

IFCC Professional Scientific Exchange Programme (PSEP) Report:

BIOCHEMICAL MARKERS OF BONE TURNOVER ON WELL-CONTROLLED POSTMENOPAUSAL WOMEN WITH TYPE 2 DIABETES MELLITUS.

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INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous group of disorders connected by raised plasma glucose concentration and disturbance of carbohydrate and lipid metabolism. This syndrome is characterized by hyperglycemia, which is caused by an absolute or relative lack of insulin and/or diminished insulin action on target tissues (insulin resistance) and occurrence of the late complications (including diabetic microangiopathy and macroangiopathy) (1).

The World Health Organization (WHO) has prepared a long list of classification schemes of DM; to date the most widely accepted is Type 1 DM, Type 2 DM, other specific types of DM, and gestational DM (2). There is some controversy about the effects of diabetes mellitus on bone remodeling and bone mineral density (BMD) (3,4).

Osteopenia is recognized in diabetic patients but the complications of diabetes mellitus and bone are often neglected. However, a reduced bone mass and an overall twofold increase in fracture rate has been shown to occur in Type 1 diabetes mellitus (5). The situation in Type 2 diabetes mellitus is less clear-cut, with reports of increased, decreased or unaltered bone mass reflecting the underlying heterogeneity of Type 2 diabetes (6).

The question whether diabetes mellitus is a risk factor for osteoporosis and/or osteoporosis is a complication of diabetes mellitus remains to be answered.

Osteoporosis is a group of skeletal disorders characterized by reduction in bone mass per unit of bone volume. A World Health Organization (WHO) panel has proposed that women with bone density values more than 2.5 SD below the young adult mean value should be considered as osteoporotic. If they also have one or more fragility fractures, they would be classified as suffering from severe, or established, osteoporosis.

Women with bone density values between 1 and 2.5 SD below the young adult mean values would be classified as osteopenic (7). This may occur if the rate of bone resorption exceeds that of bone formation, implying an uncoupling of the phases of bone remodeling (8).

The incidence of osteoporosis increases dramatically with age, becoming widely prevalent in the elderly, in whom it has become a major public health problem (9). However, the precise mechanism of the disorder remains unresolved.

Deficiency of the female hormone 17- β estradiol, caused either by the menopause or removal of ovaries, results in accelerated bone loss and as a consequence, bone mass declines after menopause. This decline is a major factor contributing to the high rate of disabling bone fractures in postmenopausal women (10).

Primary osteoporosis is by far the most common metabolic disorder of the skeleton (11).

Studies using bone markers suggest that there is accelerated bone remodelling at menopause and that bone formation may increase overall, but that the rate is inadequate to replace the bone lost by resorption (12).

TABLE I. BIOCHEMICAL MARKERS OF BONE TURN OVER

Formation markers	
Serum	Urine
Propeptides of type I collagen	
C-Propeptides	
N-Propeptides.	
Osteocalcin	
Alkaline Phosphatase	
Total activity	
Bone specific enzyme	
Resorption markers	
Tartrate -resistant acid Phosphatase	Hydroxyproline
Hydroxy-lysine glycoside	
Collagen Cross-links	Collagen Cross-links
Total pyridinolines	Total pyridinolines
Pyr and or /Dpd	Pyr and or /Dpd
Free pyridinolines	Free pyridinolines
Pyr and/or Dpd	Dyr and/or Dpd
Cross-linked N-and C-telopeptides	Cross-linked N-and C-telopeptides.

BONE METABOLISM

Bone is constantly undergoing a metabolic process called remodelling. This includes a degradation process or bone resorption, which is mediated by the action of osteoclasts. Next is a building process or bone formation, which is mediated by the action of osteoblasts. Remodeling is required for the maintenance and overall health of bone and is tightly coupled where resorption and formation are in balance. In abnormal states of bone metabolism this process becomes uncoupled and, when resorption exceeds formation, this results in a net loss of bone. The analysis and measurement of specific degradation products of bone matrix provides analytical data of the rate of bone metabolism (13).

