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**Original Article**

**A Study of the Relationship between Cystatin C and Metabolic Bone Disease in Preterm Infants**

**Short Running Title:** Cystatin and metabolic bone disease

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**Ethic Committee Approval:** Yes

The Ethical Committee of Erciyes University Medical Faculty (02.10.2015/437).

**Consent Form:** No

**What is already known on this topic?**

1. Cystatin C is a valuable marker in the diagnosis of acute kidney injury in preterm infants.
2. It was demonstrated that various conditions, such as respiratory distress, bilateral kidney anomalies, peripartum asphyxia, hemoglobin levels, and sepsis, affected Cystatin C values.

**What this study adds?**

1. It was not investigated whether Cystatin C levels are altered in premature infants with osteopenia.
2. Therefore, it is not known whether the use of CysC levels as markers of renal insufficiency in osteopenic infants is reliable.

**Abstract**

**Objective:** Cystatin C (CysC) is commonly used as a marker of renal failure. The aim of this study was to investigate serum CysC levels in osteopenia of prematurity (OP) and determine whether CysC could be safely used as a marker of renal insufficiency in infants with OP.

**Methods:** The study included 50 preterm infants (≤ 32 gestational weeks). Calcium (Ca), phosphorous (P), and alkaline phosphatase (ALP) serum levels were measured in postnatal week 9, and bone density was measured concurrently by quantitative ultrasonography. Patients with a Z score < -2 were considered to have OP.

**Results:** The mean serum CysC levels in preterm infants in postnatal week 9 were 1.50 ± 0.19 mg/L in this study. Serum CysC levels were not correlated with speed of sound (SOS) values, Z scores, serum Ca, P, or ALP levels. Serum CysC levels were 1.50 (1.35–1.61) mg/L and 1.58 (1.28–1.70) mg/L in infants with OP and without OP respectively, and there was no significant between-group difference.

**Conclusion:** The results of this study suggest that OP does not affect CysC levels in preterm infants.

**Keywords:** Cystatin C, metabolic bone disease, osteopenia, premature, speed of sound, renal failure

**Introduction**

Premature infants are at risk of developing bone disease due to a low bone mineral content. Neonatal rickets or osteopenia of prematurity (OP), also known as metabolic bone disease in premature infants, is one of the most frequent problems encountered in neonatal units. OP adversely affects linear growth and height in the long term while causing fractures, growth retardation, and respiratory problems in the early growth period (1).
Dual photon x-ray absorptiometry (DEXA) is the gold standard method for the radiological measurement of bone mineral density. Nevertheless, time spent screening, artifacts caused by movement, exposure to radiation, and cost limit the use of DEXA in newborns. A quantitative bedside ultrasonography (USG) assessment is a simple, inexpensive, and noninvasive method, which can be used to obtain measurements related to bone mineral density and structure. Bedside assessment devices have been designed that quantify broadband ultrasound attenuation or the speed of sound (SOS). Studies showed that quantitative ultrasound measurements correlated significantly with DEXA findings in both adults and children (2,3) and that they may be useful in evaluating structural and mechanical properties of bone (4). In preterm infants, a number of studies demonstrated the potential clinical value of bedside ultrasound assessments of bone status (5-7).

Cystatin C (CysC) belongs to the cystatin family of cysteine proteinase inhibitors. It has a low molecular weight and is produced in virtually all nucleated cells in the body. The production rate of CysC does not change in inflammatory conditions (8,9), and it is commonly measured to determine the glomerular filtration rate (10-12).

Osteoclastic bone resorption depends on the activity of various proteolytic enzymes, particularly proteinases. CysC inhibits cysteine proteinase, a proteolytic lysosomal enzyme that prevents bone resorption. The association of CysC with bone metabolism has been demonstrated in a variety of in vitro studies (11,12). Clinical studies on bone metabolism and CysC are limited to adults, with no studies conducted in infants. Elevated serum CysC levels in postmenopausal women have been linked to increased bone fractures and reported to be a potentially promising biomarker for the risk of hip fractures (13,14).

Clinical studies of CysC in preterm infants have focused mainly on the relationship between CysC and renal function and aimed to determine the reference range. It has been demonstrated that CysC was a valuable marker in the diagnosis of acute kidney injury in preterm infants (15-18). Other studies demonstrated that various conditions, such as respiratory distress (19), bilateral kidney anomalies (20), peripartum asphyxia (21,22), hemoglobin levels (21,22), and sepsis, affected CysC values (23).

