

## EARLY MODIFICATIONS OF BIOCHEMICAL MARKERS OF BONE METABOLISM IN SPINAL CORD INJURY PATIENTS A PRELIMINARY STUDY

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**ABSTRACT.** Spinal cord injury is associated with the development of a rapid and severe osteoporosis which might reflect uncoupling between bone formation and resorption. A prospective study was made in 6 spinal cord injury patients followed up to 2-3 months after onset with various markers of a) bone formation: osteocalcin and C-terminal peptide of type I procollagen, b) bone resorption: pyridinolines and C-terminal telopeptide of type I collagen, c) connective tissue metabolism: amino-terminal propeptide of type III collagen (PIIINP). Preliminary results show that early after onset, bone formation was depressed as compared to dramatically increased bone resorption. Low bone formation rate lasted two weeks before it began to raise, while bone resorption showed a continuous tendency to increase. The dramatic increase in PIIINP levels might represent some attempt of bone to repair. This paper describes the evolution of various biochemical markers of bone and connective tissue metabolism after onset of paralysis and critically reviews the use of those markers in patients with spinal cord injury.

*Key words:* spinal cord injury; bone metabolism; osteocalcin; PICP; PIIINP; pyridinium crosslinks of collagen.

### INTRODUCTION

Bone tissue is constantly renewed by two opposite but complementary activities, the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts. Bone mass results from the equilibrium between these two activities and, therefore, it is very important to quantitate them precisely in the various conditions affecting bone and generally leading to

osteoporosis. A 50% decrease in bone mineral content measured by dual photon absorptiometry over 3 years was observed in the paralyzed limbs of paraplegics (4), a study which confirmed previous ones (13, 16, 21). Total-body bone mass was recently found to be decreased by ~ 12% in paraplegia (16). In fact, all skeletal sites loose bone, except for the skull (14). Bone loss mainly occurs during the first 6 months after onset of paralysis and stabilizes between 12-16 months to reach ~ 2/3 of the original bone mass, near the fracture threshold. As many metabolic disturbances are totally non specific, recent studies have begun to focus on new biochemical markers of bone metabolism. Osteocalcin levels were found in the normal range 1 month after injury but continuously increased thereafter during the 6 months of follow-up (25); Zanone et al. found a peak at month 7 after onset (40). Tartrate-resistant acid phosphatase, a marker of osteoclastic activity, was situated in the upper range of the control values 1 month after injury and decreased thereafter (2). As new matrix components released from bone into the circulation became recently available as biochemical markers, the authors proposed to measure them prospectively in spinal cord injury patients.

### PATIENTS AND METHODS

The levels of various biochemical markers of bone metabolism were determined in 6 spinal cord injury male patients (Group A) aged  $36.2 \pm 2.2$  (m  $\pm$  1 sd). Patient V died shortly after inclusion. Two patients were followed for 2 months, and 3 of them for 3 months after onset of paralysis. In addition, 6 normal healthy male subjects (Group B) aged  $34.2 \pm 3.7$  were enrolled as controls. All patients included were free to participate and had suffered a recent acute traumatic spinal cord injury with complete or incomplete para or tetraplegia (Table I). All patients with severe concomitant metabolic diseases, history of drug or alcohol abuse

Table I. Spinal cord injury patients' characteristics

Patient No	Age	Sex	Level	Degree of completeness
I	37	M	L1	Incomplete
II	33	M	Th6	Complete
III	38	M	Th5	Complete
IV	39	M	C6-C7	Complete
V	34	M	Th10	Complete
VI	35	M	L1	Incomplete

or patients who had received drugs effective on bone within 3 months before inclusion in the study were excluded. The study was performed in accordance with the Declaration of Helsinki, 1981, and followed the Ethical Guidelines of the Swiss Academy of Medical Sciences.

#### Biological samples

Fasting blood samples (10 ml) were taken from each patient and subject of Groups A and B at entry, then at month 1, 2 and 3 after onset. The samples were immediately aliquoted and stored at  $-70^{\circ}\text{C}$  until analysis. Two-hour fasting urine samples after voiding were collected from each patient and subjects of Groups A and B at entry, then at month 1, 2 and 3 after onset. A total of 20 ml of urine was aliquoted and added with a 0.1% boric acid solution (v/v) to prevent bacterial growth then stored at  $-70^{\circ}\text{C}$  until analysis.

