Bone Turnover Markers in Osteogenesis Imperfecta and Effect of Bisphosphonate Treatment: First Egyptian Study

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ABSTRACT

Purpose: This report studies the changes in bone turnover markers in Egyptian OI patients and the effect of bisphosphonate treatment on these biomarkers. Methods: Twenty-six OI patients, and 30 controls were included. Age range of patients was 6.22 ± 3.72 years, while age range of controls was 11.1 ± 3.36 years. Serum calcium, phosphorus, parathormone, 25(OH) vitamin D, 1,25 (OH)2 vitamin D in addition to bone formation and degradation markers including: osteocalcin and procollagen type I N propeptide and urinary helical peptide, N-telopeptide, isomerized and non-isomerized C-telopeptide, pyridinoline and deoxypyridinoline were measured at baseline, 6 months and 12 months of bisphosphonate treatment. Results: All biochemical measurements except parathormone showed no significant difference. Bone formation markers and type I collagen degradation markers showed significant differences. Conclusions: Biochemical measurement of serum calcium is recommended in patients receiving bisphosphonates. Bone formation markers and markers of type I collagen degradation are valuable for monitoring the effect of bisphosphonate treatment in OI patients.

Keywords: osteogenesis imperfecta, bone formation, bone resorption, bone turnover markers, bisphosphonates, type I collagen.

INTRODUCTION

Osteogenesis imperfecta (OI) (OMIM; 610682, 610915, 259440, 112240, 614856, 613848, 610968, 616229, 616507, 613849, 615066, 615220, 613982, 610967), composes a heterogeneous group of disorders characterized by bone fragility and decreased bone mass with variable severity ranging from mild to severe or lethal types with an incidence higher than 1/10000 births1. Numerous classifications of OI have been published. The most common is Silence classification proposed in 1979 that classified OI into 4 types (type I, II, III and IV)2. A quantitative scoring (CSS) was proposed by Aglan et13 to assess clinical severity in OI. Most OI patients are due to dominantly transmitted mutations of COL1A1 and COL1A2 genes. These are responsible for the synthesis of the proalpaha-1 and proalpaha-2 polypeptide chains that form the triple helix of type I collagen. Mutations in COL1A1 or COL1A2 genes result in quantitative and/or qualitative defects in type 1 collagen synthesis by osteoblasts4-5. A considerable number of OI patients have recessive mutations in a growing list of genes that encode proteins and are involved in the posttranslational processing or modification of type I collagen, in the final quality control of procollagen formation or in osteoblast differentiation6-11. Bone is a metabolically active tissue, which undergoes continuous remodeling through two processes; bone formation and bone resorption12. Bone formation markers are enzymes or products produced by active osteoblasts or derived from procollagen metabolism and reflecting different functions of osteoblasts and bone formation. They include the measurement of alkaline phosphatase (ALP) activity, osteocalcin and procollagen type I propeptide concentrations. Bone resorption markers are degradation products of type I collagen produced during bone resorption. Bone resorption markers are either degradation products of type I collagen like Cross-links, C-telopeptide (CTX), N-telopeptide (NTX) and helical peptide (HeIp) or non-collagenous proteins and osteoclast-derived enzymes13. Antiresorptive bisphosphonates either oral or intravenous are the currently used therapy for OI with evidence on amelioration of bone mineral density (BMD), motor development, and decrease in fracture rate and pain in OI patients13,14. This is the first report to study the changes in biochemical markers of bone turnover in Egyptian patients with osteogenesis imperfecta and the effect of bisphosphonates used in treatment on these biomarkers.

PATIENTS AND METHODS

Twenty-six osteogenesis imperfecta patients, diagnosed at the Limb Malformations and Skeletal Dysplasia Clinic (LMSDC), National Research Centre, and 30 normal

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healthy controls were included after obtaining signed informed consent approved by the Medical Research Ethics Committee of the National Research Centre. Patients were classified according to Sillence et al. (1979). Age range of patients was 6.22 ± 3.72 years, while age range of controls was 11.1 ± 3.36 years. Assessment of disease severity was done using the clinical severity score (CSS). The CSS is based on five major criteria of high clinical value: number of fractures per year, motor milestones, long bone deformities, length/height standard deviation score (SDS), and bone mineral density (BMD) (Aglan et al., 2012).

