Introduction

Skeletal involvement in patients with diabetes mellitus was initially described over 50 years ago[1, 2]. The association between diabetes and osteoporosis has been extensively investigated, as both diseases are very common and of great socioeconomic relevance. However, despite numerous publications addressing this problem, many questions remain unanswered[3]. Several mechanisms may contribute to skeletal damage, including the increased urinary excretion coupled with the lower intestinal absorption of calcium[4], the inappropriate homeostatic response in terms of parathyroid hormone secretion, and also the complex alteration of vitamin D regulation[5]. Decreased or increased insulin, impaired insulin action and IGF-1 concentrations[6], sustained hyperglycemic state, and the effects of the accumulation of glycation end products on the bone tissue[6] could also play a role. Moreover, considerable disagreement still persists upon the possible influences on bone tissue exerted by gender[7], metabolic control of diabetes[8], and disease duration[9].

A possible role of the fat tissue, which is augmented in type 2 diabetes, apart from mechanical stress, the increased production of oestrogen protects against osteopenia in type 2 diabetic patients[10], It is also noteworthy that leptin, a peptide produced by adipocyte, has recently emerged as a potential candidate responsible for protective effects of fat on the bone tissue[11]. Also, adiponectin may regulate fat cell formation in bone marrow, and fat marrow is increased with ageing and in patients with osteoporosis[12].

It has been hypothesized that microangiopathy[13], as well as macroangiopathy[14] might also directly influence skeletal tissue, according to the most recent beliefs on regulation of bone metabolism[15].

As far as bone mass is concerned, in adult patients with type 1 diabetes a moderately reduced bone mineral density has been shown in both axial and appendicular skeleton. On the contrary, patients with type 2 diabetes seem to have higher bone mineral density in respect to healthy control subjects, especially when overweight women are considered[16]. No clear relationship between bone mass measurements and biochemical parameters of mineral metabolism has been shown in the different types of diabetes[17].

Cohort studies recently carried out on large samples indicate that diabetic patients (with both type 1 and type 2 disease) have a higher risk for fracture, in particular for hip fracture, the most dangerous osteoporotic complication[3]. This seems to be dependent both on qualitative and quantitative alterations of the bone, as well as on extra-skeletal factors due to the neuropathic and other microangiopathic complications of the disease[13 - 15].

According to these premises, the indiscriminate inclusion in the investigated samples of diabetic patients with different pathogenesis, or assuming different therapies, or with various disease duration, could have provided as many confounding factors in both clinical and epidemiological investigations. The different design of the studies that investigated populations of varying ages and genders also contribute to the conflicting results. Finally, the assessment of bone mass with various techniques (measuring either more cortical or more trabecular bone) could have been detrimental to a unified conclusion[3]. Therefore, The present study was undertaken to clarify the influence of glycemic control on bone turnover using biochemical bone markers in type 2 diabetes female patients.

Materials and Methods

This case- control study had included 60 patients and 30 health subjects selected from Out-patient Department of Uhod Hospital, Madinah, KSA, from October 2003 till August 2004. All participants gave verbal consent to participate in this study.

Inclusion criteria were patients who were overweight, with type 2 diabetes for more

than 5 years duration with BMI >25 and were under oral antidiabetic therapy metformin, or sulfonylurea agent or both. All were premenopausal females with history of multiple pregnancies and regular menstrual cycles. They had normal thyroid, kidney, and liver function tests, normal serum albumin and complete blood count. They were divided into good controlled and poorly controlled groups according to the criteria of ADA (fasting blood glucose 90–130 mg/dl and A1C <7%).

Exclusion criteria were diabetic patients under insulin therapy, with microvascualr or macrovascualr diabetic complications, patients with other known risk factors for osteoporosis other than diabetes, cigarette smokers, patients with dietary lack of calcium, patients on drugs that could affect bone metabolism or calcium metabolism during the previous 6 months, including calcium, sex steroids, corticosteroids, vitamin D metabolites, calcitonin, warfarin, vitamin K, thiazides, anticonvulsants; thyroxin and bisphosphonate. Patient who was found to have vitamin D deficiency (plasma level <14 μg/L) were also excluded from the study.

