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Seasonal differences in biochemical parameters of bone remodelling

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Abstract

***Aims*—To compare bone remodelling parameters in late autumn and early spring in 20 post-menopausal women.**

***Methods*—The parameters measured were serum osteocalcin and its apparent degree of carboxylation (measured by hydroxyapatite binding), total and bone specific alkaline phosphatase and urinary bone resorption markers, (pyridinoline and deoxypyridinoline).**

***Results*—Serum osteocalcin concentrations were lower in autumn than in spring but the degree of carboxylation was similar. Total and bone specific alkaline phosphatase activities in serum were higher in autumn than in spring. These results support previous observations. However, notable and previously unreported changes in urinary bone resorption markers were observed. Pyridinoline concentrations were lower and deoxypyridinoline higher in autumn compared with spring. The ratio of pyridinoline:deoxypyridinoline was therefore very different between the seasons.**

***Conclusions*—The results clearly demonstrate that seasonal changes in these variables of bone remodelling must be taken into consideration when designing, reporting or analysing studies of bone metabolism in vivo.**

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Keywords: bone, season, osteocalcin, pyridinium, alkaline phosphatase.

Bone remodelling by formation and resorption is a continuous process. Biochemical parameters are measured to reflect these changes and may be used as indicators of the success or failure of therapeutic regimens.[1–3] Serum osteocalcin has been widely used as a marker primarily of bone formation[4] and the pyridinium crosslinks, pyridinoline and deoxypyridinoline have, in recent years, been shown to provide valid indexes of bone resorption.[5,6] A major consideration in the application of these markers is an appreciation of the physiological variations that occur. A large number of studies have provided information on variations in markers of both bone formation and resorption, relating particularly to diurnal (circadian), day to day and age related effects.[7–10] Generally, little attention has been paid to seasonal changes, although these have been clearly demonstrated for osteocalcin.[11] The degree of carboxylation of serum osteocalcin can be estimated by hydroxyapatite binding,[12–14] and statistically significant seasonal variations in the concentrations of undercarboxylated osteocalcin have been noted in elderly women.[15]

The present report provides a detailed analysis of the seasonal changes in serum osteocalcin and its degree of carboxylation, and in urinary pyridinium crosslinks and calcium concentrations. Total and bone specific alkaline phosphatase (ALP) activities were also studied. These results were derived from a study designed to assess the effects of vitamin supplementation in elderly women with osteoporosis,[16] and gave rise to a total of 60 values for comparisons between spring and autumn.

Methods

Twenty post-menopausal women who had suffered a Colles fracture at least 18 months previously were enrolled in a study of vitamin supplementation (K and D). This group of women were osteoporotic in comparison with controls, as determined by bone mineral density (BMD) of the lumbar spine and hip and by measuring attenuation of broad band ultrasound at the os calcis. Figure 1 outlines the study design. The 20 patients (mean age (range) 61·7 (52–73) years) were in two age matched groups I and II. More details are given in an earlier publication.[16]

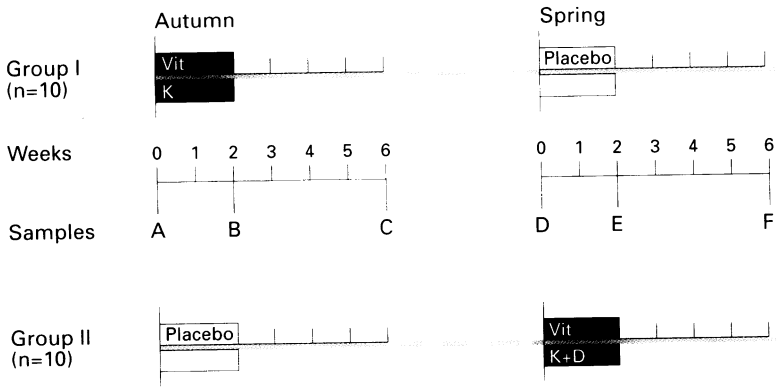

Figure 1 Study design. The study started in late November 1991 (autumn) and finished late April 1992 (spring). There was a six week "testing period" at the beginning and end of that time, with about three months "wash out" in between—the discontinuous part of the shaded horizontal line. There were two groups of patients (I and II) and six sampling days (A–F).