To assess the changes of bone turnover markers in OI patients compared to normal controls and study the effect of bisphosphonate treatment on the biochemical measurements and biochemical markers of bone turnover, measurements were done at baseline (0 M), after 6 months of treatment (6 M) and after 1 year of treatment (12 M). The treatment protocol involved intravenous infusion of zolderonic acid with a dose of 0.05 mg/kg over one hour duration and was repeated every 6 months under medical observation in hospital. Supplementary oral doses of calcium and vitamin D were given on daily bases to all patients. Fasting blood and 2nd void urine samples were collected between 9 am and 11 am, for known diurnal effect. Forty-six samples were collected from patients in the period from December 2013 to September 2015. These forty-six samples were collected as follows; 18 samples were collected from patients in the period from December 2013 to September 2015, while 12 samples were collected from patients at baseline, 6 months and 1 year after the start of bisphosphonate treatment, 14 samples were collected from patients at baseline and 6 months after the start of bisphosphonate treatment and 12 samples were collected from patients 1 year after the start of bisphosphonate treatment. 1 sample was collected from 1 patient at baseline and another collected from another patient at 6 months.

Biochemical investigations

Biochemical measurements

Serum calcium was measured by quantitative colorimetric kit (Stanbio Total Calcium LiquiColor, Stanbio Laboratory, EFK Diagnostic Company, USA). Serum inorganic phosphorus (Pi) was measured by colorimetric kit (Quinica Clinica Aplicada SA, QCA, Spain). Serum parathyroid hormone (PTH) was measured by ELISA kit (Diasource immunoassays, Belgium). Serum 25(OH) vitamin D was measured by ELISA kit (Gloryscience, USA). 1, 25 (OH)2 vitamin D was extracted from serum by solid phase extraction using chromabond cartridges (solid phase extraction cartridges, Immunodiagnostik) and then measured by ELISA kit (immunodiagnostik, Germany).

Biochemical markers of bone formation

Serum osteocalcin was measured by ELISA kit (hOST, Diasource immunoassays, Belgium) and serum procollagen I N-propeptide (P1NP) was measured by ELISA kit (USCN life, China).

Biochemical markers of type I collagen degradation

Urinary helical peptide of type I collagen (HelP), urinary N-telopeptide of type I collagen (NTX), urinary α crosslinked C-telopeptide (αCTX) and urinary β isomerized cross linked C-telopeptide (βCTX) were measured by ELISA kits (Sunred, China). Urinary pyridinoline (PYD) and deoxypyridinoline (DPD) were measured by tandem mass spectrometry according to the method of 15 with some modifications according to our instrument and calibrators (online resource).

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to test the variation in the measured parameters and the response of the patients across time. Data were presented as mean ± SE. The collected data were computerized and analyzed by Stata 8.0 (Stata Corp., Texas), p<0.05 was considered statistically significant.

RESULTS

This study included 26 OI patients and 30 normal controls. Male and female sex was equally distributed in patients, while males constituted 40% in controls. Mean age for OI patients was 6.22 ± 3.72 SD and parental consanguinity was positive in 11 patients (42.3%) and 17 controls (56.7%). Positive family history was present in 11 patients (42.3%).

Distribution of patients according to Sillence type was as follow: type I (9; 34.6%), type III (11; 42.3%) and type IV (6; 23.1%), while distribution according to clinical severity using CSS was: mild (10; 38.5%), moderate (11; 42.3%) and severe (5; 19.2%). Results of biochemical measurements including serum calcium, inorganic phosphorus, parathyroid hormone, 25 (OH) vitamin D, and 1,25 (OH)2 D for patients and controls are shown in Table 1.

Results of biochemical markers of bone formation including serum osteocalcin and procollagen I amino terminal propeptide (P1NP) for patients and controls are shown in Table 2.