Inclusion criteria for control group were age and weight matched healthy premenopausal females with history of multiple pregnancies and regular menstrual cycles, with history of balanced diet and without history of any known risk factor for osteoporosis.

After through history and clinical examination, height and weight were measured with the patient standing in light clothes and without shoes. BMI was calculated as body weight divided by height squared (kilograms per meter squared).

Ten ml fasting venous blood sample were withdrawn from each participant, 2 ml was taken on EDTA as anticoagulant for determination of glycohaemoglobin (HbA1c). Two ml blood were put on heparin, placed immediately on ice and plasma was separated and centrifuged in a cooling centrifuge, plasma was separated and stored at 20°C for estimation of osteocalcin and vitamin D, another 2 ml blood were placed on ice, serum was separated and stored at 20°C for estimation of parathyroid hormone (PTH). Another 2 ml blood were put on floride, and plasma was separated for estimation of fasting blood glucose level. The remaining 2 ml of blood was centrifuged and serum was separated for estimation of bone minerals (Ca, Ph) and kidney function test (urea and creatinine). On the same day of blood sampling, the last 24 hours urine samples were collected for measurement of urinary calcium at 8.00 AM and the morning 1st or 2nd void urine sample was stored in a dark container for estimation of deoxypyridinoline (DPd).

The FBG was measured using an enzymatic oxidation in the presence of glucose oxidase (The Kits provided by Randox GOD/PAP, Diamond Road, Crumlin, Co Antrim, United Kingdom). The HbA1c determination was based on turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood samples (The kits was supplied by BioSource International, Inc. 542 Flynn Road, Camarillo, California 93012 USA). Serum Ca, Ph, alkaline phosphatase (ALP), urea and creatinine were measured by Boechringer Mannheim 911 Hitachi automated analysis.

The serum osteocalcin level is measured with a commercially available N-MID Osteocalcin Electrochemiluminescence Immunoassay kit (Roche Diagnostics GmbH, Mannheim, Germany) [18]. DPD and PTH analysis were determined by solid-phase two site chemiluminescent enzyme immunometric assay, performed on immulite auto analyses using kit supplied by DPC (Diagnostic Product Corporation, 5700 west 96th street, USA). Plasma 25, hydroxyl Vitamin D3 (25 OH-D) was measured according to Chromosystem reagent kit for high performance liquid chromatography (HPLC).

Statistical Methods

Statistical evaluation of all data was done on IBM-PC microprocessor computer using SPSS software for windows (Statistical Package for Social Sciences version 11,

USA) for data management and analysis and the Microsoft power point for charts. Quantitative data were presented as mean ± SD. For the comparison of the three groups' means, one way analysis of variance (ANOVA) was used followed by Students' Newman Keuls test to detect significant difference. All tests were two tailed and considered significant when $p<0.05$. The coefficients of correlation between levels of glucose, minerals, and bone markers were calculated according to Pearson's method.

Results

Analysis of the age (years) and body mass index BMI (Kg/m2) in the different studied groups shows that they were age matched (46.1 ± 6.45, 49 ± 5.14 & 47.8±8. 79 years for controlled, uncontrolled diabetics and control group respectively, $p<0.05$). The mean duration of diabetes did not differ between the 2 diabetic groups (6.6±0.4 & 7.3± 1.1 years, $p<0.05$). All were overweight (29.38±6.00, 28.86±6.00 & 27.59±5.12 BMI respectively, $p<0.05$; **Figure 1**).

The fasting blood sugar levels of both diabetic groups (98.1 ± 73.57 & 239.64± 97.78 mg/dl) were significantly higher than in controls (83 ± 7.6 mg/dl, $p>0.05$ & <0.01 respectively) (figure 2). Similarly, HbA1c levels of both diabetic groups (5.9±0.8 and 9.05±3.29) were significantly higher than in controls (5.1±0.35, $p>0.05$ & $p<0.01$ respectively; **Figure 3**).

Serum calcium levels of both diabetic groups (8.87± 0.3 & 8.79 ±0.7 mg/dl) were found to be significantly lower compared with the control group, especially the uncontrolled group (9.96± 1.9 mg/dl, $p<0.05$ & $P<0.001$ respectively). However there was no difference between all studied groups concerning serum phosphorous levels (3.98 ± 0.92, 2.78 ± 0.63 & 4.3 ± 0.39 mg/dl respectively, $p>0.05$; **Figure 4**).