COLLECTION OF BLOOD AND URINE

Blood (20 ml) was collected by clean venepuncture without haemolysis, the specimen being placed on ice and centrifuged within 90

minutes of collection. Serum was separated and stored in aliquots at −20°C. Sampling was done between 1200 and 1600 hours, each patient having the same appointment time at each of the visits to control for possible circadian changes.

Urine samples were collected in supplied containers on first rising, and kept at 4°C until taken to the hospital later that day. The samples were acidified to pH 2 using hydrochloric acid and frozen at −20°C until analysed. The samples were collected on six separate occasions, three (A, B, C) in autumn and three (D, E, F) in spring. Samples B and E were collected two weeks after A and D, while C and F were collected four weeks after B and E (fig 1).

Between samples A and B, vitamin K supplement was given to half the volunteers (group I) in autumn and to the other half vitamins K and D were given in the spring (group II). Comparisons were made between samples in autumn and in spring (see later).

The remodelling indexes studied were serum osteocalcin (and its level of apparent carboxylation), urinary pyridinium crosslinks, urinary calcium and creatinine, and serum ALP (total, bone and liver).

MEASUREMENT OF OSTEOCALCIN

Serum osteocalcin concentrations were measured with the INCSTAR radioimmunoassay kit (IDS Ltd, Boldon, UK), according to the manufacturer's instructions. The intra- and interassay variations were 3–6% and 5–8%, respectively. In addition, repeated analyses of a standard serum sample stored in aliquots at −20°C showed no significant decrease in the measured osteocalcin concentration over the full time course of the study. Total osteocalcin in serum was measured and an estimate of the degree of carboxylation was obtained by determining the proportion of osteocalcin remaining unbound after mixing the serum with hydroxyapatite (Fluka Chemika, Glossop, UK), as described by Knapen *et al.*[17] Thus "bound" refers to the fraction of osteocalcin which binds to hydroxapatite in the laboratory assay, assumed to be fully carboxylated, and "free" refers to the undercarboxylated osteocalcin left in the supernatant fluid after the hydroxyapatite has been centrifuged down.

URINARY PYRIDINIUM CROSSLINKS

The urine samples were analysed for pyridinoline and deoxypyridinoline using a fully automated method with solid phase extraction and reversed-phase high-performance liquid chromatography, as described by Pratt *et al.*[18] With this assay, the overall coefficient of variation was <3% for pyridinoline and <5% for deoxypyridinoline. Analyses of well characterised standard crosslinks were done with each batch of samples, together with repeated analyses of a standard urine sample stored in aliquots at −20°C. These analyses showed that the pyridinoline:deoxypyridinoline ratio did not vary by more than 6% over the study period.

URINARY CALCIUM AND CREATININE

Both calcium and creatinine were analysed by standard techniques using a Kone autoanalyser.

ALKALINE PHOSPHATASE

Bone, liver and total ALP activities were measured colorimetrically using a Boehringer Mannheim kit and values were calculated as described previously.[18–20] Briefly, following measurement of the total ALP activity (a), the assay was repeated in the presence of 0·5 mmol/l phenylalanine (b), which inhibits ALP activity other than bone and liver, and again after heat inactivation of bone ALP at 56°C for 10 minutes (c). Total (a), chemical inhibition (b) and post-heat inactivation (c) activities were measured on the same day in such a way that samples A–C or D–F for each patient were measured in the same batch. The values obtained after heat treatment were corrected to account for the proportion of liver enzyme destroyed under these conditions.[19] The liver ALP is subtracted from the total activity. Measurement of bone plus liver activity by phenylalanine inhibition (b) was used to confirm that no individual patient had a large (>10%) contribution from the intestinal or placental isoenzymes. These were all post-menopausal women in whom no placental contribution was expected, but there could have been an intestinal or unexpected tumour source. Animal serum samples were run with each batch of reagent as quality control. For women between the ages of 50 and 70 years the measuring laboratory uses a reference interval of 110–380 U/l for total ALP activity. No patient had an activity above the upper limit of this range.