Type I collagen, a triple helical protein, is the most abundant collagen type in the body. It accounts for more than 90% of organic matrix of bone. It is derived from a larger protein, type I procollagen, which has propeptide extensions at both ends of the molecule. These N- and C propeptides are removed extracellularly by specific proteinases before the collagen molecules are assembled into fibers. The sequence removed from the carboxyterminal end of the molecule, known as the carboxyterminal propeptide of type I procollagen (CICP), can be found in blood with levels reflecting the synthesis of type I collagen (14).

TABLE 2. Age, Body Mass Index (BMI), Glucose and Glicated Hemoglobin (HbA1c) in both controls (1) and well controlled DM Type 2 postmenopausal women (2). (Mean \pm SD))

GROUPS	AGE	BMI	GLU	HbA1c
	Years	Kg/m ²	mg/dL	%
1				
N=6	58 \pm 5.7 a	25.7 \pm 3.5 b	98.3 \pm 3.5	*
2				
N=7	57.7 \pm 3.1	31.4 \pm 3.4	168.2 \pm 72	6.9 \pm 1.2

^a p \leq 0.87; ^b p \leq 0.06 ; * data not available

TABLE 3. Serum and urine concentration of calcium-phosphorous metabolism in controls (1) and well controlled DM Type 2 postmenopausal women (2). (Mean \pm SD)

GROUPS	Ca	P	Ca/Cr	RTF	Intact PTH
1	mg/dL	mg/dL	%		pg/mL
N=6	10.1 \pm 0.3	3.6 \pm 0.2	0.13 \pm 0.05 a	87 \pm 9.5 b	25.1 \pm 6.9 c
2					
N=7	10.3 \pm 0.6	3.7 \pm 0.4	0.11 \pm 0.04	80 \pm 4.2	32.1 \pm 8.1

^a p \leq 0.54; ^b \leq 0.26; ^c \leq 0.38.

TABLE 4. Serum and urine concentration of the biochemical markers of bone turnover in control (1) and well controlled DM Type 2 postmenopausal women (2) (Mean \pm SD).

GROUPS	BAP	CICP	Dpd	NTx
1	U/L	ng/mL	nM/mMcr	nMBCE/mMcr
N=6	21.9 \pm 4.5 a	114.7 \pm 30.5 b	6.3 \pm 1.0 c	37.6 \pm 14.3 d
2				
N=7	21.4 \pm 2.3	94.8 \pm 31	4.5 \pm 1.2	27.3 \pm 7.2

^a p \leq 0.81; ^b p \leq 0.45; ^c p \leq 0.05 ; ^d p \leq 0.25

Type I collagen of bone is crosslinked at the N-terminal and C-terminal ends of the molecule by specific molecules which provides rigidity and strength. Crosslinks of mature type I collagen in bone are the pyridinium crosslinks, pyridinoline (Pyd) and deoxypyridinoline (Dpd).

Biochemical assays for monitoring bone turnover rely on the measurement, in serum or urine, of enzymes or matrix proteins synthesized by osteoblasts or osteoclasts that spill over into body fluids, or of osteoclast-generated degradation product of the bone matrix itself (15). These markers can be used for research (16) and for clinical purpose (17).

In addition, calcium regulation hormones mediate calcium balance; one of them is the parathyroid hormone (PTH) (18). In healthy adults, a decrease in plasma ionized calcium concentration leads to an immediate increase of PTH secretion from parathyroid gland (19). PTH acts simultaneously on bone and kidney causing an increase in calcium through stimulation of osteocytic osteolysis, and an increase reabsorption in distal parts of the nephron. The indirect and long-term effect of increased PTH concentration is a stimulation of dietary calcium reabsorption in the gut through a PTH-induced increase in renal 1,25-dihydroxy-cholecalciferol (18). A long-term effect of PTH is a relative increase in the osteoclastic over osteoblastic activity.