Premature infants have an increased risk of both kidney failure and osteopenia, and the CysC level can be used as a diagnostic marker of renal failure in preterms. However, no studies have examined whether CysC levels are altered in premature infants with OP. Therefore, it is not known whether the use of CysC levels as markers of renal insufficiency in osteopenic infants is reliable. Based on the literature, we hypothesized that the CysC level would be altered in OP. To shed light on this issue, this study investigated the relationship of the CysC level with bone density and levels of biochemical markers of bone metabolism (serum calcium [Ca], phosphorus [P], and alkaline phosphatase [ALP]) and whether serum CysC levels were altered in OP.

Methods
This study enrolled infants born between 24 and 32 gestational weeks who were admitted to the Newborn Unit of Erciyes University Medicine Faculty. Infants with a severe congenital anomaly, congenital metabolic disease, history of perinatal asphyxia, disorder detected in a renal function test, and hypothyroidism were not included in the study. Gestational week was determined by the last menstrual period of the mother. Infants with a birth weight below 10 percentile according to the Fenton 2013 chart were deemed to be small for gestational age (SGA).

Clinical findings of OP manifest between postnatal 6 and 12 weeks (1). Thus, bone density was assessed in the 9th week using quantitative USG of the right tibia. Serum Ca, P, ALP, and CysC levels were concomitantly measured.

Serum CysC was measured using an Abbott Architect C 16000 (Abbott, US) analyzer and an enhanced nephelometric immunoassay.

SOS was measured performed using a Sunlight Omnisense 2000 (Sunlight Medical, Tel Aviv) quantitative ultrasound sonometer. Measurements were done on the right tibia. The SOS measurement is based on the fact that ultrasound waves propagate faster in bone than in soft tissue. SOS is influenced not only by bone mineralization but also by quantitative factors, such as micro-architecture, elasticity, and cortical thickness. The results are reported as meter/second. The SOS measurements are compared with mean SOS measurements of the same age group using a reference database, the Z score is automatically calculated based on the difference between the patients’ SOS scores and mean SOS scores of an age- and sex-matched group and expressed as a standard deviation by this sonometer. In this study, infants with Z scores < -2 were considered to have OP (7).
This study was approved by the ethical committee of Erciyes University Medical Faculty (02.10.2015/437).

**Statistical analysis**

Visual (histograms and probability plots) and analytical methods (Shapiro-Wilk’s test) were used to determine whether the data were normally distributed. Parametric data were presented as mean ± standard deviation. For intergroup comparisons, independent two-sample tests and the Mann–Whitney U test were conducted. Nonparametric data are presented as median (25th percentile to 75th percentile). The correlation of serum Ca, P, and ALP levels, in addition to SOS and Z scores, with CysC levels was determined by Pearson’s correlation analysis. The correlation analysis of the nonparametric data was tested by Spearman’s correlation analysis. A Chi-square test was conducted to determine the relationship between categorical variables. In all the tests, the level of statistical significance was accepted as $P < 0.05$.

**Results**

In total, 50 premature infants were included in the study. Infants’ and maternal demographic features are summarized in Table 1. In the measurements obtained in the 9th week, only 11 of the 50 patients remained in the hospital. The other 35 patients were evaluated during follow-up visits.

The mean serum CysC level of the 50 preterm infants was $1.50 \pm 0.19$ mg/L. Serum Ca, P, ALP, and CysC levels, in addition to SOS measurements, were grouped and compared according to gestational week and birth weight. In the group of infants with gestational age 26–29 weeks, the serum Ca levels ($P = 0.02$), P levels ($P = 0.01$), and SOS measurements ($P = 0.01$) were significantly higher. There was no difference in the serum CysC levels of the infant group with gestational age 26–29 weeks versus those of the infant group with gestational age 30–32 weeks. In the comparison of the infants according to birth weights, serum Ca levels ($P = 0.04$) and P levels ($P = 0.02$) were significantly higher, whereas serum ALP levels ($P = 0.04$) were lower in those with birth weight ≥ 1500 g as compared with infants whose birth weight was < 1500 g. There was no between-group difference in serum CysC levels according to birth weight (Table 2). The serum CysC level of boys and girls was $1.48 \pm 0.17$ mg/L and $1.50 \pm 0.24$ mg/L, respectively, with no significant between-group difference.

Serum CysC levels were not correlated with serum Ca, P, and ALP levels or with SOS measurements (figure 1) and Z score values. Serum CysC levels were also not correlated with birth weight or gestational age.

The Z score values of 24 of 50 (48%) infants were < -2, and these infants were diagnosed with OP. Serum CysC levels were $1.50 (1.35–1.61)$ mg/L and $1.58 (1.28–1.70)$ mg/L in infants with OP and without OP, respectively, with no statistically significant difference ($P = 0.34$). The demographic and clinical features of infants with OP and without OP are summarized in Table 3.