In the examination of serum osteocalcin and urinary pyridinium crosslinks there were 60 samples (samples A, B, C) from 20 patients in autumn and 60 samples (D, E, F) in spring. The ALP estimates were made at the end of the study and only 48 samples (16 patients) were available for analysis. In the remaining four patients at least one of the six samples (A–F) had been used up in the previous assays. This explains why in table 3 there are 48, 16 and eight pairs rather than the 60, 20, 10 pairs in the preceding tables.

EXAMINATION OF RESULTS

All sets of data were normally distributed and a paired *t* test was used in comparisons between autumn and spring values in the same individuals.

Because the samples were taken from patients also involved in a trial of vitamin supplementation the data had to be examined not only as a whole (60 samples in autumn and 60 in spring) but also in other smaller groups. There were 20 samples in autumn before the study started to be compared with 20 in spring following the winter "wash out" period and before vitamin supplementation restarted. In view of the remote possibility that the "wash out" was incomplete, comparison was made of 10 samples (A) in autumn from the placebo group II with samples D, the same group ex-

Table 1 Osteocalcin and percentage carboxylation

	*Assessment**	*Autumn*	*SD*	*Spring*	*SD*	*Paired t test*	*p value*
Osteocalcin (ng/nl)							
(60 pairs)	(i)	2·91	1·28	3·91	1·20	t=4·71	p<0·001
(20 pairs)	(ii)	2·80	1·15	3·73	1·10	t=2·56	p<0·05
(10 pairs)	(iii)	2·83	1·14	3·79	1·13	t=1·53	NS
Percentage carboxylation							
(60 pairs)	(i)	58·79	16·28	59·10	16·12	t=0·11	NS
(20 pairs)	(ii)	54·10	15·15	57·90	13·80	t=0·89	NS
(10 pairs)	(iii)	52·22	14·12	60·20	14·78	t=1·14	NS

* See Methods.

amined in the spring (fig 1). In the description of results for osteocalcin and pyridinium crosslinks there are three assessments: assessment (i): 60 pairs comparison (A + B + C cf D + E + F); assessment (ii): 20 pairs comparison (A cf D); assessment (iii): 10 pairs comparison (A (group II) cf D (group II)).

TIMINGS OF THE COLLECTIONS

The first sample (A) was collected on 25 November 1991 and the last (F) on 28 April 1992. A, B and C were collected over six weeks at the beginning and D, E and F similarly at the end. There was no sampling for 10 weeks in the middle. Samples A and D were about 16 weeks apart as were B and E, and C and F. These were the pairs used in the evaluation.