ALP levels of both diabetic groups (177± 39.88 & 287 ± 41.4 mg/dl) were significantly higher than in control group, especially the uncontrolled group (144 ± 22.54 mg/dl, $p<0.05$ & $p<0.001$ respectively; **Figure 5**).

PTH level was significantly higher in both diabetic groups (49 ±9.87 & 56.25± 12.3) compared with control group (26.9± 5.60, $p<0.01$). vitamin D levels in both diabetic groups (50.9±12.6, 45.4±18.9) were significantly lower than the controls (57.9±13.6, $p<0.05$; **Figure 6**).

A lower level of osteocalcin was found in both diabetic groups especially the uncontrolled one (4.09 ± 1.48 & 1.89 ± 0.24 ng/ml) compared with control group (6.5±1.5 ng/ml $p>0.05$ and <0.01 respectively; **Figure 7**).

Both diabetic groups showed increased levels of urinary calcium (270.66±41.7 and 300.56. ±55.67 mg) significantly than the control group (244.23 ±51.5 mg), especially the uncontrolled one ($p<0.05$ & $p<0.001$ respectively; **Figure 5**).

Similar results were detected for urinary Dpd (10.8 ± 4.6, 12.06±5.12, 6.2 ±0.8 nM/mM creatinine, $P<0.05$ & $p<0.001$ respectively); **Figure 7**).

PTH level in all diabetic patients was found to correlate positively with ALP level ($r= 0.54$, $P<0.01$) and negatively with serum calcium ($r =-0.65$, $P<0.01$). Glycemic indices (FBG, HbA1C) showed significant positive correlation with alkaline phosphatase (0.290 & 0.294 respectively, $p<0.01$ for both), 24 hours urinary calcium ($r = 0.340$, $P<0.01$&0.260, $p<0.5$ respectively) and Dpd (0.468, $p<0.01$, 0.228, $p<0.05$ respectively). Dpd correlated also with urinary calcium (0.278, $p<0.5$). Serum 25 OH- vitamin D levels were negatively correlated to PTH levels ($r=-0.290$, $P < 0.01$). OC and types of treatment (i.e. metformin with or without sulphonylurea) did not show correlation with any of the studied parameters.

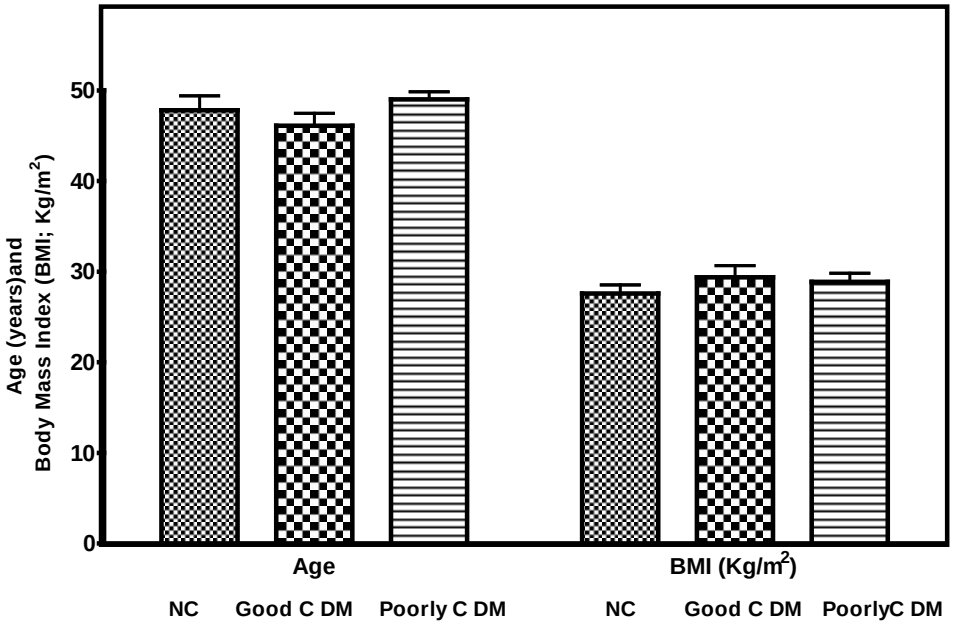

Figure 1: *Analysis of the age (years) and body mass index BMI (Kg/m²) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.05. All data were non-significant in comparison to normal control group.*
Data were expressed as mean ± S.D. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.