**Discussion**

The serum CysC level was not correlated with serum Ca, P, and ALP levels or SOS measurements and Z score values. There was no difference in the serum CysC levels of infants with and without OP. Serum CysC levels in preterm infants in postnatal week 9 were $1.50 \pm 0.19$ mg/L.

Most previous studies in the English literature reported reference values for CysC levels in preterms in the postnatal first month (24-26). However, our results showed a gradual decrease with term in preterm neonates. The levels are higher in preterm than term neonates, with the highest values found in the most immature cases (24). In the present study, mean CysC values of infants with gestational age 26–29 weeks tended to be higher than those of infants with
gestational age 30–32 weeks in the postnatal 9th week. However, this difference was not statistically significant.

In the current study, SOS and Z scores determined by USG were lower in osteopenic infants. Previous studies reported that bone SOS measurements were lower in preterm infants than term infants during early postnatal life, with SOS values of preterm infants decreasing until the age of 2 months old and not reaching the levels of term newborns until they were 12 months old when measured longitudinally (29-31). Previous research also reported that SOS values showed a significant association with birth weight and gestational age (29,30,32). In the present study, bone SOS values were lower in the infant group with gestational age 26–29 weeks ($P = 0.01$) and birth weight < 1500 g ($P = 0.01$).

A Z score of less than -2 suggests low bone density (7,33). In the present study, infants with a tibia Z score of less than -2 were considered as having OP, and we compared demographic and clinical features of infants with and without OP. According to previous estimates, OP occurs in 30–50% of infants with birth weights < 1000 g and in 23–32% of infants with birth weights < 1500 g (34,35). In the present study, 24 of the 50 (48%) patients were diagnosed with OP. The high rate of OP in our study may be explained by the timing of the SOS measurements, which were performed when the infants were 2 months old, a time when SOS values are lowest in premature infants. Previous studies reported that the incidence of OP was inversely correlated with gestational age and birth weight (34,35). In the present study, SOS values of infants were reduced in infants with fewer gestational weeks and lower birth weights. When the patient population was evaluated according to the presence or absence of OP, the gestational age and birth weight of infants with OP were lower than those without OP, but there was no statistical significance. This may be due to the Z score values, which were similar in both groups, as well as the sample size.

Previous in vitro studies confirmed that CysC prevented bone resorption (36-38). In one study, Lerner et al. showed that CysC in bone culture stimulated with parathyroid hormone and parathyroid hormone-related peptide was a potent inhibitor of mineral mobilization and matrix degradation (36). In an osteoblast cell culture system, Danjo et al. demonstrated that CysC affected bone morphogenetic protein signal pathways in osteoblasts, causing variation in osteoblasts and enabling mineralization and bone formation (37). Stralberg et al. showed that CysC inhibited osteoclast differentiation and formation (38). Clinical studies of bone resorption of CysC are limited to adults. Elevated serum CysC levels reported in postmenopausal women have been linked to an increased risk of bone fractures (39,40). Based on the literature, we speculate that changes in the CysC level may act as a protective mechanism in OP and play a role in the pathogenesis of bone resorption.

In the present study, we investigated the relationship of serum CysC levels with bone density and levels of biochemical markers of bone metabolism (Ca, P, and ALP) using quantitative USG. The results showed that serum CysC levels were not correlated with serum Ca, P, and ALP levels or with SOS measurements and Z score values.

In conclusion, CysC levels are not altered in OP. The presence of OP does not affect the safety of CysC as a marker of renal insufficiency in preterm infants.

References

**TABLES**

**Table 1. Demographic features**

<table>
<thead>
<tr>
<th>Infant demographics</th>
<th>Birth weight (g)</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1500 g (n = 29)</td>
<td>1104 ± 227</td>
<td>30–32 weeks (n = 27)</td>
</tr>
<tr>
<td>≥ 1500 g (n = 21)</td>
<td>1731 ± 239</td>
<td>26–29 weeks (n = 23)</td>
</tr>
<tr>
<td></td>
<td>(600–1450)*</td>
<td>Female (n, %)</td>
</tr>
<tr>
<td></td>
<td>(1500–240)*</td>
<td>SGA (n, %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 (56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (12)</td>
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</table>