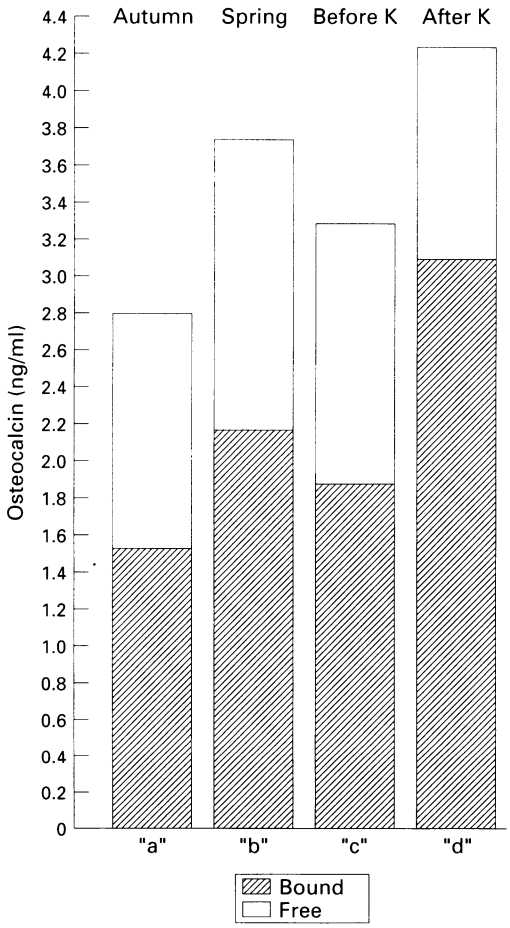

Figure 2 The vertical axis gives osteocalcin "free" or "bound" and on the horizontal axis: "a", 20 samples in autumn (groups I and IIA); "b", 20 samples in spring (groups I and IID) before supplement or placebo; "c", 20 samples before vitamin K supplementation (10 samples in autumn (group IA) and 10 samples in spring (group IID)); "d", 20 samples after vitamin K supplementation (10 samples in autumn (group IB) and 10 samples in spring (group II E)).

Results

OSTEOCALCIN (TABLE 1)

In assessment (i) serum osteocalcin values were significantly greater in the spring than in the autumn but the percentages of carboxylation were not significantly different. The same result was present in assessment (ii); similar, but no longer significant, figures were present in assessment (iii).

The values of osteocalcin for assessment (ii) were similar to the results before and after vitamin K supplementation (fig 2)—that is, the extent of the rise in total osteocalcin in the spring was similar to that after giving vitamin K. However, the two increases were different (fig 2) when examined as "free" (undercarboxylated) osteocalcin or "bound" (carboxylated). In fig 2 the rise in bound osteocalcin was significantly greater after vitamin K supplementation (p<0·001), as we reported previously.[16] In the seasonal rise in osteocalcin there is a similar proportion of "free" compared with "bound" osteocalcin in spring as compared with autumn.

URINARY PYRIDINIUM CROSSLINKS (TABLE 2)

The values for the two pyridinium crosslinks move in opposite directions when compared between autumn and spring. Pyridinoline was lower in the autumn than the spring, whereas deoxypyridinoline was higher in autumn than in spring. The result is that the ratio of pyridinoline:deoxypyridinoline was strikingly different between autumn and spring. Of the 60 pairs, 59 were higher in the spring and the remaining value was equal. This finding was present at a very high level of significance in all three assessments (i), (ii) and (iii).

CALCIUM (TABLE 2)

There were no differences in urinary calcium measured as mmol/mmol creatinine between autumn and spring. The spring value, although higher than the autumn value, was not significantly different.

ALKALINE PHOSPHATASE (TABLE 3; AS CAN BE SEEN IN THIS TABLE THE ASSESSMENTS IN THIS SECTION ARE RENUMBERED (I) (II) (IIIX AND Y) FOR REASONS GIVEN BELOW)

In assessment (i) (A + B + C cf D + E + F) total ALP was raised in the autumn com-

Table 2 Urinary pyridinium crosslinks

Pyrinium crosslinks (nmol/mmol creatinine)	*Assessment**	*Autumn*	*SD*	*Spring*	*SD*	*Paired t test*	*p value*
Pyridinoline							
(60 pairs)	(i)	62·47	16·12	71·07	15·55	t= 4·29	p<0·001
(20 pairs)	(ii)	64·06	18·84	70·38	17·53	t= 1·39	NS
(10 pairs)	(iii)	67·77	22·39	70·10	18·08	t= 0·33	NS
Deoxypyridinoline							
(60 pairs)	(i)	22·38	7·20	19·12	5·86	t= 4·25	p<0·001
(20 pairs)	(ii)	22·67	8·12	19·43	6·83	t= 2·11	p<0·05
(10 pairs)	(iii)	21·01	11·75	17·78	9·25	t= 1·31	NS
Pyridinoline:deoxypyridinoline ratio							
(60 pairs)	(i)	2·86	0·40	3·84	0·58	t=15·25	p<0·001
(20 pairs)	(ii)	2·87	0·49	3·76	0·56	t= 7·69	p<0·001
(10 pairs)	(iii)	2·99	0·63	3·71	0·44	t= 5·16	p<0·001
Urinary calcium (mmol/mmol creatinine)							
(60 pairs)	(i)	0·49	0·26	0·53	0·34	t= 1·0	NS
(20 pairs)	(ii)	0·48	0·32	0·47	0·39	t= 0·2	NS
(10 pairs)	(iii)	0·44	0·22	0·44	0·20	t= 0·02	NS

* See Methods.