#### Analytical assays

Human osteocalcin was determined in serum by IRMA using a new commercially available kit (ELSA-OSTEO<sup>®</sup>, CIS Bio International) with Mabs which recognize the intact [1-49] human molecule and its main [1-43] fragment (15). Carboxy-terminal peptide of type I procollagen (PICP) was measured in serum by a commercially available specific RIA (PICP RIA<sup>®</sup>, Orion Diagnostica) (24). Amino-terminal peptide of type III procollagen (PIIINP) was measured in serum by a commercially available IRMA kit (PIIINP RIA-gnost<sup>®</sup>, Behringwerke, Hoechst Diagnostica) (29). Pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen (ICTP) was measured in serum using a new commercially available specific RIA (ICTP RIA<sup>®</sup>, Orion Diagnostica) (30). The four markers levels in serum were expressed in  $\mu\text{g/l}$ . Total hydroxyproline (HOP) was measured in urine after acid hydrolysis according to the method of Kirivikko et al. (22) and expressed in  $\mu\text{mol/mmol}$  creatinine. Pyridinoline (Pyr) and deoxy-pyridinoline (D-Pyr) were measured by post-column fluorescence detection after reverse-phase high precision liquid chromatography (RP-HPLC) of a cellulose-bound extract of acid-hydrolysed urine according to the method described by Eyre (11) and modified by Uebelhart et al. (35). Pyr and D-Pyr levels were expressed in  $\mu\text{mol/mol}$  creatinine. Urinary creatinine was determined by the modified Jaffe analysis and expressed in  $\text{mol/l}$ .

## RESULTS

#### Markers of bone resorption

Pyr/creat. values were dramatically increased as compared with controls ( $51.4 \pm 21.9 \mu\text{mol/mol}$ ) beginning

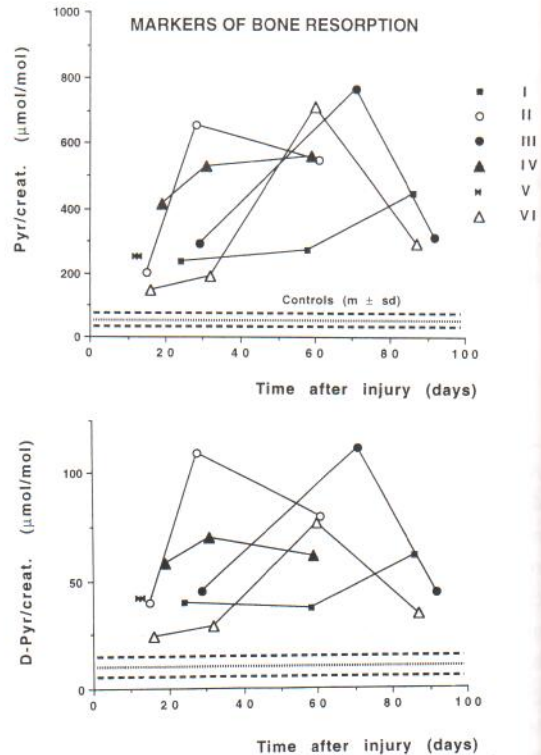


Fig. 1. Upper panel: Two-hour fasting urinary levels of pyridinoline corrected for creatinine (Pyr/creat.), and lower panel: Deoxy-pyridinoline corrected for creatinine (D-Pyr/creat.) in spinal cord injury patients from inclusion up to 2-3 months after onset. The horizontal lines represent the range of respectively fasting Pyr/creat. and D-Pyr/creat. of age and sex-matched controls (mean value given as a pointed line and  $\pm 1$  sd as dashed lines).

at week 1 after onset and remained high during the whole follow-up period. D-Pyr/creat. values also showed a very significant increase from week 1 after onset up to 3 months of follow-up as compared to controls ( $10.0 \pm 4.5 \mu\text{mol/mol}$ ) (Fig. 1). HOP/creat. levels were elevated in some patients after week 3 as compared with reference values ( $31.0 \pm 17.1 \mu\text{mol/mmol}$ ), but great individual variations were found. ICTP levels were dramatically increased as compared with controls ( $3.4 \pm 0.74 \mu\text{g/l}$ ) from week 1 after onset and showed further increase up to month 3 (Fig. 2).

