Disordered bone metabolism in hereditary spherocytosis patients

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ABSTRACT
Aim: This study was planned to evaluate bone health in patients with hereditary spherocytosis. Materials and methods: In this prospective study, a total of 30 hereditary spherocytosis patients which followed in the Pediatric Hematology and Oncology Department of KSU Medical Faculty and 30 patients for control group were included. Patient and control group were chosen equal in age and sex. Hemogram and biochemical tests (serum calcium, phosphorus, alkaline phosphatase, parathormone, vitamin D) and osteocalcin were studied from the patient and control groups. Also DXA examination was performed in the patient group. Results: There was a significant difference in hemogram parameters between the two groups due to hemolytic anemia in hereditary spherocytosis patients. In the patient group, osteocalcin was 6.88 ± 4.35 ng/ml, vitamin D was 17.74 ± 7.76 ng/ml and in the control group osteocalcin was 11.93 ± 8.92 ng/ml, vitamin D was 24.04 ± 11.70 ng/ml. There was a statistically significant difference between the vitamin D and osteocalcin levels of the two groups (p = 0.017 and 0.008, respectively). Bone density was assessed in the patient group. In patients DXA results showed lower Z-scores than the normal population according to age and sex. Conclusion: Hereditary spherocytosis patients should be followed closely in terms of development, puberty, bone health as they are in other hemolytic anemias. Nutritional recommendations, vitamin D supplementation, physical activity should be advised to protect bone health.

KEYWORDS
Hereditary spherocytosis; hemolytic anemia; bone health; vitamin D; osteocalcin

Introduction
Chronic hemolytic anemia is a rare disease characterized by abnormal destruction of erythrocytes. Intrinsic hemolytic anemias divided into categories like membranopathy (Hereditary spherocytosis etc.), hemoglobinopathy (sickle cell anemia etc.), and enzyme deficiency (pyruvate kinase deficiency etc.). Extrinsic hemolytic anemias caused by autoimmunity and microangiopathy [1]. In patients with sickle cell anemia and thalassemia, the impairment in bone metabolism was demonstrated. In these patients there are changes in parameters related to bone metabolism and bone densities [2–4]. Vitamin D and growth hormone deficiency, chronic blood transfusion, iron toxicity, endocrine pathologies cause deterioration in bone metabolism [5]. In mouse studies, hemolytic anemia alone has been shown to cause bone health decline, decreased bone density, and a decrease in osteocalcin levels in the bone formation markers [6].

In Hereditary spherocytosis patients, rarely seen in the pediatric patient population, the number of data is very small. This study was planned to evaluate bone health in patients with hereditary spherocytosis.

Materials and methods
The design of the study
The study started after the permission of the Ethics Committee for Clinical Research of the KSU Medical Faculty. Prospectively designed research started on 15 June 2017 and completed on 01 December 2017. All parents were informed about the purpose and content of the study and written approval was obtained. A total of 30 hereditary spherocytosis patients who were followed up at the Department of Pediatric Hematology and Oncology at KSU Medical Faculty were included in the study. Patients with comorbidities other than hereditary spherocytosis were not included in the study. Thirty patients without chronic diseases such as hypothyroidism, obesity, malnutrition, neurological disease and who were of similar age and gender were enrolled as the control group from General pediatrics and Child Endocrinology outpatient clinics.
**Collection and analysis of samples**

Heights and weights of the patients and controls were measured. Body mass indexes are calculated because age range is wide. Body mass indexes of all patients calculated by; (BMI): weight (kg) / height² (meter) formula. The range of body mass indexes calculated according to percentile values Olcay Neyzi in Turkish children between 0 and 18 years old [7]. Hemogram and biochemical tests (serum calcium, phosphorus, alkaline phosphatase, parathormone, vitamin D) were studied in patients and the control group.

Hemogram parameters from plasma of blood samples were analyzed using SYSMEX XN 3000 (Japan) device; bun (blood urea nitrogen), creatinine, AST, ALT, uric acid, ALP, total bilirubin, indirect bilirubin, direct bilirubin, LDH, sodium, calcium, phosphorus and albumin levels were measured spectro-photometrically by ADVIA 1800 biochemistry analyzer (Germany); ferritin, parathormone measured with the chemiluminescent method using CENTAUR XP (Germany) device; vitamin D3 levels were studied by means of a chromatographic method with an Ultra HPLC 3000 device (Thermo Sci. USA). The instruments used in all studies are calibrated both in terms of technique and method, and standard curve graphics are used in the calculation of unknown samples.