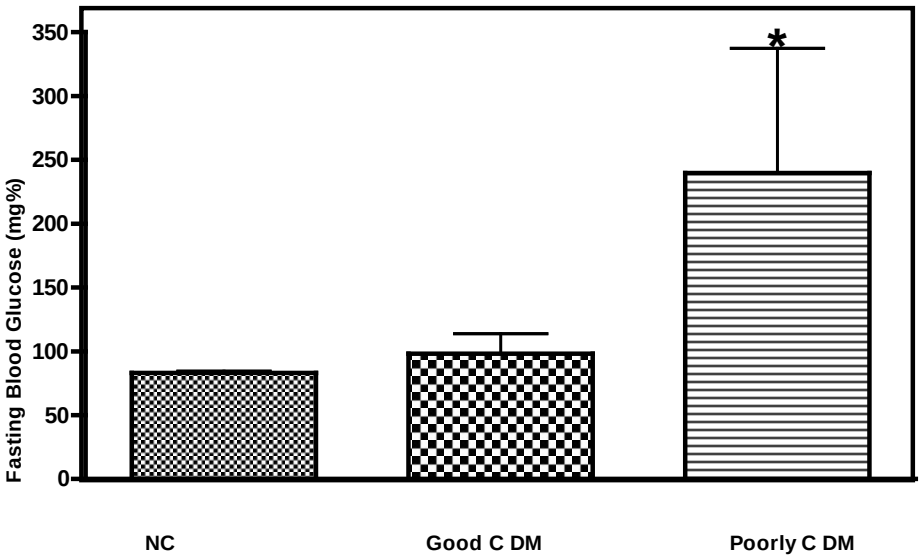

Figure 2: *Fasting blood glucose (mg%) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ± S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

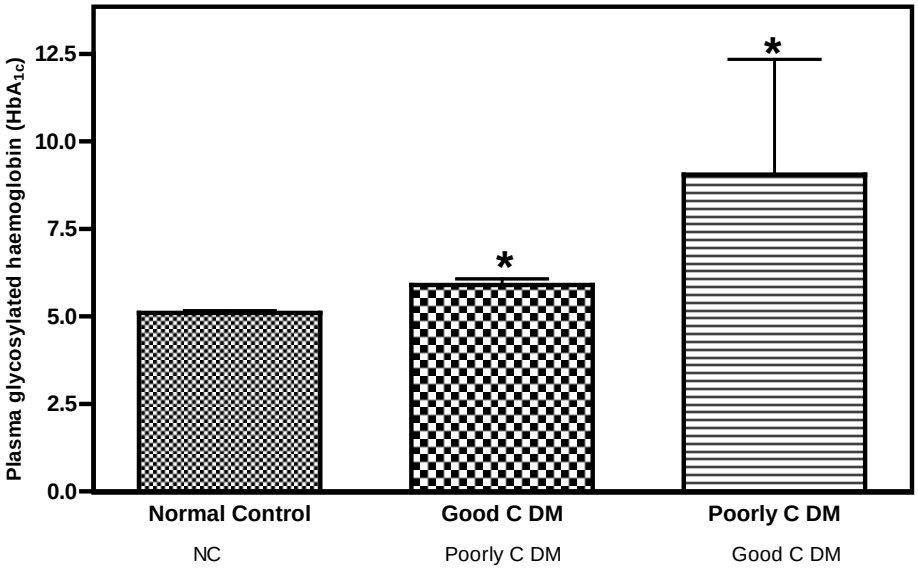

Figure 3: *Plasma glycohaemoglobin (%) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ± S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