#### Markers of bone formation

Osteocalcin levels were low or depressed at week 1 after onset as compared with controls ( $21.5 \pm 10.3 \text{ ng/ml}$ ) and then increased regularly up to month 3 of follow-up. PICP levels were located in the normal

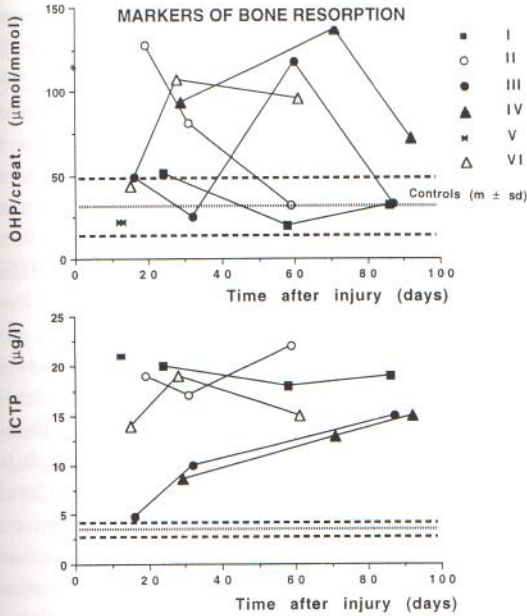


Fig. 2. Upper panel: Two-hour fasting urinary levels of hydroxyproline corrected for creatinine (OHP/creat.), and lower panel: Serum levels of the cross-linked carboxy-terminal telopeptide of type I collagen (ICTP) in spinal cord injury patients from inclusion up to 2-3 months after onset. The horizontal lines represent the range of respectively fasting urinary OHP/creat. and serum ICTP levels of age and sex-matched controls (mean value given as a pointed line and  $\pm$  1 sd as dashed lines).

range ( $123.8 \pm 40.9$  ng/ml) during the whole follow-up period except for 1 patient who showed increased levels 2 months after onset (Fig. 3).

*Markers of connective tissue remodelling*

PIIINP levels were dramatically increased as early as week 2 after onset as compared with controls ( $0.78 \pm 0.17$  ng/ml) and continued to increase regularly up to 3 months of follow-up (Fig. 4).

**DISCUSSION**

Total HOP is a widely used measurement of bone collagen degradation (28) but has a great number of limitations. Excreted HOP represents only 10% of that produced by tissue catabolism, and many other connective tissues and the diet can change the proportion of HOP which is metabolized. Urinary HOP is generally measured by a colorimetric reaction, but a major concern in this method is the interference by

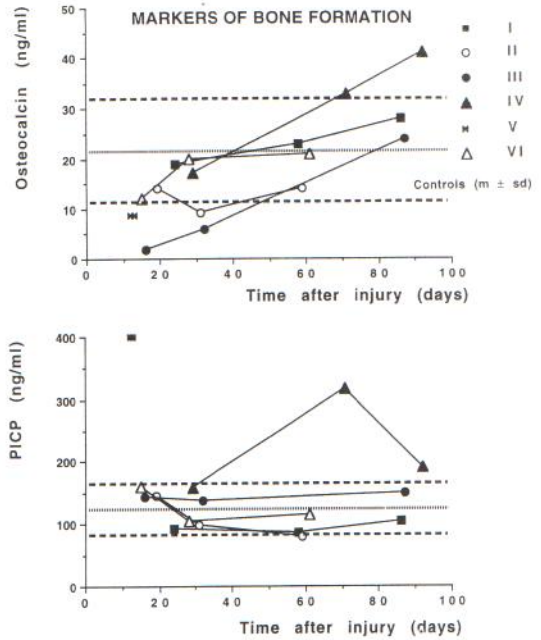


Fig. 3. Upper panel: Serum levels of osteocalcin (OC), and lower panel: Serum levels of the carboxy-terminal peptide of type I procollagen (PICP) in spinal cord injury patients from inclusion up to two or three months after onset. The horizontal lines represent the range of respectively serum OC and serum PICP levels of age and sex-matched controls (mean value given as a pointed line and  $\pm$  1 sd as dashed lines).

urinary molecules with the measured chromophore (22). To measure bone collagen degradation it is suggested to determine HOP in a 2-hour fasting urine sample corrected by creatinine. The sensitivity of the method can be improved by using High Pressure Liquid Chromatography (HPLC) procedures,

