinical purpose. Second, we interpreted our in vivo results using evidence from an animal in vitro study that demonstrated the release of CK from cultured osteoclasts via the pharmacological action of BPs. Third, in the OI children who continued neridronate therapy, we observed a progressive increase of serum CKbb level without evidence of a plateau. In children who discontinued therapy, we did not observe recovery to pre-treatment levels at the end of the follow-up time. Therefore, further follow-up will be necessary to completely evaluate the variations of serum CKbb in relation to therapy.

In conclusion, this study provides evidence of the cumulative effect of neridronate administration in increasing serum CKbb levels and the reversible effect after its discontinuation. These data could be employed in verifying the usefulness of serum CKbb as a biochemical marker for clinical purpose in patients receiving prolonged BP treatment, especially regarding the prevention of over-treatment risk. This knowledge will be of particular interest considering the lack of consensus on criteria to initiate treatment, determine treatment duration, and evaluate

of the present study (1 to 3 years). Vertical bars represent SD. NER start start of neridronate treatment, NER stop stop of neridronate treatment. The shadow area shows the range of serum CTX normal values

long-term safety, especially in the pediatric population. Moreover, the decrease of serum




CKmb suggests a systemic effect of these drugs, whose implications are not yet known, and needs to be investigated in future studies.

Compliance with ethical standards

Conflicts of interest

## References

None.

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