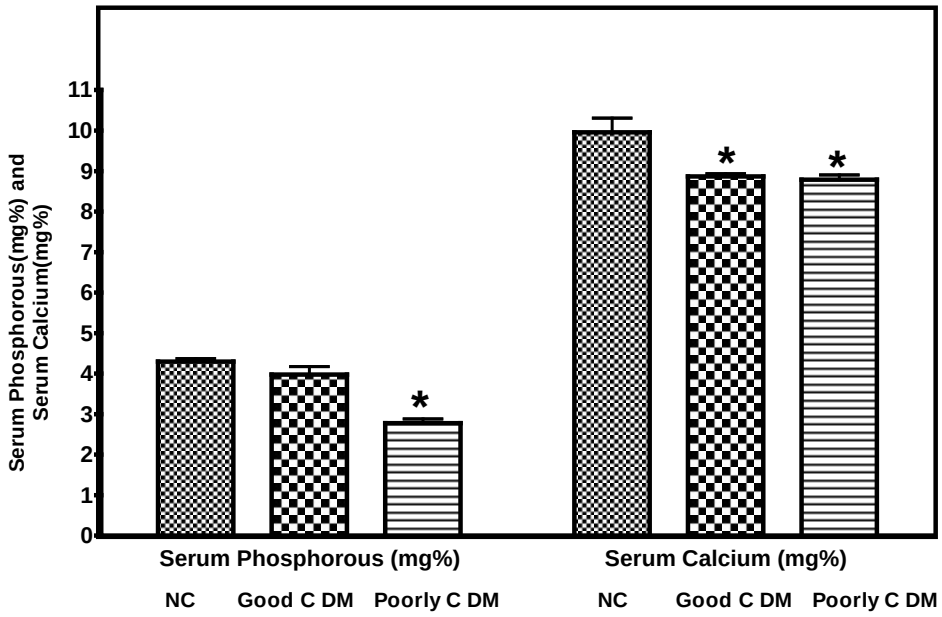

Figure 4: *Serum calcium (mg%0 and serum phosphorous (mg%) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ±S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

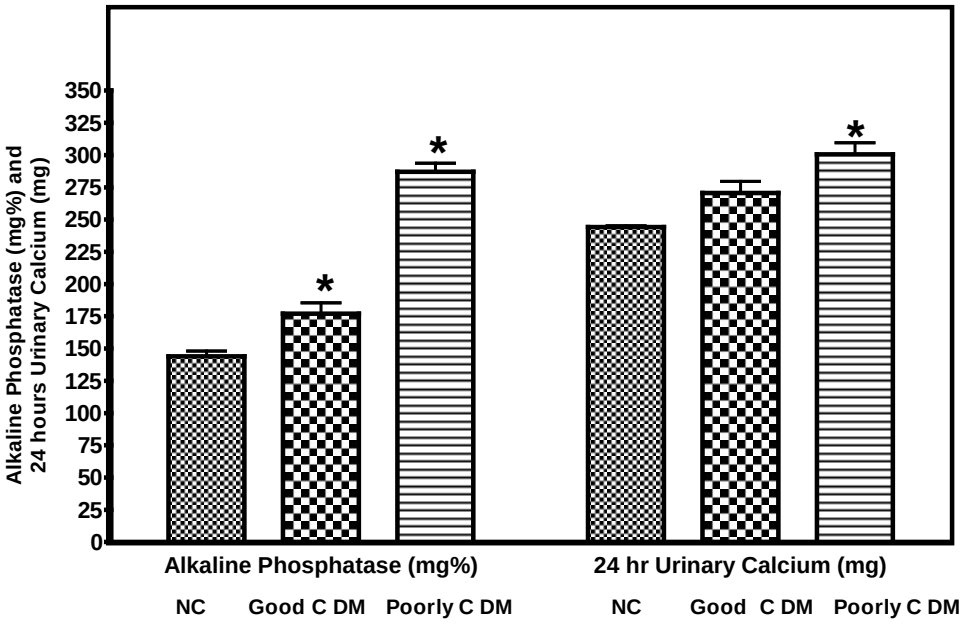

Figure 5: *Alkaline phosphatase (mg%) and 24 hours urinary calcium (mg) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ± S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