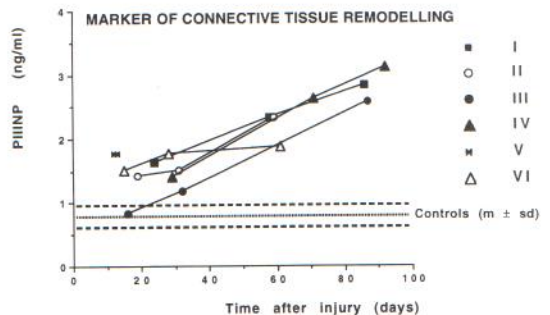


Fig. 4. Serum levels of the amino-terminal peptide of type III procollagen (PIIINP) in spinal cord injury patients from inclusion up to two or three months after onset. The horizontal lines represent the range of serum PIIINP levels of age and sex-matched controls (mean value given as a pointed line and  $\pm$  1 sd as dashed lines).

but its clinical application is limited (6;34). Osteocalcin, or Bone Gla Protein, a marker of bone formation is a small protein of 5,800 Kd and makes up to 20% of bovine and 10% of human non collagenous proteins of bone (26). Its biological function has not yet been fully determined, but it appears to be unique to bone and to be produced by the osteoblasts (23). Serum osteocalcin is an index of bone turnover when bone formation and resorption are coupled and a specific marker of bone formation when the two processes are uncoupled (27). Osteocalcin levels are significantly correlated with histomorphometric bone formation indexes measured on iliac crest biopsies (5). As antibodies directed against bovine osteocalcin cross-reacted with the human moiety, most RIA systems were so far developed with bovine osteocalcin as a tracer, standard and immunogen. Recently, a new immunoradiometric assay (hIRMA) based on monoclonal antibodies raised against human osteocalcin has been developed (15) and seems to be more sensitive to detect variations in bone turnover. The assay recognizes human intact osteocalcin as well as a large N-terminal fragment, both released during the synthesis of new bone matrix by osteoblasts. Used in our study, this human assay could show significant variations in serum from paraplegics.

Osteocalcin can be considered so far as the most powerful marker of bone formation and turnover and its clinical use should be rapidly extended. The carboxy-terminal peptide of type I procollagen (PICP) is a large globular glycoprotein of 100,000 daltons Mw which is cleaved during collagen biosynthesis by specific propeptidases. PICP can be measured in serum by a specific RIA (24). The biochemical background and clinical application of PICP has been reviewed recently (8, 18, 31), but no data were available so far in paralyzed patients. Our data did not show any significant increase in serum PICP and strongly suggest that the neo-synthesis of type I collagenous matrix might be delayed after paralysis eventually due to the 3-month remodelling time of the bone multicellular unit and to the half-life of type I collagen which is  $\sim 55$  days.

To illustrate the relevance of our data, it was recently showed that in the periosteum isolated from tibiae and femurs of growing rats submitted to unilateral sciatic neurectomy a significant decrease in mRNA levels for osteocalcin, alkaline phosphatase and eventually the prepro-alpha I subunit of type I collagen occurred after 7 and 14 days (37). Interestingly,

based upon these preliminary results, markers of bone formation were not influenced by the level and/or the degree of completeness of the spinal cord injury. The amino-terminal peptide of type III procollagen (PIIINP) is a glycoprotein of 42,000 daltons Mw in human which can be measured in serum by different assays (29). Type III collagen is a major component of interstitial tissues; it accounts for 5% of adult bone collagens, and significantly more in fetal bone. PIIINP has initially been proposed as an indicator of liver fibrosis degree. Interestingly, prospective studies performed in systemic sclerosis (19) and in Paget's disease of bone (33, 38) have demonstrated that PIIINP levels correlated with disease activity and collagen synthesis.