Measurement of osteocalcin levels was done with Elabscience (catalog no: E-EL-H1343) brand kit. In each patient, an average of 3 cc blood was collected to standard tubes without anticoagulants. Immediately after taking the blood, the serums were separated by centrifugation in the biochemistry laboratory and the separated samples were stored at −80°C until the moment of study. At the time of the study, all frozen blood samples were dissolved in the room temperature and liquidated, then taken to analysis. Bone mineral density measurements of patients were automatically carried out in the device using the standard curve graphic. The results were reported in ng/ml.

Bone mineral density measurements of patients over 3 years of age in the patient group were determined by DXA method using the Hologic QDR4500 Elite brand device in the KSU radiology outpatient clinic. The lumbar vertebra Z-score was automatically calculated with the Hologic QDR4500W program by entering the age, sex, height and weight of the patients on the computer.

**Statistics**

Analysis of the data obtained from study was made with SPSS 16 (Statistical Package for Social Sciences) package program. Mean, standard deviation, frequency and percentage distributions were used as descriptive statistics. Values are given as mean ± SD. The homogeneity of the variants was tested with the Kolmogorov–Smirnov Test and the homogeneity of variance test-Levene statistic. Independent sample T-test was used for independent samples in the analysis of data with normal distribution and homogeneous variants. ‘Mann–Whitney U Test’ was used in the analysis of non-homogeneous data that did not fit normal distribution. Comparisons of categorized variables according to groups were examined by Pearson Chi-square test. Correlations between the variables were determined by Pearson or Spearman correlation coefficient. The probability of error (P-value) for statistical significance was chosen as 0.05. Test results were considered significant when \( P < 0.05 \).

**Results**

**Demographic data**

A total of 60 children (30 patients and 30 control groups) were included in the study. There were 13 female (43.3%) and 17 male (56.7%) patients in the patient group. The mean age of the patient group was calculated as 7.4 years (5 months 18 years). There were 13 female (43.3%) and 17 male (56.7%) patients in the control group. The mean age of the control group was calculated as 7.5 years (5 months 17 years). There was no statistically significant difference between the BMIs examined but there was a significant difference in the age distribution. Between the groups that were evaluated BMI percentages by age, there was a significant difference (Table 1) (Pearson Chi-square p = 0.006).

In our study, a 16-year-old patient with hereditary spherocytosis complained of amenorrhea. In the patient group during physical examination, there were no hepatosplenomegaly in 10 patients. Fifteen

| Groups | Percentile | <5p | 5–15p | 15–25p | 25–50p | 50–75p | 75–85p | 85–95p | >95p |
|--------|------------|-----|-------|--------|--------|--------|--------|--------|------|
| Patient Number | | 6 | 1 | 2 | 10 | 0 | 4 | 5 | 2 |
| % | | 20.0 | 3.3 | 6.7 | 33.3 | 0 | 13.3 | 16.7 | 6.7 |
| Control Number | | 1 | 3 | 4 | 7 | 6 | 1 | 3 | 2 |
| % | | 3.3 | 26.7 | 13.3 | 23.3 | 20.0 | 3.3 | 6.7 | 3.3 |

Pearson Chi-square p: 0.006.
patients had splenomegaly in various sizes, average 6.2 cm (1–10). The other 5 patients were splenectomized. Of the thirty patients, only 3 (10%) had cholecystectomy.

**Biochemical parameters**

Hemogram parameters according to groups are given in Table 2. There was a statistically significant difference between Hb, Hct, RBC, RDW, MCHC values in the hemogram parameters examined in the patient and control groups. There was a statistically significant difference between WBC, neutrophil and eosinophil counts too (Table 2).

Biochemical parameters according to groups and parameters related to bone metabolism by groups are given in Tables 3 and 4, respectively. There were statistically significant differences in the biochemical parameters between the two groups for vitamin D, osteocalcin, total bilirubin, direct bilirubin, LDH, ferritin values examined; but there was no significant difference between Ca, P, ALP, PTH values.

Vitamin D was 17.74 ± 7.76 ng/ml in the patient group and 11.93 ± 8.92 ng/ml in the control group. There was a statistically significant difference (p = 0.017 and 0.008, respectively) between the levels of vitamin D and osteocalcin between the patient and the control group (Table 4). A total of 19 patients (63.3%) had vitamin D deficiency (<20 ng/ml) in the patient group. Vitamin D deficiency was detected in 11 patients (36.6%) in the control group.