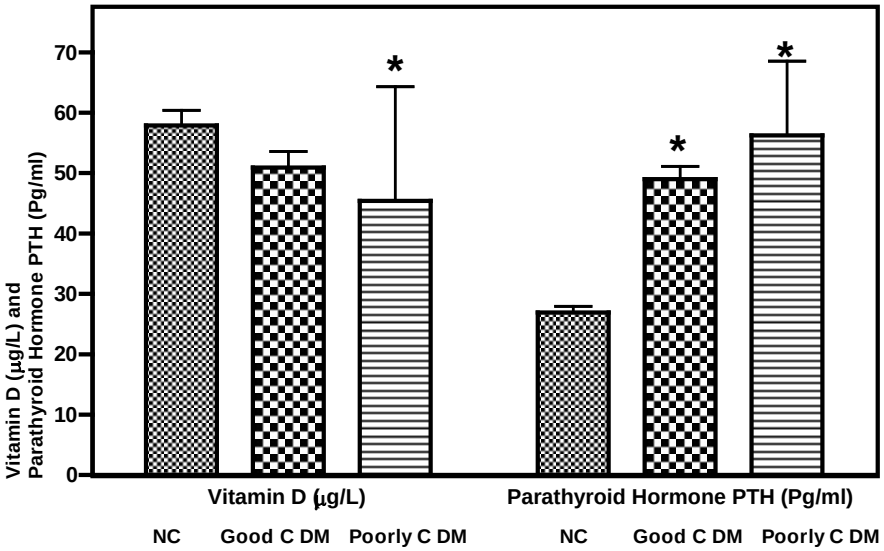

Figure 6: *Vitamin D (□g/ml) and parathyroid hormone (Pg/ml) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ± S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

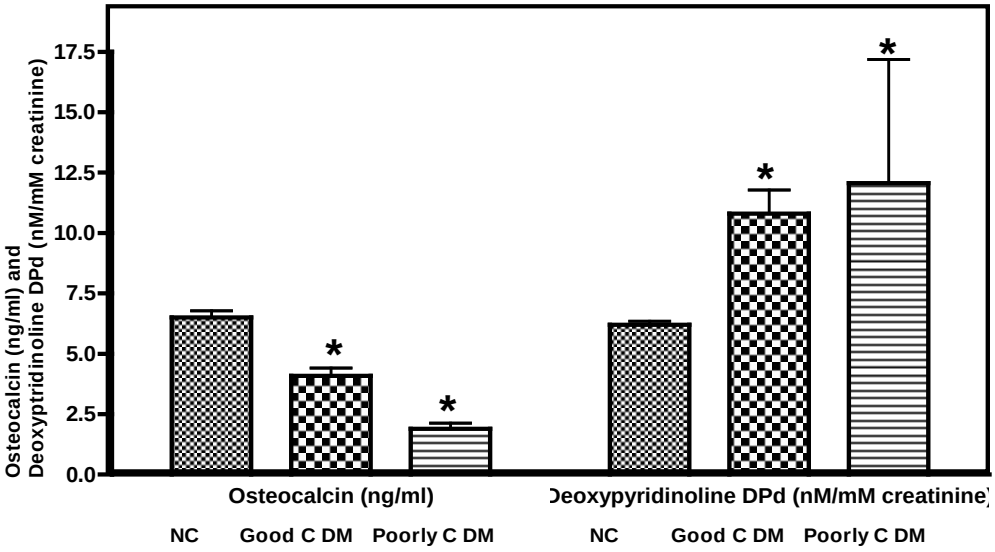

Figure 7: *Osteocalcin (ng/ml) and deoxypyridinoline (nM/mM creatinine) in the different studied groups; normal control, good controlled type 2 Diabetes mellitus and poorly controlled type 2 Diabetes mellitus patients using Students` Newman Keuls multiple comparison test at level of significance p<0.01.*
*Data were expressed as mean ± S.D. *Significant change difference compared with normal control group at p<0.05. NC=normal control, n= 30; Poorly C DM= poorly controlled type 2 Diabetes mellitus, n= 38; Good C DM= good controlled type 2 Diabetes mellitus, n= 22.*