As no previous data on PIIINP levels in paraplegia are available, our data showing increased serum levels are potentially very important. Based upon our current knowledge of type III collagen function, PIIINP might be a sensitive marker to monitor some reparative response of bone or surrounding tissues after paralysis. Further data are of course necessary to confirm this hypothesis. The extracellular matrix of bone tissue is stabilized by the formation of covalent crosslinks between adjacent collagen molecules. Two mature crosslinks of the 3-hydroxypyridinium family have been identified by their natural fluorescent properties in most connective tissues (11). Pyridinoline (Pyr), also known as hydroxylysylpyridinoline, is the major mature component present in all of these tissues, since deoxy-pyridinone (D-Pyr), also known as lysylpyridinoline, is present mainly in bone tissue and dentine (9). The age-related changes in Pyr and D-Pyr content of human bone indicate that a maximum is reached by 10-15 years of age, then stays essentially the same range throughout adult life (10). Pyr and D-Pyr are excreted in urine as free and peptide-bound forms and can be measured by fluorescence detection after RP-HPLC of a cellulose-bound extract of hydrolyzed urine (11) or by an ELISA procedure which detects free Pyr or D-Pyr in urine (available from Metra Systems Inc., Palo Alto, CA, USA). Urinary levels of both compounds are elevated during childhood and adolescence, then decrease and stay rather constant throughout life (1). Increased urinary levels of Pyr and D-Pyr have been found in various metabolic bone diseases and osteoporosis (12, 32, 35, 36).

Urinary excretion of pyridinolines correlates with histomorphometric parameters of bone resorption measured on iliac bone biopsies (7). Pyr and D-Pyr

are more sensitive markers of bone resorption than OHP as demonstrated in Paget's disease of bone and primary hyperparathyroidism (3, 35). Due partly to their low Mw, these compounds cannot be detected easily in serum samples, but a recent assay using narrow-bore ion-paired RP-HPLC was able to measure as low as 28 fmol Pyr and 58 fmol D-Pyr (20).

The recent development of an ELISA directed against the cross-linked amino-terminal telopeptide of type I collagen (NCTP) and a RIA directed against the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) further increases the sensitivity and the specificity of the assay (17, 30). No such data are available so far in humans but our data clearly show that both Pyr and D-Pyr measured in 2-hour fasting urine as well as ICTP measured in serum were dramatically increased in spinal cord injury patients early after onset and continued to increase thereafter.

Importantly, no correlation could be found in this study between the level and/or the degree of completeness of spinal cord injury and the level of markers of bone resorption. A previous study measuring crosslinks of collagen in primates restrained in a semi-recumbent position demonstrated that mature crosslink levels remained constant as compared with an increase in the reducible crosslinks with time (39). The difference from our results may be mostly accounted for by the absence of paralysis, as compared with our spinal cord injury patients.

So far, urinary Pyr and D-Pyr and serum ICTP can be considered as the most sensitive and specific markers of bone resorption, and since immunoassays are now commercially available, their measurement should represent a very attractive alternative to the poorly sensitive and non specific HOP determination.

## CONCLUSIONS

There is clearly a crucial need of sensitive and specific markers of bone turnover which could help to discriminate the patients at risk of developing osteoporosis and help orienting new therapeutical trials intended to correct for the musculoskeletal deleterious effects of disuse. Most sensitive and specific markers of bone resorption are pyridinium crosslinks of collagen measured in urine and/or serum. Most sensitive markers of bone formation are osteocalcin and amino-terminal peptide of type I procollagen. Since commercial kits become available, a more

generalized use of these non invasive tools to assess bone metabolism should be triggered.