Table 3 Total, bone plus liver, bone, and liver alkaline phosphatase activities

Alkaline phosphatase (U/l)	*Assessment**	*Autumn*	*SD*	*Spring*	*SD*	*Paired t test*	*p value*
Total							
(48 pairs)	(i)	131·77	44·31	105·21	33·85	t=6·12	p<0·001
(16 pairs)	(ii)	08·36	36·46	102·56	32·38	t=1·01	NS
(8 pairs)	(iiix)	145·00	42·92	110·13	33·16	t=5·80	p<0·001
(8 pairs)	(iiiy)	153·63	43·62	110·13	33·16	t=3·61	p<0·01
Bone + liver							
(48 pairs)	(i)	127·85	42·59	103·42	33·95	t=5·64	p<0·001
(16 pairs)	(ii)	110·50	35·21	109·88	36·09	t=0·10	NS
(8 pairs)	(iiix)	141·56	49·32	101·94	33·00	t=5·48	p<0·001
(8 pairs)	(iiiy)	148·56	47·16	101·94	33·00	t=5·97	p<0·001
Bone†							
(48 pairs)	(i)	53·04	27·80	34·40	19·18	t=4·05	p<0·001
(16 pairs)	(ii)	46·06	31·93	30·94	17·14	t=1·67	NS
(8 pairs)	(iiix)	57·25	20·76	33·88	19·63	t=3·18	p<0·05
(8 pairs)	(iiiy)	67·63	36·03	33·88	19·63	t=2·45	p<0·05
Liver†							
(48 pairs)	(i)	78·73	34·89	70·81	26·84	t=1·49	NS
(16 pairs)	(ii)	62·31	29·32	71·63	24·65	t=1·17	NS
(8 pairs)	(iiix)	87·75	30·95	76·25	20·33	t=1·11	NS
(8 pairs)	(iiiy)	86·00	41·46	76·25	20·33	t=0·74	NS

* See Methods: 48 pairs = A + B + C cf D + E + F (groups I and II); 16 pairs = A cf D (groups I and II); 8 pairs x = B cf D (group II); 8 pairs y = C cf D (group II). † The subtraction is made deliberately from the total (see text).

pared with spring. This is mainly due to the B and C autumn samples having significantly higher bone ALP activities. The figure for liver ALP was greater in autumn than in spring, but this was not significant. None of the patients had raised ALP activities from intestinal or other sources. The differences between measurement of total compared with bone plus liver are very small; the mean difference in autumn is 3·92 U/l and in spring is 1·79 U/l, these activities not being significantly different. In assessment (ii) (table 3 A cf D) there was no difference and the autumn rise in assessment (i) was due to rises later in autumn in samples B and C. In assessment (iiix and y) the B and C rises in group II in autumn were compared with group II D and they were significantly greater for bone or bone plus liver ALP.

Discussion

The seasonal changes in osteocalcin, pyridinium crosslinks and ALP were incidental findings in a study of vitamin supplementation in post-menopausal osteoporotic women who had sustained a Colles fracture two to three years before.

The seasonality of serum concentrations of 25 hydroxyvitamin D3 (25(OH)D) has been studied extensively.[22–27] In the Northern hemisphere, this has a peak in July and a nadir in January. In the young the concentration of the active D3 metabolite calcitriol (1,25(OH)^2 D) does not become deficient in winter, as it has been reported to do in the elderly.[23] There is an extensive literature showing that parathyroid hormone (PTH) is seasonally high when the serum concentration of 25(OH)D3 is low.[24–26] Krall *et al*[27] also confirmed the inverse relation between serum 25(OH)D concentrations and PTH, concluding that intakes over 220 IU of vitamin D per day may be sufficient to keep 25(OH)D concentrations constant, thus preventing a seasonal rise in the PTH concentration with its associated risk to the skeleton. It is likely that sunlight is a "time setter" for bone remodelling and there is increasing evidence that BMD varies by season.[28–30] Vitamin D status and PTH concentration may well be key factors in the observed changes, but such biochemical changes, which are well documented in the literature, were not studied here. In the study by Bergstralh *et al*[28] the average BMD of the lumbar spine was 1·4% higher in the late summer than in the winter months. There are other reports supporting this finding. Krølner[30] found BMD greatest in mid-August. It may be that the balance of bone remodelling in autumn/winter is in favour of bone resorption whereas in spring/summer it is in favour of bone formation.