Discussion

From results, it is plausible to assume that both the resorption and formation of bone are altered in premenopausal type 2 diabetes and these alterations are directly influenced by the degree of diabetic control.
Both diabetic groups were associated with an increase in Dpd, a marker of bone resorption and a decrease in OC, a marker of bone formation. Also, Dpd was positively correlated with glycemic indices. The decrease in OC among diabetic patients has been previously reported[9, 20, 21] and is interpreted to represent a reduction in bone formation. In contrast, other researchers found that bone ALP, another bone formation marker, tends to be elevated in diabetic patients[22, 23]. Bone alkaline phosphatase and osteocalcin are both synthesized by osteoblast and are currently considered the most sensitive markers to assess bone formation[23]. In this work, total alkaline phosphatase was done and not the specific bone one and it was found to be higher in all diabetic patients versus control group (**Figure 5**) with positive correlation with glycemic indices.
The detected elevation in ALP level could be explained by the prolonged exposure to PTH which eventually increases osteoblastic activity. While reduction of the osteocalcin level could result from inhibition of osteoblast function due to impaired insulin secretion and increase in insulin resistance leading to hyperglycemia[24].
Diabetes-associated hyperglycemia may modulate osteoblast gene expression, function and bone formation[25]. Sustained hyperglycemia alone causes suppression of osteoblast proliferation and its response to parathyroid hormone and 1 alpha, 25-dihydroxyvitamin D. Hyporesponse of osteoblast to 1 alpha, 25-dihydroxyvitamin D, was also confirmed in diabetic patients as reflected by a reduction in an incremental response of serum OC during 1, 25-dihydroxyvitamin D administration[26]. It is not known whether decreased OC is a predictive factor for fractures in diabetes,

but its levels have been found to return toward the normal range after glycemic control. Since OC is known to be glycosylated, it is also possible that the measurement would be affected by high glucose conditions[9]. Therefore, in poorly controlled diabetics, OC may not properly reflect bone formation. Such a possibility is corroborated by our observation that OC was not correlated with any other metabolic bone markers or with indices of glycemic control.

Elevated Dpd in diabetics could be explained by the fact that bone blood flow is increased in the distal limb of diabetic patients, which is believed to increase osteoclastic activity[27]. Also Endre and Robert[28] reported acute reduction in collagen synthesis and increase osteoclastic bone resorption in diabetic patients with a net increase in minerals released from bone into extracellular fluid. Elevated urinary Dpd in diabetics, especially those with poor metabolic control in whom the rate of bone resorption is increased, exposes growing diabetic patients to the risk of bone loss. In harmony with our results were those of others[7, 21]. On the other hand, Gerdhem and his co-workers[17], reported reduction in urinary Dpd level on comparing type 2 diabetes patients to control group, in spite of the reduction in osteocalcin level in those patients. This was attributed to the fat that only the bone formation phase was affected in diabetics, while the resorption phase remained unaltered.

The mechanism whereby bone turnover is affected by glycemic control status is not clear at the moment. Results show a significant increase in urinary calcium in both diabetic groups more significant in the uncontrolled group. Also, urinary Ca excretion was correlated with both the glycemic indices and bone resorption marker Dpd. Increased urinary calcium, phosphate, and magnesium have been found in diabetic patients with poor metabolic control, whose renal excretion rates correlated positively with the degree of hyperglycemia and glycosuria[5]. Hypercalciuria has been traced back both to the osmotic diuresis promoted by glycosuria and to renal hemodynamic changes induced by prostaglandin excess[18]. The increased renal leak appears to be associated with lower duodenal calcium absorption. It has been speculated that hyperphagia, although it determines higher calcium intake, may limit the efficacy of active calcium transport, inducing an overall decrease in intestinal absorption[4]. The reduced concentrations of the binding protein calbindin D-9K in the duodenal mucosa[29] could contribute to calcium malabsorption.

In type 2 diabetes patients, the mean serum calcium level was significantly lower than the control group, mean serum phosphorus level was also lower than the control group, yet the difference was statistically insignificant (**Figure 4**). These results agreed with others[30, 31]. The reduction in serum calcium level is most probably due to several factors: reduction in insulin level which impairs bone formation due to its stimulatory action on osteoblast proliferation, and impairment of calcium homeostasis, also hyperglycemia increase calcium and phosphorus excretion in urine which is proportional to the degree of glucosuria[1].