Results of biochemical markers of type I collagen degradation including urinary helical peptide of type I collagen (HelP), N-telopeptide of type I collagen (NTX),

Table 1: Biochemical measurements for patients and controls.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean ± SE</th>
<th>N=14</th>
<th>N=14</th>
<th>N=18</th>
<th>N=30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Ca (mg/dl)</td>
<td>9.5 ± 0.19</td>
<td>9.4 ± 0.24</td>
<td>9.4 ± 0.16</td>
<td>9.91±0.09</td>
<td></td>
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<tr>
<td>Serum Pi (mg/dl)</td>
<td>5.1 ± 0.23</td>
<td>5.1 ± 0.23</td>
<td>5.2 ± 0.25</td>
<td>5.52±0.13</td>
<td></td>
</tr>
<tr>
<td>Serum PTH (pmol/ml)</td>
<td>31.5 ± 3.4</td>
<td>54.7 ± 4*</td>
<td>34.3 ± 4.9</td>
<td>25±0.5</td>
<td></td>
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<tr>
<td>Serum 25 (OH) D (µg/l)</td>
<td>33 ± 2.7</td>
<td>28.9 ± 2.5</td>
<td>37.5 ± 3.5</td>
<td>53.6±3.62</td>
<td></td>
</tr>
<tr>
<td>Serum 1.25 (OH)2 D (pg/ml)</td>
<td>65.5 ± 2.5</td>
<td>69.9 ± 4.9</td>
<td>57 ± 3.4</td>
<td>58.7±3.1</td>
<td></td>
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</table>

*Significant from other time points and controls at P<0.05. Statistical analysis was carried out by one-way ANOVA.
α crosslinked C-telopeptide (αCTX), β isomerized cross linked C-telopeptide (βCTX), αCTX/βCTX ratio, pyridinoline (PYD), deoxypyridinoline (DPD) and PYD/DPD ratio of patient and controls are shown in Table 3.

**DISCUSSION**

In osteogenesis imperfecta, bone is characterized by excessive number of osteoblasts with impaired activity resulting in defective bone matrix deposition. Also, the skeletal turnover is generally increased. These features have been confirmed by the detection of high levels of bone turnover markers. In this study, biochemical measurements, markers of bone formation and markers of type I collagen degradation were studied in 26 patients with osteogenesis imperfecta (OI) and 30 normal controls with matched age and sex.

Results showed no statistically significant difference in serum calcium that was almost within reference range in all patients except 5 patients, who had slightly lower concentrations. Serum phosphorous and 1,25 (OH)2 vitamin D concentrations were within reference range for all patients at all intervals. Although, no significant difference was found statistically, 25 (OH) vitamin D was lower than reference range in 4 patients, while it was higher than 100 µg/l in 5 patients. Both low and high serum levels of 25 (OH) vitamin D in OI patients were reported in the literature. Planet et al. (2016) found low serum 25OHD concentrations in many children and adolescents with OI. On the other hand, Zerwekh (2008) suggested that high serum concentrations of 25 OHD may be due to the fact that serum 25OHD test accounts for both endogenous and exogenous sources of vitamin D. In this study, all patients were on vitamin D oral supplement.

Serum parathyroid hormone (PTH) concentrations were comparable at baseline to controls which increased significantly at 6 months and returned to baseline concentration at 1 year. However, only one patient had a higher concentration of PTH than reference range. On the other hand, 2 patients at 6 months’ time point and 1 patient at 1 year time point had lower level of serum PTH than reference range. In a study carried out by Ipach et al., in 2012, 84 % of OI patients had low PTH levels with no statistically significant changes during the treatment period that lasted at least for 18 months. In another study PTH was normal in OI patients at baseline and only fine changes were reported during bisphosphonate treatment. It was proposed that calcium and vitamin D supplements might contribute to the normalization of the biochemical markers.

Bone formation markers are either markers of osteoblast function like osteocalcin and bone specific alkaline phosphatase, or markers specific to type I collagen formation like P1NP.

Our results showed that serum osteocalcin concentrations were significantly higher at baseline than controls. Bisphosphonate treatment returned osteocalcin level to that of the controls at 6 months and 1 year time points. In a study carried out by Ipach et al. (2012), 60 % of the studied patients had low serum osteocalcin with no significant change in the osteocalcin level during treatment P. Another study carried out by 19 showed normal range of serum osteocalcin in all types of OI and controls.

However, a study carried out on adult OI patients (75.3 % Silence type I, 9% type III and 15.5 % type IV) reported high level of osteocalcin in 11% of the included patients which was nearly the same percent of type III, the severe form of OI20. Another study, where OI patients were chosen according to severity (35.7% were severe and 46% were moderate), serum osteocalcin level was decreased significantly over time. It was suggested that the significant decrease of the osteocalcin level may be correlated to the higher percentage of the severe group in that study21. Despite the relatively low percentage of the severe group in this study (19.2%), our results supports the suggestion that the high level at baseline may be due to the severity of the disease.