**DXA scanning**

A total of 24 (14 male, 10 female) patients underwent DXA evaluation in the patient group and a Z-score calculated. The Z-score average was −1.64 ± 1.08 (−3.4–0.2). A total of 10 patients had a Z-score under −2 (41.7%), 8 patients had a Z-score between −1 and −2 (33.3%), 6 patients had a Z-score greater than −1 (25%). There was no significant difference between boys and girls.

**Correlation analyzes**

There was a statistically significant correlation between hemogram parameters and hemolytic findings such as Hb, Hct, total bilirubin, LDH in Pearson correlation analysis.

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**Table 2. Hemogram parameters according to groups.**

| Parameter | Patient | Control | P-value |
|-----------|---------|---------|---------|
| WBC(10³/L) | 9.88 ± 3.11 (4.81–17.80) | 8.27 ± 2.07 (5.14–12.79) | 0.022* |
| Neutrophil (10³/L) | 4.46 ± 2.10 (1.24–10.77) | 3.47 ± 1.40 (1.02–6.92) | 0.036* |
| Lymphocyte(10³/L) | 4.30 ± 1.82 (2.00–7.96) | 3.93 ± 1.48 (1.54–8.35) | 0.807 |
| Monocyte(10³/L) | 0.64 ± 0.26 (0.23–1.41) | 0.58 ± 0.14 (0.09–0.58) | 0.344 |
| Eosinophil(10³/L) | 0.39 ± 0.28 (0.05–1.36) | 0.23 ± 0.19 (0.01–0.23) | 0.011* |
| RBC (10⁹/L) | 4.01 ± 0.93 (2.35–6.07) | 4.97 ± 0.38 (4.25–6.07) | <0.001* |
| Hb (g/dl) | 10.81 ± 2.74 (5.6–15.7) | 12.91 ± 1.61 (9.7–17.3) | 0.001* |
| Hct (%) | 30.80 ± 7.35 (17.2–44.6) | 37.59 ± 4.17 (30.9–51.9) | <0.001* |
| MCV (fl) | 76.81 ± 6.13 (67.9–90.7) | 75.52 ± 5.03 (64.4–85.5) | 0.377 |
| MCH (pg) | 26.89 ± 2.55 (22.6–32.8) | 25.93 ± 2.20 (20.2–28.9) | 0.124 |
| MCHC (g/dl) | 34.99 ± 1.23 (32.4–37.6) | 34.29 ± 1.29 (31.4–36.6) | 0.037* |
| RDW (%) | 10.76) 9.93 ± 0.37 (9.33–10.83) | 9.93 ± 0.37 (9.33–10.83) | 0.547 |
| Plt (10⁹/L) | 21.13 ± 4.86 (12.2–29.8) | 13.69 ± 1.34 (12.0–19.2) | <0.001* |
| MPV (fl) | 9.49 ± 0.78 (8.2–11.9) | 9.78 ± 0.64 (8.5–11.1) | 0.128 |

*p < 0.05 was considered statistically significant.

**Table 3. Biochemical parameters according to groups.**

| Parameter | Patient | Control | P-value |
|-----------|---------|---------|---------|
| Tbil (mg/dl) | 2.13 ± 1.11 (0.34–3.97) | 0.56 ± 0.31 (0.22–1.47) | <0.001* |
| Dbil (mg/dl) | 0.57 ± 0.26 (0.09–1.07) | 0.26 ± 0.11 (0.08–0.54) | <0.001* |
| LDH (U/L) | 315.50 ± 101.62 (133–606) | 254.40 ± 63.57 (152–393) | 0.007* |
| Ferritin (ng/ml) | 248.78 ± 389.51 (15–1630) | 21.80 ± 14.68 (4–68) | <0.001* |

*p < 0.05 was considered statistically significant.