My training was focused in learning the methodologies for the determinations of the biochemical markers of bone turnover as described in this report:

BONE FORMATION MARKERS:

Bone alkaline phosphatase (BAP), a tetrameric glycoprotein, is a prominent product of osteoblast and osteoblast precursors (20). The function of BAP has not been fully elucidated, though its role in skeletal mineralization has been confirmed. The measure of bone formation provided by BAP is indirect, depending presumably on a spillover over of excess or spent enzyme from active osteoblast and probably also pre-osteoblast, lining cells, and perhaps osteocytes (20,21).

Bone specific alkaline phosphatase (BAP) immunoassay is a microtitre strip format utilizing a monoclonal anti-BAP antibody coated on the strip to capture BAP in the sample. The enzyme activity of the captured BAP is detected spectrophotometrically with p-nitrophenyl phosphate (pNPP) substrate (20).

The carboxy-terminal propeptide of type I procollagen (CICP) is known as the sequence removed from the carboxyterminal end of the type I procollagen molecule, and its levels reflects the synthesis of type I collagen (22).

C-terminal propeptide of type I collagen in serum is a sandwich enzyme immunoassay in a microtitre plate utilizing a monoclonal anti-CICP antibody coated on the plate, a rabbit anti-CICP antiserum, a goat anti-rabbit alkaline phosphatase, and a pNPP substrate to quantify CICP in human serum (14).

BONE RESORPTION MARKERS:

Deoxypyridinoline (Dpd) is formed by the enzymatic action of lysine oxidase on the amino acid lysine. Dpd is released into the circulation during the bone resorption process (15,23). Dpd is excreted unmetabolized in urine and is unaffected by diet (15), making it suitable for assessing resorption. Dpd assay is a competitive immunoassay in a microtitre stripwell format utilizing a monoclonal anti-Dpd antibody coated on the strip. The Dpd concentration in the specimen is determined spectrophotometrically and calculated from a standard calibration curve. Assay values are corrected for urinary dilution by urinary creatinine analysis (24).

Cross-linked N-telopeptides of type I collagen (NTx) molecule is a specific biochemical marker of human bone resorption due to the unique amino acid sequences and orientation of the cross linked- $\alpha 2$ (I) N-telopeptide (15). Generation of the NTx molecule is mediated by osteoclast on bone, and is found in the urine as stable end-product of degradation. NTx is an immunoassay utilizing a monoclonal anti NTx coated on the strip to capture NTx in the sample. NTx concentration in the specimen is determined spectrophotometrically with 3,3', 5'-tetramethylbenzidine in dimethylsulfoxide, and calculated from a standard calibration curve. Assay values are corrected for urinary dilution by urinary creatinine analysis (25).

MATERIALS AND METHODS

Our aim was to determine the biochemical markers of bone turnover in a group of healthy postmenopausal women compared to a group of diabetic type 2 postmenopausal women without showing any symptoms of osteoporosis at the time of the clinical evaluation.

SUBJECTS: We studied 16 postmenopausal women who were divided by groups as follows:

- a). Group 1 included 7 clinically healthy postmenopausal women with less than 75% risk factors of osteoporosis evaluated by a questionnaire (11). From this group, one showed high concentrations of all biochemical markers of bone turnover. The results were outside the reference range and therefore she was excluded from group 1 for the final analysis.
- b). Group 2 included 9 postmenopausal women with type 2 diabetes. From this group, 7 of them were well-controlled type 2 diabetic postmenopausal women and 2 of them were poorly controlled. We excluded the two poorly controlled type 2 diabetic postmenopausal women from group 2 for the final analysis. Both subjects showed very high concentration of BAP, Dpd and NTx. All participants were voluntary subjects from the diabetic clinics at the Maciel Hospital and at the Military Hospital in Montevideo, Uruguay. The diabetic women who met all eligibility criteria were selected from the laboratory database and invited to participate in the study from both institutions. Control subjects were recruited from the Gynecology clinic at the Military Hospital. The Military Hospital Ethics and Scientific

Committees and the Diabetes Clinic at the Hospital Maciel approved the study. The subjects were seen at the hospital laboratory and informed consent was obtained from all of them.