Procollagen type I N-terminal propeptide (P1NP) is a bone formation marker of type I collagen synthesis. Serum P1NP in this study showed significantly higher concentrations in all patients at different time points than in controls indicating a high rate of procollagen type I synthesis in OI patients in agreement with previous studies that reported an increase in bone formation markers (osteocalcin and P1NP) and bone resorption (α CTX) markers in children with OI22.

The P1NP level decreased gradually at 6 months time point and significantly at 1 year time point. In a former study on OI adult patients, changes in the level P1NP as a marker of collagen formation was determined at baseline, 6 M and 12 M time points during treatment with bisphosphonate, results yielded significant decrease in P1NP level at 6 months with no further significant change after 6 months23. Helical peptide of type I collagen (HelP) is the most recent isolated bone resorption marker. It is a fragment of type I collagen molecule corresponding to the 620-633 residues of the helicoidal region of the α 1 chain24. In this study HelP concentration was significantly higher at baseline and 6 months than its concentration in controls. At baseline, the level was significantly higher than at 6 months and 1 year. It decreased significantly at 6 months.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean ± SE</th>
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<tbody>
<tr>
<td>Serum osteocalcin (ng/ml)</td>
<td>37.5 ± 2.8*, 34 ± 3.5, 30.7 ± 2.8, 28.6±1.18</td>
</tr>
<tr>
<td>Serum P1NP (ng/ml)</td>
<td>1172.3 ± 58.5*, 1071.8 ± 68.9*, 872.7 ± 40.7**, 503.3±28.7</td>
</tr>
</tbody>
</table>

*Significant from controls and † significant from other time points at P<0.05. Statistical analysis was carried out by one-way ANOVA.
but still higher than the control level. It decreased gradually from 6 months to 1 year to be comparable with control level. These results are consistent with a previous study carried on adult OI patients where HeLP was higher in patients than in healthy controls. The authors stated that determination of HeLP level is unlikely to be affected by changes in collagen telopeptide isomerization and crosslinking, thus, it may provide a more reliable index of overall bone resorption in OI25. The results of our study further suggest that HeLP could be a good bone resorption marker for the monitoring of bisphosphonate treatment.

Urinary N-telopeptide level (uNTX) was significantly higher at all time points than in controls. Also, at baseline the level was significantly higher than at 6 months. After 6 months, the level decreased gradually but non-significantly to be comparable to controls level at 1 year. In a study by Forin et al. (2005), uNTX level was above the normal range in 16 OI children out of 29 and the highest levels were observed in type III patients. A dramatic decrease in the level of uNTX in all patients was observed at the end of their study after bisphosphonate treatment, which is the same as in our results. Additionally, in another study a higher baseline level of urinary NTX between PYD/DPD ratio was observed in our results where urinary levels of PYD and DPD were decreased sharply during the first three months of treatment and then decreased gradually throughout the remaining 2 years26.

Collagen type I molecules undergo racemization and isomerization during post-translational modification of the aspartic acid residue in the C telopeptide, consequently it is called CTX. Isomerization is the transfer of peptide bond between the aspartic acid and the neighboring amino acid from the α-carboxyl group to the β- or γ-carboxyl group, giving rise to αCTX and βCTX isomers. Isomerization forms a kink in the peptide backbone, which may change the properties of collagen molecule. These changes include effect of degree of isomerization on the activity of cathepsin K (EC 3.4.22.38), a collagenolytic enzyme. It may also interfere with the formation of enzymatic cross-links, consequently it affects bone strength indirectly27. In our study urinary levels of αCTX, βCTX and their ratio αCTX/βCTX were significantly higher in patients at all time points than in controls. No significant change was observed throughout the study period.

A previous study on adult OI patients reported high level of αCTX, low level of βCTX and consequently high level of αCTX/βCTX ratio28. However, urinary βCTX level was found to be high in children with OI in another study29, reported that there was no association between αCTX/βCTX ratio and biochemical markers of bone formation and resorption. They stated that this ratio could provide additional information about bone collagen maturation which is related to bone strength.