In our study serum osteocalcin concentrations in spring were about one third greater compared with the autumn values. There is a definitive paper by Thomsen *et al*[11] who measured osteocalcin monthly in 15 normal young individuals aged 27–39 years and found a zenith in February and a nadir in summer, the size of the seasonal variation being 23%: the osteocalcin values were highest when the vitamin D level would be expected to be lowest. The findings in the present study are consistent with those.[11] Our spring timing is just past their peak and our autumn timing is only two to three months beyond the nadir.

Vitamin D controls the synthesis of osteocalcin in vitro in cultures of osteosarcoma cells.[31] As in the present study the percentage of undercarboxylation was found to be similar in autumn and spring by Szulc *et al*[15]: however, in that study Szulc *et al* made monthly readings and the level of undercarboxylation was greater in winter than summer and raised by vitamin D and calcium. The mean value of osteocalcin rises by 0·93 ng/ml in spring compared with autumn and it is 0·95 ng/ml greater after vitamin K supplementation than before. These two increases are dissimilar because the seasonal rise is due to an increase in both free and bound osteocalcin while the mean values for per cent carboxylation were similar in autumn and spring. In the rise after vitamin K the free level falls and the bound level increases, so that the rise in total osteocalcin is due entirely to the bound value (fig 2).

Urinary pyridinium crosslinks provide a measure of bone resorption with distinct advantages in terms of sensitivity and specificity over urinary hydroxyproline. Pyridinium crosslinks are formed during maturation of collagen fibres and are released during the breakdown of bone, providing a measure of bone resorption.[1] The values of pyridinoline and deoxypyridinoline have been previously reported to be highly correlated,[1] but they move in opposite directions when examined by season. There is already known to be a circadian rhythm, the value being highest usually at night.[6] The changes in the pyridinoline:deoxypyridinoline ratio are difficult to interpret but undoubtedly the observed differences may be an important consideration in the design and interpretation of future studies. The changes in this ratio are unlikely to be the result of degradation of collagen in tissues other than bone.[5,6,32] The amount of deoxypyridinoline formed will depend on the degree of hydroxylation of the specific lysine residues in the collagen helix that are involved in crosslinking. This is therefore controlled intracellularly during the synthesis of bone collagen by the activity of lysyl hydroxylase. As calcium ions are known to inhibit this enzyme,[33] it is conceivable that slight seasonal differences in ionised calcium concentrations in bone might contribute to changes in the crosslink ratio. This may also be associated with the changes in the calciotropic hormones discussed previously. However, the unknown time-lag between synthesis of collagen and its degradation as mature bone matrix is an additional factor confounding the interpretation of these observations.

Total ALP activities were higher in the autumn and lower in the spring. During the six weeks of the autumn study the activities rose significantly between late November and early January. This confirms the 1947 study by Tuba *et al*[34] and a subsequent study in 1981 from Dundee.[35] Bone ALP activity was also significantly different and was the major contributor to the seasonality of total ALP activities. This seems to be consistent with an association with the calciotropic hormones, vitamin D, PTH, and calcitonin. Surprisingly, the changes in serum ALP activities were in the opposite direction to those for osteocalcin when autumn was compared with spring.

These studies were made at only two points in the year (late autumn and early spring), approximately three months apart. The examination of the results in the three different assessments suggests that the changes reported are due to season and should not be attributed to vitamin supplementation.

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