Exclusion criteria were renal impairment with a serum creatinine >1.40 mg/dL and the presence of microalbuminuria. They also could not be under steroid replacement or thyroid hormone therapy. It was also requested that they have good metabolic control with a % HbA1c 8.5 or less, at least 4 years of being diagnosed as Type 2 diabetic, and showing no alteration of the calcium-phosphorous metabolism (Table 2). All subjects were Caucasian and physically active, with an average age of 55.5 (± 4.0) years old, 9.2 (± 6.4) years after menopause and BMI of 25.7 \pm 3.5 (Group 1) and 31.4 \pm 3.4 (Group 2).

METHODS: We collected a 4-hour urine sample (5:00am - 9:00am) and a fasting serum sample (8:30 am) on the day of the urine collection. Serum and urine creatinine, calcium, phosphorous and serum glucose, were determined on a Hitachi 750 (Roche Diagnostics, Uruguay). We also determined %HbA1c by IMx (Abbott, Uruguay) and microalbuminuria by nephelometry (Beckman Array 360 System, Relab, Uruguay) for all diabetic subjects. These tests were run at the Laboratory of the Military and Maciel Hospital in Montevideo, Uruguay.

Bone turnover was assessed by measuring the following bone markers: in serum, collagen type I C-terminal propeptide (CICP) and bone specific alkaline phosphatase (BAP) and in urine, deoxypyridinoline (Dpd) crosslink [Quidel Corp (Metra™) Santa Clara, CA, USA] and cross-linked N-telopeptides of type I collagen (NTx) (Ostex International, Inc. Seattle, WA, USA). Intact PTH was measured by two-site immunoradiometric assay (IRMA) from Nichols Institute Diagnostic (San Juan Capistrano, CA, USA). Standard curves, controls, and unknowns were run in duplicate for all assays. The average intra-assay % CV was between 8.0-10.0. Bone turnover assays and PTH were run at the GCRC Biochemistry Core Lab at Cincinnati Children's Hospital Medical Center in Cincinnati, OH, USA.

STATISTICAL ANALYSIS

Results are expressed as mean \pm SD of the data. The data were analyzed by Student's t-test. *P* values less than 0.05 were considered statistically significant.

RESULTS

The media for both groups, controls (Group 1) and well-controlled DM Type 2 postmenopausal women (Group 2) was calculated and it is shown in Table 2, 3 and 4.

SUMMARY

All subjects had normal GFR that correlated to their ages (data not shown). Both groups were homogenous by age (group 1: 58 \pm 5.7, group 2: 57.7 \pm 3.1; *P* < 0.87) and BMI (group 1: 25.7 \pm 3.5, group 2: 31.4 \pm 3.4; *P* < 0.06).

No significant differences were observed between groups for Ca/Cr ratio, % RTF, PTH, and biochemical bone markers (BAP, CICP, NTx). On the other hand, significant difference was found between group 1 and group 2 for urinary Dpd concentration, which was higher in group 1 than in group 2 with a *p* < 0.05, but inside the adult reference range. Therefore, we assumed that from a metabolic point of view there is no evidence of greater bone resorption in postmenopausal women with well-controlled type-2 diabetes compared to the group of healthy postmenopausal women. However we found in the diabetic group of subjects, there were two of them showing concentrations of

HbA1c ≥ 9.8 %, Ca/Cr ≥ 0.50 , Dpd, NTx and BAP two fold or greater than reference ranges; CICP was inside the reference range. For these subjects PTH values were inside the reference range but RTF was lower (RTF < 40%) in both subjects.

These preliminary findings suggested that the well-controlled group of diabetic postmenopausal women do not show an increase of biochemical bone markers when compared with healthy postmenopausal women. These findings are in accordance with those of Isaia GC *et al.* (26) who found similar results in control vs. well-controlled diabetic postmenopausal women. However if the diabetic subjects are poorly-controlled, higher bone turnover could be found. Okazaki R *et al.*, (27) reported that glucose metabolism might influence bone turnover and the improvement of glucose metabolism could decrease the biochemical markers, reflecting a decrease of bone turnover. Therefore, as other investigators have suggested it, the mechanism whereby bone turnover could be affected by glycemic control should be elucidated (28,29,30,31,32).

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