It is tempting to speculate from these data that renal hypercalciuria by osmotic diuresis caused stimulation of bone resorption caused by secondary hyperparathyroidism. PTH level was found to be higher in both diabetic groups than in control group especially in uncontrolled patients (figure 6). Other workers are in agreement with our results[32 - 34]. Physiologically, the reduced intestinal absorption, together with the increased urinary calcium excretion, should induce a compensatory increase of parathyroid hormone (PTH) secretion. Also, the reduction of serum-ionized calcium due to higher concentration of the complexed ion[35] should promote PTH secretion in these patients. On the other hand, some other studies[17, 36] reported no change in values of PTH in diabetics compared to non-diabetics. Other researchers reported a lower PTH level in type 2 diabetes patient than the control group[31, 37, 38] and they explained their findings by the inverse correlation noted between blood glucose and PTH suggesting

that hyperglycemia per se may have an inhibitory action on the synthesis and secretion of PTH. This is consistent with experimental studies in bovine parathyroid cell culture. This state of hypoparathyroidism has been reported to be correlated with the duration of diabetes and the degree of hyperglycemia[39].

The discrepant data of PTH are partly related to the different assays employed for its measurement, which can detect either active or inactive fragments of the hormone molecule. However, overall, parathyroid hormone secretion seems to be lower than expected for the homeostatic needs[40]. This state of 'functional hypoparathyroidism' has also been confirmed by dynamic challenge studies, such as during citrate-induced hypocalcemia[40], or hyperinsulinemic hypoglycemia[41], or following an oral glucose tolerance test[42]. Functional hypoparathyroidism has been related to magnesium deficiency[43] and has been considered responsible for the low bone turnover[44].

In agreement with other studies[17, 44] Vitamin D was found to be lower in both diabetic groups than in control group (more significant in uncontrolled patients; **Figure 6**). Several studies have investigated the possible role of vitamin D in the pathogenesis of skeletal involvement. It has been shown that the balance among the major vitamin D metabolites is altered. For instance, 24, 25-dihydroxyvitamin D levels are markedly reduced and not correlated with those of 25-hydroxyvitamin D, in contrast to that in normal subjects[45]. Others described variations of vitamin D metabolism included the decreased synthesis of vitamin D-binding protein by the liver, decreased renal 1α-hydroxylase activity, and reduced vitamin D-receptor concentrations. The latter could induce peripheral vitamin D resistance[46]. Calcitriol receptors have been demonstrated on islet cells of the pancreas, and vitamin D deficiency may alter insulin secretion. This could explain reports of a significant inverse correlation between glucose levels after oral glucose load and serum 25-hydroxyvitamin D concentrations in elderly men[47]. This finding is relevant, since circulating 25-hydroxyvitamin D levels are considered the best index of nutritional vitamin D status[48]. On the other hand, normal 25-hydroxyvitamin D levels were detected in some studies in diabetic patients[49]. Different results on vitamin D status may be due to several factors such as the assay employed, the cutoff value utilized for defining hypovitaminosis, and the physiological seasonal variations of vitamin synthesis[49].

The fact that the glycemic indices correlates with the bone resorption marker DPD, alkaline phosphatase and urinary calcium suggests that the better glycemic control would be associated with the lower bone loss. However, the presence of altered bone turnover in good controlled patients needs to be explained. Bone turnover is regulated by many local cytokines, cell-cell, and cell-matrix interactions as well as systemic hormones, and hyperglycemia may affect any of these local microenvironments that regulate bone turnover. McCarthy et al.[6] reported that advanced glycosylation endproducts (AGEs) stimulate production of interleukin-6, a bone resorbing cytokine, in human and mouse osteoblast-like cells in culture. Because the formation of AGEs is considered irreversible, this mechanism could partially explain the altered bone turnover in the presence of good glycemic control. Further studies are needed to clarify these issues.

Conclusion

Our data give evidence of altered bone minerals, vitamin D metabolism and increase bone turnover in patients with Type 2 diabetes mellitus. These alterations were already present in patients with good metabolic control. On the other hand, the greatest alteration was observed in poorly controlled patients. Therefore, we can conclude that loss of skeletal tissue in diabetes could reflect the underlying disease with strong influence of hyperglycemia. Therefore, the regimens having stimulatory effect on bone turnover, such as intermittent PTH therapy and vitamin D, are

recommended to treat diabetic osteopenia, besides improvement of diabetic control state.

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