**Table 4. Parameters related to bone metabolism by groups.**

| Parameter | Patient | Control | P-value |
|-----------|---------|---------|---------|
| ALP (U/L) | 192.87 ± 113.90 (55–687) | 239.53 ± 81.74 (48–370) | 0.073 |
| Ca (mg/dl) | 9.87 ± 0.43 (8.62–10.76) | 9.93 ± 0.37 (9.33–10.83) | 0.547 |
| Phosphorus (mg/dl) | 5.00 ± 0.64 (3.5–6.0) | 5.00 ± 0.59 (3.4–5.9) | 1.000 |
| Osteocalcin (ng/ml) | 6.88 ± 4.35 (1.24–17.83) | 11.93 ± 9.82 (0.50–33.29) | 0.008* |
| Vitamin D (ng/ml) | 17.74 ± 7.76 (4.32–35.25) | 24.04 ± 11.70 (3.32–38.69) | 0.017* |
| Parathormon (pg/ml) | 36.34 ± 16.59 (18.4–92.0) | 35.69 ± 14.00 (12.4–70.2) | 0.246 |

*p < 0.05 was considered statistically significant.
analysis of the data. There was a statistically significant relationship between ALP ($r = -0.446$, $p = 0.029$) and Z-score. For the correlation analysis of unequally distributed variants such as lymphocytes, RDW, ALT, total bilirubin, direct bilirubin and ferritin tests Spearman correlation analysis was used. A negative correlation between ferritin and vitamin D levels was observed by Spearman correlation analysis ($r = -0.402$, $p = 0.028$). There was a negative correlation between lymphocyte count and total bilirubin ($r = -0.482$, $p = 0.007$) and direct bilirubin ($r = -0.643$, $p < 0.001$).

Discussion

There are many studies about osteoporosis in hemolytic anemia in the literature. However, most of these studies have focused on thalassemia and lytic anemia. In our study, we examined hereditary spherocytosis patients about their bone health. Our results showed that vitamin D and osteocalcin levels in hereditary spherocytosis patients were found to be lower than the normal population. And also, in patients DXA results showed lower Z-scores then the normal population according to age and sex.

DXA is the gold standard for BMD measurement and it is a noninvasive technique. According to the Official Positions of the International Society for Clinical Densitometry, Lumber Spine and TBLH (total body less head) are the preferred skeletal sites for BMD measurements in children and adolescents. The hip is not the preferred place for growing children due to variability in skeletal development. A Z-score of $-2.0$ or lower in BMD is defined as ‘below the range expected for age’ [10]. In our study, a total of 24 patients (14 males, 10 females) with hereditary spherocytosis were evaluated by DXA method in the lumbar vertebra region. The Z-score average was $-1.64 \pm 1.08$ ($-3.4-0.2$). Ten of the patients (41.7%) had Z-scores under $-2$, Z-scores of 8 patients (33.3%) were between $-1$ and $-2$, and 6 patients (25%) had Z-scores greater than $-1$. In the study of 25 sickle cell anemia patients, Lal et al. [3] found a median Z-score $-2.3$ ($-5.1-4.3$) for lumbar spine BMD. Schündeln et al. [5] in a 45 hemolytic anemia patients study (14 with hereditary spherocytosis, 17 with sickle cell anemia, 2 with HbSC (sickle hemoglobin C), 6 with thalassemia major, 1 with thalassemia minor, 2 with glucose-6-phosphate dehydrogenase deficiency, 1 with paroxysmal nocturnal hemoglobinuria and 2 with unknown hemolytic anemia) were examined DXA and Z-score in a total of 14 patients and found a mean value of $-0.74 \pm 1$ ($-2.5-0.7$). Of these 14 patients, one had hereditary spherocytosis and had a Z-score of 0.7 (5). Our results were similar to these previous studies in the literature [3,5]. And also, in this study a 16-year-old patient with hereditary spherocytosis complained of amenorrhea. In sickle cell anemia patients, a delay of 1–2 years in puberty has been reported, which causes the Z scores to worsen during puberty because bone mineral accumulation is severely affected by pubertal growth and hormonal changes [11].

In our study, there were also significant differences between the two groups in terms of white blood cell counts aside from anemia and bone metabolism findings. Total white blood cell, neutrophil and eosinophil counts were statistically significantly higher in the patient group compared to the control group.

Valderrábano et al. [12] found a statistically significant association between bone loss assessed by DXA and anemia, a decrease in lymphocyte count, and an increase in the neutrophil count in their study of 2571 patients over the age of 65. There is a close relationship between hematopoietic cells and bone. Regions and receptors at the cellular level are common [13]. Additional parameters can be studied that can clarify this relationship in future studies.

In our study, mean level of osteocalcin in the patient group was $6.88 \pm 4.35$ ng/ml and $11.93 \pm 8.92$ ng/ml in the control group, and statistically significant difference was found between the two groups ($p = 0.008$). The majority of the patients in our study were in the prepubertal period, with an average age of 7.5 years. A total of 42 children (70%) from the patient and control groups were younger than 10 years old, 13 children (21.7%) were between 10–15 years old and the remaining 5 children (8.3%) were between 15 and 18 years old.