There is no sufficient data about urinary levels of αCTX, βCTX and their ratios in children with OI, previous studies were mostly carried on adult OI patients. In normal children, due to high bone remodeling, equilibrium of isomerization could not be reached29.

Enzymatic cross-linking is a post-translational modification of type I collagen giving rise to pyridinium and pyrrolic molecules within the N- and C-telopeptide. Pyridinium cross-links are pyridinoline (PYD) and deoxypyridinoline (DPD). It is thought that post-translational modification plays a role in the mechanical properties of bone. High urinary levels of free PYD and DPD were reported to be associated with increased risk of fractures in postmenopausal women, while the correlation between PYD/DPD ratio and risk of fracture was inconclusive29. Few studies have found increased PYD/DPD ratio in OI patients than in healthy controls25. This was obvious in our results where urinary levels of PYD and DPD were significantly higher in OI patients at all time points than in controls.

During treatment of the studied patients, the levels of urinary PYD and DPD were decreased significantly at 6 months with no further decrease at 1 year. Ipach et al. (2012) reported an increase in the urinary level of PYD in 60 % and of DPD in 80% of OI patients. The high levels of PYD and DPD were decreased significantly due to bisphosphonate therapy31. The PYD/DPD ratio in this study was higher than controls at all time points. Bisphosphonate therapy did not affect the level of PYD/DPD ratio, which is consistent with32, who stated that bisphosphonate treatment did not affect PYD/DPD ratio. In order to study the effect of bisphosphonate treatment on bone turnover markers in different clinical phenotypes, patients were divided into 3 groups according to severity; mild, moderate and severe; using CSS. Preliminary

### Table 3: Biochemical markers of type I collagen degradation in patients and controls.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean ± SE</th>
<th>Controls</th>
<th>Baseline</th>
<th>6 months</th>
<th>1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary HeLP (mg/mmol creat.)</td>
<td>781.12 ± 1.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>402.83 ± 47.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>321.5 ± 22.9</td>
<td>131.±22.02</td>
<td></td>
</tr>
<tr>
<td>Urinary NTX (nmol/mmol creat.)</td>
<td>1447.7 ± 2.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>761.12 ± 1.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>486.93 ± 40.8</td>
<td>359.±64.31</td>
<td></td>
</tr>
<tr>
<td>Urinary αCTX (µg/mmol creat.)</td>
<td>6102.6 ± 3.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6539.1 ± 7.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5514.7 ± 4.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2738.2±283.5</td>
<td></td>
</tr>
<tr>
<td>Urinary βCTX (µg/mmol creat.)</td>
<td>5789.8 ± 5.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5554 ± 4.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4792 ± 2.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3148±316.3</td>
<td></td>
</tr>
<tr>
<td>αCTX / βCTX</td>
<td>1.13 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.09 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.18 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83±0.04</td>
<td></td>
</tr>
<tr>
<td>Urinary PYD (nmol/mmol creat.)</td>
<td>386.27 ± 30.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>245.67 ± 33.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>288.45 ± 28.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.21±8.08</td>
<td></td>
</tr>
<tr>
<td>Urinary DPD (nmol/mmol creat.)</td>
<td>63.97 ± 5.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.98 ± 5.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.51 ± 3.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.53±8.16</td>
<td></td>
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<tr>
<td>PYD / DPD</td>
<td>6.74 ± 0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.02 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.76 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.12±0.32</td>
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</table>

<sup>*a</sup>Significant from other time points and <sup>*</sup> significant from control at P<0.05. Statistical analysis was carried out by one-way ANOVA.
statistical analysis indicated variable response to treatment in many of the measured bone turnover markers in the different clinical groups. However, due to the small sample size in each group, further studies on larger number of patients are ongoing.

CONCLUSIONS
Biochemical measurement of serum calcium is recommended in OI patients receiving bisphosphonates to avoid hypocalcemia which is a side effect of the treatment. Serum osteocalcin and P1NP as bone formation markers are valuable for monitoring the effect of bisphosphonate treatment in OI patients; however, P1NP is a better marker as it is directly related to the biosynthesis of type I collagen. Markers of type I collagen degradation as bone resorption markers are valuable and less invasive markers for monitoring treatment in different clinical groups of OI.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENT
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REFERENCE