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## REFERENCES

1. Beardsworth, L. J., Eyre, D. R. & Dickson, I. R.: Changes with age in the urinary excretion of lysyl- and hydroxylslypyridinoline, two new markers of bone collagen turnover. *J Bone Minet Res* 5: 671-679, 1990.
2. Bergmann, P., Willems, D., Wilmet, M. & Heilporn, A.: La demineralisation osseuse chez le paraplegique. *Readaptation/Revalidation 1-2*: 122-127, 1991.
3. Bettica, P., Moro, L., Robins, S. P., Taylor, A. K., Talbot, J., Singer, F. R. & Baylink, D. J.: Bone-resorption markers galactosyl hydroxylysine, pyridinium crosslinks, and hydroxyproline compared. *Clin Chem* 38: 2313-2318, 1992.
4. Biering-Sorensen, F., Bohr, H. H. & Schaadt, O. P.: Longitudinal study of bone mineral content in the lumbar spine, the forearm and the lower extremities after spinal cord injury. *Eur J Clin Invest* 20: 330-335, 1991.
5. Brown, J. P., Malaval, L., Chapuy, M. C., Delmas, P. D., Edouard, C. & Meunier, P. J.: Serum bone-gla protein: a specific marker for bone formation in postmenopausal osteoporosis. *Lancet* i: 1091-1093, 1984.
6. Dawson, C. D., Jewell, S. & Driskell, W. J.: Liquid-chromatographic determination of total hydroxyproline in urine. *Clin Chem* 34: 1572-1574, 1988.
7. Delmas, P. D., Schlemmer, A., Gineyts, E., Riis, B. & Christiansen, C.: Urinary excretion of pyridinoline crosslinks correlates with bone turnover measured on iliac crest biopsy in patients with vertebral osteoporosis. *J Bone Miner Res* 6: 639-644, 1991.
8. Ebeling, P. R., Peterson, J. M. & Riggs, B. L.: Utility of type I procollagen propeptide assays for assessing abnormalities in metabolic bone diseases. *J Bone Miner Res* 7: 1243-1250, 1992.
9. Eyre, D. R., Koob, T. J. & Van Ness, K. P.: Quantitation of hydroxypyridinium crosslinks in collagen by high-performance liquid chromatography. *Anal Biochem* 137: 380-388, 1984.
10. Eyre, D. R., Dickson, I. R. & Van Ness, K.: Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues. *Biochem J* 252: 495-500, 1988.
11. Eyre, D. R.: Collagen cross-linking amino-acids. In *Methods in Enzymology*, vol. 144 (ed. L. W. Cunnham), pp. 115-139, Academic Press Inc., New York, 1987.
12. Eyre, D. R.: New biomarkers of bone resorption. *J Clin Endocrinol Metab* 74: 470 A-470 C, 1992.
13. Finsen, V., Indredavik, B. & Fougner, K. J.: Bone mineral and hormone status in paraplegics. *Paraplegia* 30: 343-347, 1992.