<table>
<thead>
<tr>
<th>Maternal demographics</th>
<th>Age (year)</th>
<th>Gestational diabetes(n, %)</th>
<th>Hypertension (n, %)</th>
<th>PPROM (n, %)</th>
<th>Prenatal steroid (n, %)</th>
<th>Hypothyroidism (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29.2 ± 6.1</td>
<td>4 (8)</td>
<td>3 (6)</td>
<td>10 (20)</td>
<td>36 (72)</td>
<td>5 (10)</td>
</tr>
</tbody>
</table>

* Data are given as (minimum-maximum). SGA, small for gestational age; PPROM, premature prolonged rupture of membranes
Table 2. Measurements according to grouping by gestational week and birth weight

<table>
<thead>
<tr>
<th></th>
<th>30–32 weeks</th>
<th>26–29 weeks</th>
<th>P</th>
<th>≥ 1500 g</th>
<th>&lt; 1500 g</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 27)</td>
<td>(n = 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>9.95 ± 0.39</td>
<td>9.23 ± 0.93</td>
<td>0.02</td>
<td>9.85 ± 0.32</td>
<td>9.45 ± 0.95</td>
<td>0.04</td>
</tr>
<tr>
<td>P (mg/dl)</td>
<td>6.02 ± 0.87</td>
<td>5.08 ± 1.46</td>
<td>0.01</td>
<td>6.19 ± 0.78</td>
<td>5.16 ± 1.37</td>
<td>0.02</td>
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<tr>
<td>ALP (IU/L)</td>
<td>366.40 ± 114.36</td>
<td>467.39 ± 258.21</td>
<td>0.07</td>
<td>347.23 ± 116.10</td>
<td>460.38 ± 232.07</td>
<td>0.04</td>
</tr>
<tr>
<td>CysC (mg/L)</td>
<td>1.51 ± 0.18</td>
<td>1.48 ± 0.21</td>
<td>0.51</td>
<td>1.51 ± 0.19</td>
<td>1.49 ± 0.20</td>
<td>0.74</td>
</tr>
<tr>
<td>SOS (m/s)</td>
<td>2875.63 ± 146.11</td>
<td>2780.22 ± 97.64</td>
<td>0.01</td>
<td>2874.90 ± 157.59</td>
<td>2800.48 ± 105.84</td>
<td>0.05</td>
</tr>
<tr>
<td>Z score</td>
<td>-1.68 ± 1.26</td>
<td>-2.23 ± 0.82</td>
<td>0.08</td>
<td>-1.69 ± 1.35</td>
<td>-2.11 ± 0.87</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Ca, calcium; P, phosphorus; ALP, alkaline phosphatase; CysC, cystatin C; SOS, speed of sound

Table 3. Demographic and clinical features of infants with OP and without OP.

<table>
<thead>
<tr>
<th></th>
<th>Without OP</th>
<th>With OP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>n = 26</td>
<td>n = 24</td>
<td></td>
</tr>
<tr>
<td>GA (week)</td>
<td>30.5 (29.4–32.0)</td>
<td>29.0 (28.2–31.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1432 ± 399</td>
<td>1297 ± 371</td>
<td>0.22</td>
</tr>
<tr>
<td>Female (n)</td>
<td>15</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>2666 ± 951</td>
<td>2556 ± 938</td>
<td>1.69</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>33.8 ± 3.0</td>
<td>32.7 ± 3.1</td>
<td>0.36</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>48.0 (44.2–51.0)</td>
<td>47.5(43.7–53.0)</td>
<td>0.73</td>
</tr>
<tr>
<td>SGA (n)</td>
<td>4</td>
<td>2</td>
<td>0.66</td>
</tr>
<tr>
<td>BPD (n)</td>
<td>5</td>
<td>13</td>
<td>0.01</td>
</tr>
<tr>
<td>TPN duration (day)</td>
<td>18.0 (10.0–34.0)</td>
<td>23.0 (11.0–30.0)</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Maternal

<table>
<thead>
<tr>
<th></th>
<th>Without OP</th>
<th>With OP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prenatal steroid (n)</td>
<td>19</td>
<td>17</td>
<td>0.79</td>
</tr>
<tr>
<td>Hypothyroidism (n)</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes (n)</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>PPROM (n)</td>
<td>7</td>
<td>3</td>
<td>0.39</td>
</tr>
</tbody>
</table>

OP, osteopenia of prematurity; GA, gestational age; * at the moment of SOS measurement; SGA, small for gestational age; BPD, bronchopulmonary dysplasia; TPN, total parenteral nutrition; PPROM, premature prolonged rupture of membranes
Figure 1. The serum CysC levels and SOS measurements of all infants.

CysC cystatin C, SOS speed of sound. There was no correlation between serum CysC levels and SOS measurements (Rho = 0.04, P = 0.75).