There is no specific range for osteocalcin level in childhood and interpretation of the values of children and adolescents is difficult because it depends on many factors such as age, gender, pubertal stage, race, diet and health, and the specificity of the tests. Osteocalcin levels reach high values in developing children especially in puberty compared to adult population. Peak values of osteocalcin reaches $115.6 \pm 21.3$ ng/mL between 9 and 13 years of age in girls during puberty and $117.8 \pm 22.3$ ng/mL between 10 and 15 years in boys. It has been shown that the level of osteocalcin rapidly decreases in all children after puberty [14].

Osteocalcin synthesis is induced by 1,25-dihydroxy-vitamin D3 in osteoblasts and is higher in childhood, reaching peak in puberty. It is difficult to compare osteocalcin values between studies due to different test methods. Lal et al. [3] in the study with sickle cell anemia patients found the median osteocalcin value $12.2$ ng/ml and range was $2.2-17.9$ in
patients aged 10–12 years. Seydewitz et al. [15] found the median prepubertal osteocalcin level to be 34.4 ng/ml and the range was 17.1–66.7 [3,15]. Schündeln et al. [5] in the 45 hemolytic anemia patients study found the mean level of osteocalcin was 75 ± 51.1 (17.5–247) ng/ml in the patient group while the mean level of osteocalcin was 115.3 ± 35.2 (72.6–186) ng/ml in the control group and was found to be statistically significant lower. In the same study, the level of osteocalcin was 45.6 ± 17.6 (17.5–76.6) ng/ml in sickle cell anemia patients and 90.0 ± 46.7 (37.5–204) ng/ml in hereditary spherocytosis patients [5]. In order to be used clinically in the future, it is necessary to standardize the laboratory tests and establish normal ranges.

Long-term vitamin D deficiency causes poor mineralization of the bone and clinically results in widespread, chronic pain, muscle weakness, abnormal BMD symptoms, and low energy fractures. Vitamin D deficiency is very common in sickle cell anemia patients [16]. In many countries, the prevalence of vitamin D deficiency (25 (OH) vitamin D <20 ng/mL) and insufficiency (25 (OH) vitamin D between 20 and 30 ng/mL) in thalassemic patients has been reported much despite of high sunlight intake and routine vitamin D prescription [17].

In our study, vitamin D was found 17.74 ± 7.76 ng/ml in the patient group and 24.04 ± 11.70 ng/ml in the control group (p = 0.017). Vitamin D deficiency was found in 19 patients (63.3%) in patient group, while vitamin D deficiency was detected in 11 patients (36.6%) in the control group. Schündeln et al. [5] in the study of 45 hemolytic anemia patients, found mean vitamin D value 19.1 ± 5.7 (12.8–30.2) ng/ml in patients with hereditary spherocytosis, and mean vitamin D value in patients with sickle cell anemia was 9.3 ± 7.4 (1–25.2) ng/ml. In 86.7% of patients with sickle cell anemia and 61.5% of patients with hereditary spherocytosis, vitamin D level was found below 20 ng/ml [5]. Our results were consistent with the vitamin D deficiency in previous study in hemolytic anemia patients [5]. It should be aimed that vitamin D is kept above 30 ng/ml in hemolytic anemia patients who are already at risk of osteoporosis [18].

In our study, ferritin value was above 200 ng/ml in 8 patients (27%) with frequent blood transfusion history in the patient group and negative correlation between ferritin and vitamin D levels was observed by Spearman correlation analysis (r = −0.402, p = 0.028). Iron overload impairs osteoid maturation and inhibits local mineralization to form focal osteomalacia. In addition, integration of iron in calcium hydroxyapatite affects the growth of crystals, which causes mineralization failure [9]. Increased ferritin has been shown to inhibit osteoblast function in animal studies in vitro, and increased ferritin is associated with decreased BMD in the general population [19].

In conclusion, vitamin D deficiency was more common in hereditary spherocytosis patients than in the control group. The level of osteocalcin, a bone-forming indicator, was found to be lower in hereditary spherocytosis patients than in the control group. The Z-score of BMD in hereditary spherocytosis patients according to age and sex was found to be lower than the normal population. Hereditary spherocytosis patients should be followed closely in terms of development, puberty, bone health as they are in other hemolytic anemias. Nutritional recommendations, vitamin D supplementation, physical activity should be advised to protect bone health.

Disclosure statement
No potential conflict of interest was reported by the authors.

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