14. Garland, D. E., Stewart, C. A., Adkins, R. H., Hu, S. S., Rosen, C., Liotta, F. J. & Weinstein, D. A.: Osteoporosis after spinal cord injury. *J Orthop Res* 10: 371-378, 1992.
15. Garnero, P., Grimaux, M., Demiaux, B., Preaudat, C., Seguin, P. & Delmas, P. D.: Measurement of serum osteocalcin with a human-specific two-site immunoradiometric assay. *J Bone Miner Res* 7: 1389-1398, 1992.
16. Geusens, P., Nijs, J., Lovey, N., Borghs, H. & Dequeker, J.: Bone loss in the radius, lumbar spine, femoral neck and total body after paraplegia in men. *J Bone Miner Res* 7: S-326, 1992.
17. Hanson, D. A., Weis, M. A. E., Bollen, A. M., Maslan, S. L., Singer, F. R. & Eyre, D. R.: A specific immunoassay for monitoring human bone resorption: quantification of type I collagen cross-linked N-telopeptides in urine. *J Bone Miner Res* 7: 1251-1258, 1992.
18. Hassager, C., Jensen, L. T., Johansen, J. S., Riis, B. J., Melkko, J., Podenphant, J., Risteli, L., Christiansen, C. & Risteli, J.: The carboxyterminal propeptide of type I procollagen (PICP) in serum as a marker of bone formation in postmenopausal osteoporosis. *Metabolism* 40: 205-208, 1991.
19. Hørslev-Petersen, K., Ammitzbøll, T., Engstrøm-Laurent, A., Bentsen, K., Junker, P., Asboe-Hansen, G. & Lorenzen, I.: Serum and urine amino-terminal type III procollagen peptide in progressive systemic sclerosis: relationship to scleroderma involvement, serum hyaluronan and urinary collagen metabolites. *J Rheumatol* 15: 460-467, 1988.
20. James, I., Crowley, C. & Perrett, D.: Assay of pyridinium crosslinks in serum using narrow-bore ion-paired reversed-phase high-performance liquid chromatography. *J Chromatogr Biomed App* 612: 41-48, 1993.
21. Kiratli, B. J.: Skeletal adaptation to disuse: longitudinal and cross-sectional study of the response of the femur and spine to immobilization (paralysis). Ph.D. Thesis. The University of Wisconsin-Madison, 1989.
22. Kivirikko, K. I., Laitinen, O. I. & Prockop, D. J.: Modifications of a specific assay for hydroxyproline in urine. *Anal Biochem* 19: 249-255, 1967.
23. Lian, J. B. & Friedman, P. A.: The vitamin K dependent synthesis of gamma-carboxyglutamic acid by bone microsomes. *J Biol Chem* 253: 6623-6626, 1978.
24. Melkko, J., Niemi, S., Risteli, L. & Risteli, J.: Radioimmunoassay of the carboxyterminal propeptide of human type I procollagen. *Clin Chem* 36: 1328-1332, 1990.
25. Pietschmann, P., Pils, P., Woloszczuk, W., Maerk, R., Lessan, D. & Stipicic, J.: Increased serum osteocalcin levels in patients with paraplegia. *Paraplegia* 30: 204-209, 1992.
26. Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J. & Raman, N.: Characterization of a gamma-carboxyglutamic acid containing protein from bone. *Proc Natl Acad Sci (USA)* 73: 1447-1451, 1976.
27. Price, P. A. & Nishimoto, S. K.: Radioimmunoassay for the vitamin K dependent protein of bone and its discovery in plasma. *Proc Natl Acad Sci (USA)* 77: 2234-2238, 1980.
28. Prockop, D. J.: Isotopic studies on collagen degradation and the urine excretion of hydroxyproline. *J Clin Invest* 43: 453-460, 1964.
29. Risteli, L. & Risteli, J.: Noninvasive methods for detection of organ fibrosis. In *Connective Tissue in Health and Disease*, Vol. 1, (ed. M. Rojkind), CRC Press, Boca Raton, 1989.
30. Risteli, J., Elomaa, I., Niemi, S., Novamo, A. & Risteli, L.: Radioimmunoassay for the pyridinoline cross-linked carboxyterminal telopeptide of type I collagen: a new serum marker of bone collagen degradation. *Clin Chem* 39: 635-640, 1993.
31. Risteli, L. & Risteli, J.: Carboxyterminal propeptide of type I procollagen. A new direct indicator of bone matrix formation. *Ital J Miner Electrolyte Metab* 6: 1-6, 1992.
32. Robins, S. P., Black, D., Paterson, C. R., Reid, D. M., Duncan, A. & Seibel, M. J.: Evaluation of urinary hydroxypyridinium crosslink measurements as resorption markers in metabolic bone diseases. *Eur J Clin Invest* 21: 310-315, 1991.
33. Simon, L. S., Krane, S. M., Wortman, P. D. et al.: Serum levels of type I and III procollagen fragments in Paget's disease of bone. *J Clin Endocrinol Metab* 58: 110, 1984.
34. Teerlink, T., Tavenier, P. & Coen Netelenbos, J.: Selective determination of hydroxyproline in urine by high-performance liquid-chromatography using precolumn derivatization. *Clin Chim Acta* 183: 309-316, 1989.
35. Uebelhart, D., Gineyts, E., Chapuy, M. C. & Delmas, P. D.: Urinary excretion of pyridinium cross-links: a new marker of bone resorption in metabolic bone disease. *Bone Miner* 8: 87-96, 1990.
36. Uebelhart, D., Schlemmer, A., Johansen, J. S., Gineyts, E., Christiansen, C. & Delmas, P. D.: Effect of menopause and hormonal therapy on the urinary excretion of pyridinium crosslinks. *J Clin Endocrinol Metab* 72: 367-373, 1991.
37. Wakley, G. K., Portwood, J. S. & Turner, R. T.: Disuse osteopenia is accompanied by downregulation of gene expression for bone proteins in growing rats. *Am J Physiol* 263: E1029-E1034, 1992.
38. Wilder-Smith, C. H., Raue, F., Holz-Gottswinter, G. & Ziegler, R.: Procollagen-III peptide serum levels in Paget's disease of the bone. *Klin Wochenschr* 65: 174-178, 1987.
39. Yamauchi, M., Young, D. R., Chandler, G. S. & Mechanic, G. L.: Cross-linking and new bone collagen synthesis in immobilized and recovering primate osteoporosis. *Bone* 9: 415-418, 1988.
40. Zanone, X., Castanier, M. & Chantraine, A.: Dosage de l'osteocalcine chez les blessés médullaires: Etude préliminaire. *Readaptation/Revalidatie* 1-2: 89-93, 1991.

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